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Délivré par :
Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :
Núria Solà Tapias
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The involvement of the three main Inflammatory Bowel Disease pathways and the secretion of trypsin proteolytic activity on intestinal epithelial cells

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Directeur/trice(s) de Thèse :
Dr. Nathalie Vergnolle
Dr. Frédérick Barreau

Jury :
Dr. Alain Couvineau, Directeur de Recherche. Rapporteur
Pr. Sophie Thenet, Professeur à l'Ecole Pratique des Hautes Etudes. Rapporteur
Pr. Vincent Lagente, Professeur Université Rennes 1. Président du Jury
Dr. Nathalie Vergnolle, Directrice de Recherche. Directrice de thèse
Dr. Frédérick Barreau, Chargé de Recherche. Directeur de thèse
Abstract

Crohn’s disease (CD) and Ulcerative colitis (UC) are two forms of Inflammatory Bowel Disease (IBD), a chronic inflammatory pathology affecting the digestive tract. Patients suffer from relapsing flares, diarrhea, abdominal pain and bleeding. Although the molecular mechanisms of IBD are poorly understood, recent data suggest that IBD occurs in genetically predisposed individuals developing an abnormal immune response to intestinal microbes after, being exposed to specific environmental triggers. Genetic studies have reported more than 170 polymorphisms susceptible to be involved in IBD pathogenesis. The strongest associations have highlighted three main pathways altered in IBD including bacterial sensing (NOD2, CD), autophagy (ATG16L1 and IRGM, CD) and endoplasmic reticulum stress (ER-Stress) (XBP1, UC). The role of intestinal barrier function is also strongly implicated in IBD pathogenesis, and is modulated by factors present in the lumen derived from microbiota, food or at a molecular level, by factors such as proteases. In IBD pathophysiology, the inflammatory process is characterized by impaired intestinal biology including disruption of tight junctions and leaky gut, decreased amount of Paneth and Goblet cells, and translocation of luminal antigens triggering inflammation. Previous studies have demonstrated an increased level of active serine proteases in the stools and tissues of IBD patients, supposing that proteases originate from infiltrated immune cells, pancreatic secretion or microbiota. However, our team has reported that intestinal epithelial cells are a major source of serine proteases, in particular trypsin-like enzymes, are released by a stressed epithelium in pathogenic context such as irritable bowel syndrome.

In this project, we aimed at better understanding whether the three main pathways involved in IBD (Nod2, autophagy, ER-stress) could be linked to an epithelial release of trypsin and reciprocally, if epithelial trypsin is able to induce or modulate these three IBD pathways.

We confirmed that trypsin-like activity was significantly higher in biopsies from UC and CD patients compared to healthy controls. In Caco-2 monolayers cultured in transwells, secreted trypsin-like proteolytic activity remained stable upon NOD2 stimulation but decreased under autophagy induction. Thapsigargin (Tg) stimulation a well-known ER-stress inducer, enhanced the apical release of trypsin-like activity in Caco2 cells. Activity-based probe assay identified a unique band at 33-KDa in ER-Stress-induced Caco-2 supernatants. This band showed specificity for Trypsin-3 in western blot. In UC patients, immunochemistry of colonic biopsies showed that Trypsin-3 was detectable mainly in epithelial cells, and up-regulated compared to biopsies from healthy controls and CD. Similarly, only UC patients displayed altered ER-stress with increased XBPs mRNA levels. In Caco-2 cells, ER-Stress induction provoked increased paracellular permeability, CXCL8 release, antimicrobial peptides (AMP) (TFF-3 and HBD2), and mucus (MUC2) dysregulation. Serine protease inhibitor AEBSF inhibited Tg-induced increased permeability and AMP dysregulation, while CXCL8 increase was aggravated. In Caco-2, Tg-induced ER-Stress increased PAR2 and -4 mRNA expression, PAR4 control levels were restored in the presence of AEBSF. ER-Stress-associated increased paracellular permeability was suppressed by PAR2 and/or -4 antagonist treatment, while CXCL8 was aggravated. Trypsin-3 didn't induce ER stress in Caco2.

Our data showed that in intestinal epithelial cells, ER-Stress increased trypsin-3 expression and trypsin proteolytic activity, which is responsible for altered barrier function and dysregulated AMP and mucin expressions. We identified PAR2 and -4 activation as possible
mechanisms by which ER-Stress contributed to epithelial pathophysiology. **Trypsin-3** appears as a candidate protease overexpressed upon ER-Stress and in UC patients epithelium.
Résumé


Les protéases jouent un rôle fondamental dans la digestion du bol alimentaire mais également dans le maintien de l’homéostasie intestinale en activant ou dégradant divers motifs moléculaires, ou in induisant des signaux spécifiques aux cellules par l’activation de quatre récepteurs : les PARs (Protease-Activated Receptor). Dans les MICI, un excès d’activité protéolytique de type trypsine est observé. L’origine de cette activité est théoriquement attribuée aux cellules immunitaires, à une surproduction pancréatique ou au microbiote, mais les cellules épithéliales intestinales semblent également être une source majeure de protéases.

L’objectif de mon projet de thèse visait à étudier l’impact des principales voies impliquées dans les MICI sur l’homéostasie des protéases épithéliales et le rôle de celles-ci dans la déstabilisation de la fonction de barrière.

Nos résultats ont confirmé un excès de protéases à sérine dans les cellules épithéliales de patients atteint de MC ou de RCH. In vitro, sur des monocouches de cellules Caco-2, l’induction de l’autophagie diminuait la libération apicale deprotéase de type trypsine, alors que le senseur bactériens NOD2 n’avait aucun effet. À l’inverse, une stimulation du Stress du réticulum endoplasmique (SRE) par la Thapsigargin, induisait une libération accrue de protéases actives de type trypsine au pôle apical des cellules. L’utilisation d’ABP (Activity-based probe), emprisonnant les protéases actives de type trypsine dans des surnageants apicaux de Caco-2 stimulées par la Thapsigargin, a montré une importante sécrétion d’une protéase unique au poids moléculaire de 33-KDa. Par western blot, la présence augmentée de Trypsine-3 était identifiée dans ces surnageants, de même que dans les colonocytes de patients atteints de RCH comparé à des échantillons contrôles ou CD. Seul les colonocytes de patients RCH présentaient également une induction du SRE. Sur les monocouches de Caco-2, l’induction du SRE augmentait la perméabilité paracellulaire, la sécrétion de CXCL88 et l’expression de PAM, de mucine et des récepteurs PAR2 et -4. Les inhibiteurs de protéases de type trypsine supprimaient l’augmentation de la perméabilité et l’expression des PAM, de la mucine 2 et des récepteurs PAR2 et -4 induite par le SRE, et aggravaient la sécrétion de CXCL8. Les antagonistes sélectifs des récepteurs PAR2 et/ou PAR4 inhibaient l’augmentation de la perméabilité et l’expression des PAM, de la mucine 2 et des récepteurs PAR2 et -4 induite par le SRE, mais aggravaient la sécrétion de CXCL8. Enfin, la Trypsine-3 ne modifiait pas les marqueurs de SRE.

En conclusion, l’induction d’un SRE dans les cellules épithéliales déclenche une libération apicale de Trypsine-3 et d’activité trypsine, responsable de l’altération de la fonction de barrière de la monocouche cellulaire. Nous avons identifié l’implication des récepteurs PAR2 et -4 (tous deux...
activables par la Trypsine-3) dans la rupture de l’homéostasie de l’épithélium intestinal. La Trypsine-3 semble être spécifiquement surexprimée dans les colonocytes de patients RCH, cette surexpression pourrait être liée à une induction anormale du SRE.
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“Everything is toxic, it all depends on the dose”
The involvement of the three main Inflammatory Bowel Disease pathways and the secretion of trypsin proteolytic activity on intestinal epithelial cells

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<tbody>
<tr>
<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>Agr2</td>
<td>Anterior Gradient 2</td>
</tr>
<tr>
<td>AIEC</td>
<td>Adherent-invasive Escherichia coli</td>
</tr>
<tr>
<td>AJs</td>
<td>Adherent junctions</td>
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<td>AMPs</td>
<td>Antimicrobial peptides</td>
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<td>ASUC</td>
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<td>Azathioprine</td>
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<td>BiP</td>
<td>Binding immunoglobulin protein</td>
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<td>BIR</td>
<td>Baculovirus inhibitor repeat</td>
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<td>Caco-2</td>
<td>Cancer coli-2</td>
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<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
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<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>CDI</td>
<td>Clostridium difficile infection</td>
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<tr>
<td>CFU</td>
<td>Colony-forming units</td>
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<td>CHOP</td>
<td>CCAAT/enhancer-binding protein homologous protein</td>
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<td>CMA</td>
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<td>CSs</td>
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<td>DAG</td>
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<td>EBSS</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>eIF2a</td>
<td>Eukaryotic translation initiation factor 2</td>
</tr>
<tr>
<td>EPIC</td>
<td>Investigation into Cancer and Nutrition</td>
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<td>EPS</td>
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<td>ERAD</td>
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<td>ERK</td>
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<td>FMT</td>
<td>Fecal microbiota transplants</td>
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<td>Acronyms and abbreviations</td>
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<tr>
<td><strong>GADD34</strong></td>
<td>Damage-inducible protein 34</td>
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<td><strong>GALT</strong></td>
<td>Gut-associated lymphoid tissue</td>
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<tr>
<td><strong>GI</strong></td>
<td>Gastrointestinal tract</td>
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<tr>
<td><strong>GPCRs</strong></td>
<td>G-protein-coupled receptors</td>
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<td><strong>GPI</strong></td>
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<td><strong>GRP78</strong></td>
<td>Glucose-regulated protein 78</td>
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<tr>
<td><strong>GWASs</strong></td>
<td>Genome-wide association studies</td>
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<tr>
<td><strong>IBD</strong></td>
<td>Inflammatory Bowel Disease</td>
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<tr>
<td><strong>IBS</strong></td>
<td>Irritable bowel syndrome</td>
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<tr>
<td><strong>IECs</strong></td>
<td>Intestinal epithelial cells</td>
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<tr>
<td><strong>IFN-γ</strong></td>
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<td>Inositol-requiring enzyme-1α /-β</td>
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<td><strong>MAMPs</strong></td>
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<td>Matrix metalloproteases</td>
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<td>Macrophage-stimulating protein</td>
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<td><strong>NOD2</strong></td>
<td>Nucleotide-binding oligomerization domain 2</td>
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<td><strong>PERK</strong></td>
<td>Protein kinase-like ER kinase</td>
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<td>Bacterial peptidoglycan</td>
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<td><strong>PKC</strong></td>
<td>Protein kinase C</td>
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<td>Peyer’s patches</td>
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<td>Acronym</td>
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Chapter 1

Biology of the intestine

Content at a Glance

- General overview of the GI tract
- Biology of the intestine
  - Histology of the intestine
  - Cellular organization of the intestine
  - The five cellular cell types
1. General overview of the gastrointestinal tract

The gastrointestinal tract (GI) is an essential tube which progresses through the middle of the body, from the mouth until the anus, including the following segments: mouth, oesophagus, stomach, small and large intestine and anus (Figure 1). The main function of the digestive tract is to digest and absorb nutrients and water from the ingested food as energy source for the body (Sherwood 2010).

The part from the small intestine to the anus is considered as the lower part of the gastrointestinal tract. The small intestine, also called small bowel, starts right after the stomach and is divided in three part including the duodenum, the jejunum and the ileum. The large bowel is subdivided into the colon, the cecum, the appendix and the rectum and is the most distal segment of the GI tract. With about 1.5 m of length and with a diameter of 6-7.5 cm the principal role of the large bowel is to absorb water and minerals and to store the waste remains as feces.

The cecum is considered as the first part of the large bowel. The ileum, last division of the small intestine, empties into the cecum acting as a junction between small and large intestine. At the bottom of the cecum, the appendix, a finger like projection, is part of the immune tissue of the gut, carrying lymphocytes. The colon (Figure 1) is divided in four parts – ascending colon, transverse colon, descending colon and the sigmoid colon – then straightens out to form the rectum (Kiela and Ghishan 2016). The colon absorbs water and mineral from food residues, the remains are eliminated as feces.

2. Biology of the intestine

2.1 Histology of the intestine

Although small differences are found between the small and large intestine, both share a common structure. In cross-sections, the intestine wall has four layer of specific tissues including the innermost mucosa, the submucosa, the muscularis externa layer and the outermost serosa.

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Figure 1. Lower gastrointestinal anatomy. The lower part of the gastrointestinal tract comprises the jejunum and ileum of the small intestine and the large intestine that includes the colon, the appendix, the rectum and the anus. Extracted from National Cancer Institute website (http://www.cancer.gov).
**Mucous membrane** is the innermost layer of the mucosa, adjacent to the epithelial layer and has a protective role. Below, the mucosa middle layer is termed lamina propria (LP). It is made up of a thin layer of loose connective tissue which includes a complex network of blood vessels, lymphatics, immune cells, and other components, providing the epithelium physical support and nutrition. It also includes the gut-associated lymphoid tissue (GALT) responsible to protect from luminal microbe invasion. The underlying tissue, muscularis mucosa is a thin band of smooth muscles that separates the mucosa from the submucosa.

Beneath the mucosa, the **submucosa** provides the digestive tract with flexibility and elasticity. It is a thick layer of irregular connective tissue that contains blood, lymphatic vessels and nerves, such as the submucosal plexus, that extends into the mucosa and muscularis externa providing nutrients supplies.

Adjacent to the submucosa the **muscularis externa** is composed of smooth muscle. It is divided in two layers – inner circular layer and an outer longitudinal layer – these layers organize gut movement or peristalsis. Between the two layers is housed a neuronal network, including the myenteric plexus.

The outermost connective tissue covering the intestinal wall is the **serosa**. It consists of a thin layer of connective tissue and a thin layer of cells that secrete serous fluids that avoid organ friction (Howell and Wells 2011).

![Illustration of the four layers of the digestive tract wall](image)

**Figure 2. Illustration of the four layers of the digestive tract wall.** The innermost mucosa, the submucosa, the muscularis externa layer and the outermost serosa. adapted from (Sherwood 2010)

### 2.2 Cellular organization of the intestine

The complex morphology of the gut is organized in two parts including the **crypt** and the **villus** (Figure 3). The **crypt of Lieberkuhn** is a tight invaginated space that comprises the **stem cell** compartment, a population of proliferating epithelial cells responsible of the self-renewal of the epithelium. Gut stem cells proliferate, migrate and differentiate along the intestinal crypt and undergo apoptosis every 3-7 days to renew the highly dynamic tissue, essential to maintain epithelial homeostasis (Clevers 2013; Dehmer et al. 2011).
The villi are finger-like projections of the intestinal wall, of approximately 0.5 - 1.6 mm in length, that greatly expand gut absorptive surface area. The presence of microvilli within the villi enhances even more the absorptive surface. The villus is irrigated with capillaries and lymph vessels to mediate the transport of absorbed nutrients into the body (Figure 3) (In et al. 2016; Barker 2013). An important feature that differs from the small to the large intestine is the presence of villi. The small bowel exhibits a huge villi surface to fulfill its absorptive role. On the contrary, the lack of villi on the colon is associated with dry out of fecal bolus.

![Figure 3. Organization of the small intestine and the colon. Illustration of the colon (left) and small intestine (right). The villi are covered with mature cells, including absorptive enterocytes or colonocytes, together with mucus-secreting goblet cells. Those cells are renewed every 5 days and are shed into the lumen to become part of the excreted residues. The crypts are covered with more immature epithelial cells, including progenitors and stem cells and also secretory cells such as Paneth cells. Adapted from (Medema and Vermeulen 2011)](image)

The inner surface of the intestine is composed by a single-cell columnar layer of highly polarized intestinal epithelial cells (IECs). The villi are coated with five differentiated intestinal cell types classified in two groups – absorptive and secretory- depending on their functions. In the small intestine the most common cell type is enterocytes, considered as the absorptive lineage and the three remaining cell types are secretory cells including Paneth cells, goblet cells and enteroendocrine cells (Figure 3 and 4). Three other minority of cell types belong to the intestine monolayer including M cells, cup cells and tuft cells (Figure 3 and 4) (Coskun 2014; Maloy and Powrie 2011). The lack of Paneth cells, and the presence of colonocytes, as colonic absorptive cells instead of enterocytes, constitute the two main differences between small and large intestine (Noah, Donahue, and Shroyer 2011).
2.3 The five different cell types

A) Enterocytes and colonocytes

Enterocytes and colonocytes, represent 80% of both small and large intestine. Their main function is to select and absorb nutrients apically and export them basally. The apical cell surface is composed by a microvillus brush border and a thick layer of glycocalyx, a glycoprotein gel-forming layer composed of several carbohydrates as a backbone molecule and mucins (Noah, Donahue, and Shroyer 2011). Glycocalix is a meshwork of approximately 0.5 µm that projects from the apical cell surface of enterocytes overlaying the intestinal surface, providing extra absorption and including crucial enzymes needed for the final step of digestion (Maury et al. 1995). Besides, enterocytes and colonocytes are also capable of producing mucins and antimicrobial peptides (Pelaseyed et al. 2014; Bahar and Ren 2013). Antimicrobial peptide content is described in chapter 2 section 3.1. Mucus layer content is described in chapter 2 section 2.2.

B) Goblet cells

The most abundant secretory lineage, goblet cell, is responsible of producing a wide variety of mucins forming a tightly attached mucus layer in the intestine. The mucus layer is the first line of defence against physical and chemical damage generating a physical barrier between luminal bacteria and the epithelial cells. The presence of mucous-producing cells is richer in the colon than in the small intestine, increasing the proportion from the duodenum (4%) to the descending colon (16%) providing extra-lubrication to the stool passage towards the colon (Barker 2013). The distal colon is protected by two different mucus-layers, including an inner dense layer and an outer loose layer, while small intestine is covered by a single mucus-layer. The inner layer, impermeable to bacteria, is 50-500 µm thick and it is basically made up of the gel-forming MUC2 mucin skeleton (Noah, Donahue, and Shroyer 2011; van der Flier and Clevers 2009; Malin E V Johansson et al. 2011). Mucus layer content is described in chapter 2 section 2.2.

C) Paneth cells

Paneth cells, located at the basis of the crypts of the small intestine, are secreting cell types specialized in producing an array of antimicrobials into the mucus layer, besides crypt development. The biological function of antimicrobial peptides (AMPs) is to target microorganisms of the lumen, both resident microbiota and harmful pathogens. Paneth cells, found in the crypt of the small bowel, are characteristic for their extensive endoplasmic reticulum and Golgi due to their intense secretory activity and they contribute to the stem cell niche (Buckley and Turner 2017; Bevins and Salzman 2011). The presence of Paneth cells’ granules, including AMP, intestinal trefoil-factor -described in chapter 2 section 1.2- and growth factors, are crucial for crypt formation in the small gut in (Porter et al. 2002). Paneth cells are absent in the colon. AMPs are described in chapter 2 section 3.1.

D) Enteroendocrine cells

Enteroendocrine cells are a minority proportion of the overall epithelial cell population – 1% approximately- randomly located along the intestinal wall. Although at least 15 subtypes of enteroendocrine cells have been described, they all share the same morphology. Apically, they possess microvilli extended to the luminal content, and their cytoplasm is characterized by the presence of secretory vesicles. Those cells sense ingested food of the luminal surface and respond
by diffusing hormone peptides, apically or basally, that activate nerve fibres and/or travel through the bloodstream. Moreover, the secretion of peptide hormones is pivotal for the physiological and homeostatic function of the gut (Cummings and Overduin 2007; Gunawardene, Corfe, and Staton 2011).

E) Other minor cells

There are three additional minor cell types present in the intestine including microfold (M) cells, cup cells and tuft cells. M cells specifically located into the follicle-associated epithelium (FAE) of both isolated lymphoid follicle or Peyer patches, two major component of the GALT, driving the foreigner material by transepithelial transport from the lumen to the lymphoid tissue within the mucosa. They interact with immune cells of Peyer’s patches controlling the immune response and tolerance (Kucharzik et al. 2000). Known as brush cells, Tuft cells cover a small fraction -0.4% - of the intestinal surface. These cells, present in the airways and the digestive system, have a unique morphology and distinctive features typical of chemosensory cell type involved in the defence towards parasites (Gerbe, Legraverend, and Jay 2012). Finally cup cells, restricted to the ileum, represent up to 6% of total epithelial cells, and likewise M cells, they express vimentin (Fujimura and Iida 2001).

Figure 4. Illustration of the epithelial cells that are present in the intestine.
Chapter 2

Central role of the colonic barrier

Content at a Glance

The main role of intestinal mucosa
Physical barrier
  The junctions of the epithelial barrier
  Mucus layer of the intestine
Bio-chemical barrier
  Antimicrobial peptides
  Immunity of the colonic mucosa
1. The main role of intestinal mucosa

The gastrointestinal mucosa has two critical functions: act as a physical barrier and as a selective filter between the inner and the outer environment.

The human gut mucosa is exposed to a wide diversity of resident microbes and harmful antigens. Therefore, to handle a peaceful coexistence between the healthy intestine and their commensal bacteria and to avoid the passage of foreign antigens into the inner body, the host is equipped with a wide variety of mechanisms. Besides, mucosa epithelium act as a selective filter allowing the translocation of essentials nutrients, ions and water from the lumen into the body. To keep a flawless intestinal homeostasis, the integrity of all protective mechanisms of the mucosal barrier, including physical and biochemical defences, need to be harmonized. First, a well-integrated epithelia monolayer is essential to avoid the leakage of the intestine. Epithelial integrity is regulated by a set of adhesive complex of proteins including Tight Junctions (TJ), Desmosomes and adherens junctions (AJs) (Figure 5). The adhesion complexes give integrity and selective permeability to the gut monolayer (Snoeck, Goddeeris, & Cox, 2005). Second, a physical thick layer of mucus covers most of the epithelia of the intestinal tract, especially the colon. It is composed of mucins secreted by goblet cells, providing the first contact to harmful antigens, toxins and other materials in the intestinal lumen. Chemical defences, such as antimicrobial peptides (AMPs), produced by enterocytes, Paneth cells and goblet cells confer additional protection (Halpern MD, 2015). Lastly, specific immune mechanisms of surveillance present in IECs and other immune cells carry innate microbial receptors that controls and monitor the luminal bacteria. In order to recognize microbial pathogens, IECs among other cells, carry the pattern recognition receptors (PRR), essential to initiate the innate immune signal that drives the necessary response to kill harmful microbes (Wlodarska, Kostic, & Xavier, 2015). Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains are the two major forms of PRR known.

Figure 5. Intestinal epithelial barrier. A single layer of epithelial mucus separates the intestinal lumen from the submucosa. Epithelial barrier integrity prevents bacterial translocation by secreting a broad spectrum of antimicrobial factors and a thick mucus layer that cover all intestinal surface. In addition intercellular interactions are essential for cell sealing. Adapted with permission from (Halpern MD, 2015).
2. Physical barrier

2.1 The junctions of the epithelial barrier

In order to fulfill the intestinal barrier challenge, IECs are held together by three intracellular adhesion complexes, Tight Junctions (TJs), Desmosomes and adherens junctions (AJs) (Figure 6). These cell-cell interconnections, likely formed between two different cell types, are crucial for the paracellular transport and barrier integrity providing mechanical properties to the barrier function (Antoni, Nuding, Wehkamp, & Stange, 2014). An important feature of the intestinal epithelium is the high polarization of the IECs. Due to the asymmetric organization of specific protein along the cell, such as TJs, AJs or ion distribution, the composition between the apical and the basolateral pole differs (Cereijido, Contreras, Shoshani, Flores-Benitez, & Larre, 2008). IECs polarization contributes to intestinal functions like absorption and secretion. Increased intestinal permeability is associated to several GI disease such as inflammatory bowel disease (IBD), mentioned in chapter 4 (Zeissig et al., 2007).

Figure 6. Intercellular adherent complexes. IECs are held together by a set of adhesive complex of proteins - Tight Junctions (TJ), Desmosomes and adherens junctions (AJs). Adapted from (Henderson, Van Limbergen, Schwarze, & Wilson, 2011).

A) Tight Junctions

The TJ network is the apical-most junctional complex that delimits the border between apical and basolateral domain. They are composed by four different families of transmembrane proteins including occludin, claudin, tricellulin and junctional adhesion molecules (JAM) (Figure 7). Occludin, claudin, tricellulin are structures proteins with two extracellular loops and two cytoplasmic domains. The intracellular tail of TJs connects with cytosolic scaffold proteins, such as the zonula occludens (ZO) proteins family, which consecutively binds to the actin cytoskeleton. In order to sustain the contractile tension caused by the TJs, the cytoskeleton, is closely interconnected to a peri-junctional ring of myosin II light chain (MLC). The phosphorylation of MLC via myosin light chain kinase (MLCK) induces contraction of the actin-miosin cytoskeleton leading to a TJs opening and leakage across intestinal epithelium (Feighery et al., 2008; Turner et al., 1997). Disruption of TJs integrity leads to a decrease of transepithelial electrical resistance (TEER)-mentioned in chapter 6 section 1-, a manner to measure paracellular flux.
i) Occludin

Occludin was the first transmembrane component of TJ to be discovered, in 1993. Occludin is mainly expressed in epithelia and endothelial cells, but its functions are not fully delineated. *In vivo* and *in vitro* data have shown that Occludin plays a role in the regulation of the **paracellular permeability** by maintaining the integrity of TJs network (Al-Sadi et al., 2011). Occludin is an integral membrane protein made up by four domains—two extracellular loops, two cytoplasmic domains, a long carboxyl region and a short N-terminus region. **Regulation and localization** of Occludin is arranged by phosphorylation on multiple residues—tyrosine, serine and threonine. Kinases, such as **protein kinase C** (PKC) and phosphates, regulate occludin phosphorylation and its localization to TJs regions. However, depending on the phosphorylated residues such as Tyrosine phosphorylation, it triggers dissociation of the occludin-ZO1 complex. On the contrary, non-phosphorylated molecules are mainly located at the basolateral membrane (Dörfel & Huber, 2012; Gonzalezmariscal, 2003).

Cytosolic **C-terminus** domain anchors to several TJ scaffold proteins, such as PDZ-domain containing ZO proteins, which, in turn, link to the actin-cytoskeleton (Figure 7). Intracellular scaffold proteins are localized at the cytoplasmic surfaces of junctional structures to establish specialization and localization of the junctions. Thus cytoplasmic platforms such as PDZ-domain-containing **zona occludens** proteins (ZO-1, ZO-2 and ZO-3) constitute a bridge between transmembrane proteins and the actin-cytoskeleton to mediate intracellular and extracellular signals (Figure 7). Some TJs can also interact with non-PDZ domains such as cingulin, which connects the junctional membrane protein to ZO-1 (Groschwitz & Hogan, 2009; Lee, 2015; Umeda et al., 2004).

![Figure 7. Tight Junction complex.](image)

**Figure 7. Tight Junction complex.** The TJs are the most apical junctional complexes which create a selective permeability between adjacent IECs. It is made up by a branch network of sealing strands including, occludin, claudins, tricellulin and JAMs. Extracted from (Lee, 2015).

ii) Claudins

Like Occludin, the transmembrane protein **Claudins** also form the core of the TJs and control for **ion selectivity** and **permeability**. To date, 24 Claudins have been identified and their functions differ depending on their tissue-specific expression. Like Occludin, some Claudins are regulated and localized to TJs via phosphorylation. The **two extracellular loops**, hemophilic and/or heterophilic, interconnect with neighbouring cells, establishing a selective permeability and ion
channel-forming. The **cytosolic C-terminus tail** anchors Claudins to the actin-cytoskeleton via scaffold proteins including ZO-1, ZO-2 and ZO-3 (Itoh et al., 1999). Some of the pores forming claudins reduce intestinal permeability whereas others alter paracellular selection. Claudins-1, -5 and -7, fulfill the formation of the barrier function decreasing permeability (Günzel, 2017). On the other hand, claudin-2, -15 and -16 possess the “leaking” phenotype, increasing transepithelial resistance (TEER) (Furuse, Furuse, Sasaki, & Tsukita, 2001; Overgaard, Daugherty, Mitchell, & Koval, 2011).

### iii) Tricellulin

Located in the tricellulin TJ, the newest discovered TJs tetraspanin protein, tricellulin (marvelD2) and marvelD3, together with marvelD1 (occludin) are members of the TJ-associated Marvel protein family (TAMPs). These three proteins share a conserved MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that contributes to epithelial function and TJs regulation (Oda, Otani, Ikenouchi, & Furuse, 2014; Riazuddin et al., 2006).

### iv) Junctional adhesion molecules

**Junctional adhesion molecules** (JAM) is the last component of the TJs complex. The JAM family including mostly A and B subtypes belongs to the immunoglobulin superfamily which comprises two IgG-like fold extracellular domains and one cytoplasmic tails. Two homophilic and heterophilic extracellular domains, from two different JAM, are required to stabilize cell-cell junctions to regulate the cellular function and paracellular permeability. Besides homeostatic functions, JAMs are required for cell migration and proliferation (Nava et al., 2011). Intracellular C-terminus domain interacts with the scaffolding and cytoplasmic proteins, such as ZO-1, which in turn, links to the actin cytoskeleton (Campbell, Maiers, & DeMali, 2017; Monteiro et al., 2013).

### B) Adherens junctions

Underneath the tight junction, **adherens junction**, also termed **zonula adherens**, form the major lateral cell-cell adhesion belt connecting transmembrane proteins, intracellular adaptor proteins and actin filaments. The AJs consist in two adhesive proteins units; the most commune AJs structure is formed by **cadherin-catenin** (Figure 8).

![Figure 8. Structural model of Adherens Junctions](image)

**Figure 8. Structural model of Adherens Junctions.** The most studied AJ complex is the cadherin-catenin network. The main function is to maintain the physical cohesion between IECs. Extracted from (Perry, Lins, Lobie, & Mitchell, 2010).
Classical epithelial (E)-cadherins anchor to the catenin complex – α-, β-, and p120 catenin. Through the armadillo repeats, the N-terminus domain of p120 catenin and β-catenin (also termed plakoglobin) binds directly to the intracellular domain of E-cadherins. β-catenin C-terminus domain, in turn, binds to α-catenin which links to the cytoskeleton. α -catenin has the ability to bridge the filaments of actin with E-cadherin (Figure 8). The extracellular domain of E-cadherins interacts with neighbouring cells that mediates cell-cell adhesion (Groschwitz & Hogan, 2009; Niessen, 2007). Together, these AJs complexes provide a strong mechanical connection required to maintain the integrity of the epithelial barrier (Niessen, 2007).

C) Desmosomes

The intracellular junction desmosomes are essential for mediating strong cell-cell cohesion and for maintaining a mechanical seal between cells. Thus, they are abundant in the epidermis and myocardium tissues due to their exposure to repetitive mechanical forces. Placed in the basal side of IECs, desmosomes are intracellular junctions with an extracellular tail that anchors to neighbouring desmosomes, and a cytoplasmic domain that anchors to cytoskeleton-associated proteins. Altogether, the desmosome complex forms a network that provides mechanical strength to the intestine named scaffold complex, and it consists in three units: two intracellular components and one cell to cell (Figure 9) (Hatzfeld, Keil, & Magin, 2017). Intracellularly, actin bind to the desmosomal adhesion molecules by the linkage of intermediate filaments (keratins). Thus, intermediate protein linkage is mediated by desmoplakin (linker 1) and the armadillo proteins plakoglobin and plakophilin (linker 2). To sum up, intermediate filaments bind to linker 1. Linker 1, in turn, binds to the linker 2 that binds to the desmosomal cadherins complex, conforming integrity and plasticity to the epithelium (Garrod and Chidgey 2007).

Figure 9. Desmosome scaffold complex. Desmosomes are crucial for strong cell-cell adhesion, and their failure can provoke disease. They are also involved in fundamental processes like cell proliferation, differentiation and migration. Extracted from (Garrod & Chidgey, 2008)

2.2 Mucus layer of the intestine

The mucus layer covers the whole epithelium of the GI tract keeping harmful antigens away from the epithelial monolayer. The small intestine presents a single and discontinuous mucus layer
whereas the large bowel consists in double and well defined mucus layer. Due to the high number of bacteria living in symbiosis in the colon, the mucus layer is essential to avoid the contacts of pathogens with the intestinal mucosa (Figure 10). Moreover, the lubrication ability of the mucus is crucial to improve intestinal transit (In et al., 2016).

**Figure 10. Schematic model of the mucus organization in the small intestine and colon.** Two layers of adherent mucus are present in the large bowel. Goblet cell-derived mucin are mostly present in the colon. Adapted from (M. E. V. Johansson, Larsson, & Hansson, 2011)

**Mucins,** the main protein of the mucus, are stored and secreted, by goblet cells – described at chapter 1 section 2.B –. Mucins are subdivided in two groups: the secreted mucins and the transmembrane mucins. The **secreted mucin** (MUC2, MUC5 and MUC6) form long polymers and are tissue-specifically expressed. On the other hand, **transmembrane mucins** (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, and MUC17) are adherent to the apical cell surface of IECs (Malin E V Johansson, Sjövall, & Hansson, 2013). These cell-membrane-associated mucins belong to the inner mucus layer of the distal colon and form an important element of the glycocalyx – mentioned in chapter 1 section 2.B - and epithelium protection (Malin E V Johansson et al., 2011; Thornton, 2004).

Mucin proteins are **translocated** into the endoplasmic reticulum (ER) where they are folded and form disulfide-bonded dimers. Thereafter, mucins PTS domains (tandem repeats) rich in proline (P), threonine (T), and serine (S) become densely O-glycosylated in the Golgi apparatus. This highly glycosylated mucin domains have a high capacity to bind water that contributes to the gel-forming properties of the mucus (Ijssennagger, van der Meer, & van Mil, 2016).

The mucus layer of the distal colon consists in two different mucus coats including an **inner layer** firmly adherent to the epithelium and an **outer loose mucus layer** (Figure 10) (Atuma, Strugala, Allen, & Holm, 2001; M. E. V. Johansson et al., 2011; Malin E V Johansson et al., 2011). The inner firm mucus layer, free of bacteria is transformed into another mucus layer expended in volume. The loose outer mucus layer is in contact with bacteria and can be degraded by their enzymes. Bacteria strains can bind to the outer mucus thanks to the abundant glycans present in MUC2 mucin. The major component and the skeleton of the mucus layer is the gel-forming mucin **MUC2** (Thornton, 2004).

Other important peptide produced by goblet cells that regulates the physical barrier and stabilizes mucin polymers are **trefoil-factor 3 (TFF3)** and **Resistin-like molecule-β (RLMβ)** (table 1) (Peterson & Artis, 2014). RLMβ belongs to the resistin-like molecules including four members:
RELM-α, -β, -γ and resistin. Unlike the other proteins of the family, RELMβ is tightly produced by goblet cells and secreted apically into the lumen content. Although its function is not fully defined, RELMβ is induced upon bacterial colonization and promotes MUC2 secretion and inflammation control by the stimulation of Th2 cytokines. Within the inflammatory area, RELMβ regulates macrophages and T cells resident in the gut (Artis et al., 2004; McVay et al., 2006). On the other hand, TFF3, also named intestinal trefoil factor, is predominantly expressed by goblet cell-derived peptide in the small intestine and colon, and it is abundantly secreted into the lumen surface. TFF3, belongs to the trefoil peptide family which includes the gastric peptides pS2 (also named TFF1) and spasmolytic polypeptide (also called TFF2), all of them are involved in gastrointestinal epithelial restitution (Sands & Podolsky, 1996). TFF3, is involved not just in the structural integrity of the mucus but also provides epithelial healing, IECs migration and turnover as well as resistance to apoptosis (Taupin, Kinoshita, & Podolsky, 2000).

Within the mucus layer, the apical IECs surface is covered with secretory IgA (sIgA). They serve as a first line of defence and display antibacterial properties protecting the gut mucosa from enteric toxins and harmful microorganisms. SlgA is capable of controlling the inflammation and regulating immune response to commensal microbiota, pathogens and antigens by a system known as immune exclusion (Mantis, Rol, & Corthésy, 2011). SlgA blocks the access of the bacteria to epithelial receptors, retaining the pathogen within the mucus and facilitating its expulsion by peristaltic movements of the intestine (a J. Macpherson et al. 2008). They shape the intestinal microflora by re-transporting antigens across the mucosa barrier to dendritic cells, subsets in GALT, which promotes pro-inflammatory signals associated with uptake of pathogens.

3. Bio-chemical barrier

3.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) belong to the innate immune defence and play a homeostatic key role maintaining the composition of the commensal flora and intestinal homeostasis. AMPs are found in the most exposed areas of the body to microbes, such as skin, eyes, oral mucosa, lung or intestinal mucosa. AMPs are produced by Paneth cells, goblet cells and enterocytes. This defence peptides possess a wide-spectrum of antibacterial properties towards pathogens and microbiota, both Gram-positive and Gram-negative (Dupont et al., 2015). Besides killing bacteria, some AMPs possess non-antimicrobial functions such as immune modulator. These peptides reinforce the total immune response by a range of mechanisms: as a chemoattractant to recruit immune cells, pro-inflammatory cytokines or as a Toll-Like Receptor ligand – mentioned in chapter 2. section 3.2 A- (Islam et al., 2001).

AMPs are usually cationic, not longer than 50 amino acids, and positively charged to prevent the diffusion of the peptide into the lumen. These features are ideal to trap the peptide into the mucus layer and to target the negatively charged bacteria surface (Hancock & Diamond, 2000; Zasloff, 2002). Depending on the peptide, the bactericidal mechanism of action differs. The peptide kill bacteria by (a) disruption of membrane integrity, (b) inhibiting DNA or RNA synthesis or (c) targeting specific intracellular molecules (Figure 11) (Bahar & Ren, 2013).
**Introduction**

**Figure 11. Biological function of antimicrobial peptides.** AMPs act not just as a bacteria killer but also modulate host immune response. Bactericidal AMPs bind to the bacterial membrane by electrostatic interaction and kill them by disrupting their membrane or by inhibiting crucial intracellular functions. Immunoregulatory AMPs recruit or activate immunocytes by chemoattraction or by acting as a TLR ligand that leads to the activation of pro-inflammatory downstream signaling pathways. Adapted from (Zhang & Gallo, 2016).

**Defensins** and **cathelicidins** are the two major mammalian antimicrobial peptides.

**Defensins** are cationic peptides with three intramolecular disulphide bonds. To date, three subclasses of defensins have been described (α, β and θ) (table 2). Although the α-, and β-defensins have been identified in humans (O’Neil et al. 1999), θ-defensin has been characterized from primates’ leukocytes (M E Selsted, 2004). Six human α-defensins have been characterized. Up to 4 different α-defensins are expressed by neutrophil, known as a **human neutrophil peptide-1** (HNP1, HNP2, HNP3 and HNP4). Human **defensine-5** and -6 (HD-5, HD-6) are tissue-specific only expressed by Paneth cells in the small intestine. Compared to α-defensins, four different β-defensins have been identified (hBD1-4) in human. In mice, it has been described 6 different α-defensins, named cryptdins (crypt defensins) and up to 45 different β-defensins gens (Michael E Selsted & Ouellette, 2005). They are quite abundant within the GI tract, especially in the colon, mainly expressed by epithelial cells. While hBD1 is constitutively expressed along the small and large colon, hBD2, -3, and -4 are induced by pro-inflammatory or pathogen stimuli, through PPR-activated signals – mentioned in chapter 2 section 3.2 A- which, in turn, activate transcription factor nuclear factor kappa-B (NF-κB) (O’Neil et al., 1999).

Secreted at the surface of the colonic crypts, like defensins, **cathelicidins** are another dominant class of AMPs. Although about 30 subfamilies of cathelicidin have been identified in mammalian, only LL-37/hCAP18 and CRAMP have been found in human and mice, respectively (Dürr, Sudheendra, & Ramamoorthy, 2006). These peptides carry a large spectrum of **bactericidal activity** against Gram-negative and -positive bacteria. Stored in neutrophils, macrophages and epithelial cells as secretory granules, cathelicidins are released upon leukocyte activation (Kościuczuk et al., 2012; Zanetti, 2005).

Paneth cells are the main source of AMPs in the small intestine, and beside α-defensins, they secret **phospholipase A2** (sPLA2), **RegIII**, and **lysozyme C**. AMPs are regulated and stored as inactive
Chapter 2. Central role of the colonic barrier

peptides in secretory granules. Triggered by bacteria among other stimulus, the granules are release on the lumen and are cleaved by trypsin or matrix metalloproteinase to generate the peptide mature form (Tollin et al., 2003; Wang, 2014).

**Human intestinal AMPs**

<table>
<thead>
<tr>
<th>Class</th>
<th>Antimicrobial</th>
<th>Cellular type</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Defensins</td>
<td>NHP-1, -2 and -3</td>
<td>Neutrophils/Bone marrow</td>
<td>Constitutively express</td>
</tr>
<tr>
<td></td>
<td>HD5 and HD6</td>
<td>Paneth cells</td>
<td>Constitutively express</td>
</tr>
<tr>
<td>β-Defensins</td>
<td>hBD1</td>
<td>Enterocytes</td>
<td>Constitutively express</td>
</tr>
<tr>
<td></td>
<td>hBD2, hBD3, hBD4</td>
<td>Enterocytes</td>
<td>Induction during inflammation</td>
</tr>
<tr>
<td>Cathelicidins</td>
<td>LL-37</td>
<td>IECs/inflammatory cells</td>
<td>Induction during inflammation</td>
</tr>
<tr>
<td>Other AMPs</td>
<td>RegIII</td>
<td>Paneth cells/enterocytes</td>
<td>Induction during inflammation</td>
</tr>
<tr>
<td></td>
<td>sPLA2</td>
<td>Paneth cells</td>
<td>Constitutively express</td>
</tr>
<tr>
<td></td>
<td>CCL20</td>
<td>Paneth cells</td>
<td>Increased under inflammation</td>
</tr>
<tr>
<td></td>
<td>Lysozyme C</td>
<td>Paneth cells</td>
<td>Constitutively express</td>
</tr>
<tr>
<td></td>
<td>BPI</td>
<td>IECs</td>
<td>Increased under inflammation</td>
</tr>
<tr>
<td></td>
<td>RELMβ H.s.</td>
<td>Goblet cells</td>
<td>Induction during inflammation</td>
</tr>
</tbody>
</table>

Table 1. Human intestinal AMPs. Adapted from (Muniz, Knosp, & Yeretssian, 2012)

AMPs constitute a shield towards commensal microbiota. Dysregulation of peptide production changes the composition of commensal microbiota and disrupts intestinal homeostasis. Failure of AMPs expression and secretion is associated with human disease such as obesity or Irritable Bowel Syndrome (Zhang & Gallo, 2016).

### 3.2 Immunity of the colonic mucosa

In order to protect the GI tract from viruses, bacteria, fungi, or parasites, the intestinal mucosa needs to recognize and respond to foreign organism and harmful substances. To identify and protect the GI tract from those, the intestinal mucosa is equipped with several weapons. IECs express various recognition molecules such as the **pattern recognition receptor** (PRR). These receptors recognized a broad spectrum of bacteria or microorganism structures, known as **pathogen/microbial associated molecular patterns** (PAMPs or MAMPs). PRRs are mostly constitutively expressed in innate immune cells including dendritic cells (DC), macrophages, neutrophils and also in IECs. However, these receptors can also be induced by harmful stimuli. To
date, PPRs are classified in four subclasses including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA receptors (cytosolic sensors for DNA) (Loo & Gale, 2011). TLRs and NLRs are the two major classes.

A) Toll-like receptors

TLRs are the most studied PRRs and are the first sensors of pathogens. They are type 1 transmembrane protein including an extracellular domain leucine-rich repeats (LRRs) that mediates the PAMPs/MAMPs recognitions and a cytoplasmic domain Toll-IL-1 receptor (TIR) that activates downstream signaling.

TLRs are classified in 10 family members in human and 13 receptors in mouse (TLR-1-10) and each one detects different PAMPs from pathogens (Kawai & Akira, 2011). The type of cell and location of the receptor is critical to its function. Intracellular TLR-7, -8 and -9 are expressed in the endosome to detect nucleic acids from infiltrated bacteria or virus (Figure 12). On the other hand, TLRs 1, -2, -4, -5, -6 and -10 are expressed at the cell surface and recognize a broad number of cell surface ligands from pathogens (Fukata, Vamadevan, & Abreu, 2009). With the exception of TLR3, upon specific stimulation, TLRs, recruit MyD88 (myeloid differentiation primary-response protein 88), which triggers the signalling pathways of NF-κB or mitogen-activated protein kinases (MAPK), leading to the expression of inflammatory cytokines (Abreu, 2010; Akira & Takeda, 2004).

![Figure 12. Immunity receptors on IECs. TLRs located on the plasma membrane are sensors either by PAMPs or DAMPs. Nevertheless, intracellular receptors including TLR3, -7 and -9 recognize invading viruses or bacteria. The difference between the two NODs is the double amino-terminal CARD present in NOD2. Adapted from (Strober, Murray, Kitani, & Watanabe, 2006)](image)

B) NOD-like receptors

NLR recognize a wide range of pathogen ligands within the cytoplasm of the cells. NLRs consist in three domains: the LRRs domains recognizes microbial products, a central nucleotide-binding effector domain termed NACHT (or termed NBD) and a N-terminal region that consists of death effector domain (DED) pyrin domain (PYD), caspase recruitment domain (CARD), or baculovirus inhibitor repeat (BIR) domain region for downstream signalling (Proell, Riedl, Fritz, Rojas, & Schwarzenbacher, 2008). NLRs protein family consist in 22 members, classified depending on the N-terminal domain (DED, PYD, CARD or BIR). The first NLRs described to monitor the cytosol were NOD1 (Nucleotide-binding oligomerization domain 1) and NOD2
(Nucleotide-binding oligomerization domain 2) (Figure 12). NOD1 and NOD2, encoded by the gene CARD4 and CARD15 respectively, are intracellular pattern-recognition molecules involved in the recognition of the core structures of bacterial peptidoglycan (PGN). NOD1 is triggered by d-glutamyl-meso-diaminopimelic acid (DAP), found in a Gram-negative and some Gram-positive bacteria, while NOD2 recognizes the muramyl dipeptide (MDP), widely expressed among both Gram-negative and -positive bacteria (Yoon, 2012). NOD1 and NOD2 share the common regions NACHT and LRR, in contrast, NOD1 N-terminal effector consist in a single CARD domain whereas NOD2 incorporate two (Figure 12).

Upon stimulation of specific bacteria motifs, NATCH domain oligomerizes leading to the interaction of CARD-CARD-containing kinase RIPK2, which in turn, initiates the recruitment of intramolecular adaptors. Once this recruitment is accomplished, downstream signalling by nucleus translocation of the cytosolic NF-κB takes place. In addition, stimulation of NOD1 and NOD2 can lead to the activation of MAPKs such as p38, extracellular signal-regulated kinases (ERK) and c-Jun amino-terminal kinases (JNK) (Kelsall, 2005; Kobayashi et al., 2002). Both signaling pathways lead to the translation of genes involved in inflammation.

C) Inflammasome

Inflammasome is a multi-protein complex located in the cytoplasm composed by NLR family members or absent in melanoma 2-like receptor (AIM2)(Med, 2016). When they sense PAMPs or DAMPs stimulus, NLR or AIM2 oligomerize triggering downstream signalling cascade including the proteolytic cleavage of pro-IL1 family cytokine to its mature form leading to cell death (Kumar, Kawai, & Akira, 2011; Med, 2016).

D) GALT, gut-associated lymphoid tissue

GALT consists of isolated lymphoid follicles present in all segments of the small and large intestine, or regrouping to form Peyer's patches (PPs) along the small intestine. PPs are considered as the immune sensors of the gut since they are able to discriminate between harmful antigens and commensal bacteria. GALT hold specialized cells termed M (microfold), which are able to transport luminal antigens and pathogens towards the underneath immune cells. Immune cells regulate the immune response with either tolerance or inflammation response (Jung, Hugot, & Barreau, 2010).
Chapter 3

Inflammatory Bowel Disease

Content at a Glance

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Ulcerative Colitis
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  - Age and gender disparity
  - Geographical distribution
Aetiology
  - Genetic factors
  - Environmental factors
Treatments
  - Pharmaceutical treatments
  - Surgery
  - Faecal Microbiota Transplantation
1. General Introduction

Inflammatory Bowel Diseases (IBD) are chronic idiopathic inflammation of the gut. The most common forms include Crohn’s disease (CD) and ulcerative colitis (UC). UC pathogenesis is characterized by a superficial contiguous inflammation of the mucosa and sub-mucosa surface extending proximally from the rectum, but always confined to the colon. Unlike UC, CD is typically defined by a transmural inflammation that might affect any part of the GI tract from mouth to anus, although often the inflammation is located in the terminal ileum and the colon (Van Assche et al., 2010).

IBD are complex diseases involving environment and genetic factors. It is believed to occur in genetically predisposed individuals whom immune response to intestinal microbes is abnormal after being exposed to specific environmental factors (Siegel et al., 2016). More than 163 loci are known to confer susceptibility to IBD. Nevertheless, genetic mutations are not enough to explain the increased IBD prevalence in developed countries, evidencing that environmental factors play also a crucial role in IBD pathophysiology (Abraham & Cho, 2009; Lees, Barrett, Parkes, & Satsangi, 2011).

The incidence rates of UC and CD differ geographically. The highest incidences of IBD have been reported in Westernized countries including North America, northern Europe and Australia. IBD have emerged in previously low-incidence nations, such as in the Eastern Europe and Asia, a rise of incidence has also been observed in the pasts years (Jacques Cosnes, Gowerrousseau, Seksik, & Cortot, 2011). This incidence increase may be linked to rapid socio-economic changes transforming the societies. Some examples are the changes from agricultural to manufacturing industry, the urban lifestyle, the diet or the increased pollution (Molodecky et al., 2012).

2. Crohn’s Disease

CD is a heterogeneous, relapsing inflammation condition present throughout the entire GI tract, from the mouth to the anus. The most common locations affected are the colon and the terminal ileum. From the oral to mid-ileum, the affected portions are less than 5% of cases (Artide, 2004; Laube et al., 2017). It is a heterogeneous disease comprising different and complex phenotypes depending on the age of onset, the location of the inflammation and the symptoms of the disease.

The diagnosis is based on a combination of clinical medical history, medical examinations, symptoms, serologic tests, endoscopy and histopathology exams (van Hogezaand, Witte, Veenendaal, Wagtmans, & Lamers, 2001). Quantification of Fecal markers such as calprotectin and lactoferrin are used to determine intestinal inflammation. Because these markers are also increased in UC, they are not used to diagnose the disease, but to monitor the severity of IBD and predict the relapses in patients (Sipponen et al., 2008). Clinical features of CD are persistent diarrhoea, abdominal pain, fever, occasional bleeding, weight loss and fatigue (Chang, Chang, Chang, & Chang, 2017). Complication for the disease course includes colonic ulcerations, known as fistulas and increased risk for colon cancer.

Patients with CD are classified according to the Montreal classification considering the age of onset, disease location and behaviour (table 2) (Satsangi, Silverberg, Vermeire, & Colombel, 2006). CD is featured by a discontinuous and transmural inflammation, most commonly involving the ileocecal area. Anatomical criteria of severity are characterized as deep ulcerations damaging the
muscle layer, or mucosal detachments with also ulcerations limited to submucosa but prolonged to more than one third of a colonic segment (Van Assche et al., 2010).

Vienna and Montreal classification for Crohn’s disease

To solve out the issue of sub-classification of CD by phenotype, the investigators have proposed a disease classification and established a unified clinical, serological and molecular classification of IBD. In 1998, the World Congress of Gastroenterology (WCB), hold in Vienna, claffified CD regarding the age of onset, the disease location and the behaviour of the disease (table 1). In 2005, WCG took place in Montreal, where Vienna classification was slightly modified. Montreal revision has not changed the three predominant parameters, but have made some modifications in each of these categories (table 2) (Satsangi et al., 2006).

3. Ulcerative colitis

UC affects the rectum and a variable extent of the colon involving most of the times only the innermost mucosa. It manifests as continuous portions of inflammation and ulceration and is characterized by atypically relapsing and remitting course (Dignass et al., 2012). The UC inflammation is defined to the mucosal surface, although in severe course of the disease, it may penetrate. It exists different varieties of UC depending on the inflammation location. Disease placed only at the most distal part of the colon and rectum is referred as ulcerative proctitis; inflammation from the descending colon is termed limited or distal colonic left-sided; and disease comprising the entire colon is named pancolitis (Koutroubakis, 2010; Whitlow, 2004).

Mild symptoms of UC include a progressive loosening of the stools, chronic abdominal pain and bloody diarrhoea. UC patients experience a gradual onset of symptoms from mild to severe. Approximately 20% of patients develop at least one severe acute exacerbation, sometimes requiring hospitalization. Severe symptoms comprises, loss of appetite that results in weight loss and lack of nutrients, mucus and blood in the stools, fever and anaemia (Edwards & Truelove, 1963; Head, Jurenka, & Ascp, 2003).

To diagnose UC and exclude other similar diseases such as CD, a patient history is required. Other tests required early in the diagnostic include a complete blood count (CBC) and a fecal occult blood, both use to check intestinal blood loss and anaemia. Colonoscopy or sigmoidoscopy are other techniques used to confirm the disease (Dignass et al., 2012). Patients with Acute and severe ulcerative colitis (ASUC) are diagnosed with bloody stool frequency ≥6 per day, together with any sign of systemic toxicity like tachycardia >90 bpm, temperature >37.8 °C, haemoglobin<105 g/l or an erythrocyte sedimentation rate >30 mm/hm. These patients with severe colitis are often hospitalized (Hindryckx, Jairath, & D’Haens, 2016).

Montreal classification incorporates an assessment of disease extent and severity. Montreal criteria of disease extent of UC define colitis macroscopic disease as proctitis, left-sided and pancolitis (table 3). Montreal classification of severity is characterized as remission. Mild, moderate and severe (table 4). ASUC classification is defined by the Truelove and Witts criteria (Satsangi et al., 2006; Spekhorst et al., 2014).
Table 2. Vienna and Montreal classification

<table>
<thead>
<tr>
<th></th>
<th>Vienna</th>
<th>Montreal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>A1 below 40 y</td>
<td>A1 below 16 y</td>
</tr>
<tr>
<td></td>
<td>A2 above 40 y</td>
<td>A2 between 17 and 40 y</td>
</tr>
<tr>
<td></td>
<td>A3 above 40 y</td>
<td>A3 above 40 y</td>
</tr>
<tr>
<td>Location</td>
<td>L1 ileal</td>
<td>L1 ileal</td>
</tr>
<tr>
<td></td>
<td>L2 colonic</td>
<td>L2 colonic</td>
</tr>
<tr>
<td></td>
<td>L3 ileocolonic</td>
<td>L3 ileocolonic</td>
</tr>
<tr>
<td></td>
<td>L4 upper</td>
<td>L4 isolated upper disease†</td>
</tr>
<tr>
<td>Behaviour</td>
<td>B1 non-stricturing, non-penetrating</td>
<td>B1 non-stricturing, non-penetrating</td>
</tr>
<tr>
<td></td>
<td>B2 stricturing</td>
<td>B2 stricturing</td>
</tr>
<tr>
<td></td>
<td>B3 penetrating</td>
<td>B3 penetrating</td>
</tr>
<tr>
<td></td>
<td>p perianal disease modifier†</td>
<td></td>
</tr>
</tbody>
</table>

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.
†”p” is added to B1–B3 when concomitant perianal disease is present.

Table 3. Montreal classification of extent of UC

<table>
<thead>
<tr>
<th>Extent</th>
<th>Anatomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Ulcerative proctitis</td>
</tr>
<tr>
<td>E2</td>
<td>Left sided UC (distal UC)</td>
</tr>
<tr>
<td>E3</td>
<td>Extensive UC (pancolitis)</td>
</tr>
<tr>
<td></td>
<td>Involvement limited to the rectum (that is, proximal extent of inflammation is distal to the rectosigmoid junction)</td>
</tr>
<tr>
<td></td>
<td>Involvement limited to a proportion of the colon distal to the splenic flexure</td>
</tr>
<tr>
<td></td>
<td>Involvement extends proximal to the splenic flexure</td>
</tr>
</tbody>
</table>

Table 4. Montreal classification of severity of UC

<table>
<thead>
<tr>
<th>Severity</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>Clinical remission. Asymptomatic</td>
</tr>
<tr>
<td>S1</td>
<td>Mild UC. Passage of four or fewer stools/day (with or without blood), absence of any systemic illness, and normal inflammatory markers (ESR)</td>
</tr>
<tr>
<td>S2</td>
<td>Moderate UC. Passage of more than four stools per day but with minimal signs of systemic toxicity</td>
</tr>
<tr>
<td>S3</td>
<td>Severe UC. Passage of at least six bloody stools daily, pulse rate of at least 90 beats per minute, temperature of at least 37.5°C, haemoglobin of less than 10.5 g/100 ml, and ESR of at least 30 mm/h</td>
</tr>
</tbody>
</table>

4. Epidemiology

4.1 Incidence and prevalence

In the latest 19th century, Wilks and Moxon reported, for the first time, the term of UC. Historically, in early 20th century Dr. Burrill Crohn, Dr. Leon Ginzburg and Dr. Gordon Oppenheimer published a research article identifying the two different IBD subtypes: CD and UC (Arora & Malik, 2016; Crohn, Burril. Ginzburg, 1932). Since the middle of the 20th century, IBD rates appeared as a growing problem in the western world and at the turn of the 21st century, prevalence of UC or CD in industrialized countries is up to 0.5% of the total population and the incidence range is from 10 to 30 per 100,000 (Molodecky et al., 2012).
Introduction

**Incidence rate:** Incidence is a measure of probability of occurrence of new cases of a disease in a population within a specific period of time. Incidence is calculated as: number of new cases of a disease in a specified period of time divided by the size of the population initially disease free.

**Prevalence rate:** Prevalence rate measure how commonly a disease or specific condition occurs in a population at a particular point in time (years). The prevalence is calculated by dividing the number of cases with the disease or condition at a given time point by the total number of individuals examined.

The **annual incidence rates of CD** in developed countries are estimated to be: 20.2 per 100,000 person-years in North America, 12.7 per 100,000 person-years in Europe and 29.3 per 100,000 person-years in Australia. In contrast, developing countries like Asia has a low incidence rate of 0.54 per 100,000 person-years (Molodecky et al., 2012; Ng, Tang, et al., 2013; Wilson et al., 2010). In addition, **annual incidence rates of UC are:** from 7.6 to 19.5 per 100,000 person-years in North America, from 1.7 to 13.6 per 100,000 person-years in Europe and from 0.3 to 5.8 per 100,000 person-years in Asia (Burisch, 2014). Whereas in the past, UC had higher prevalence than CD, in the past few decades CD incidence has raised. **Prevalence** of UC in North America is 170–250 per 100,000 person-years and 43–294 per 100,000 person-years in Europe. For CD the prevalence in North America are 25–300 per 100,000 person-years (Bernstein et al., 2006; Pinchbeck, Kirdeikis, & Thomson, 1988).

**4.2 Age and gender disparity**

Despite the fact IBD can occur at all ages, the onset peak of age is generally between 20-30 for CD and 30-40 for UC. Some studies have reported a second peak of CD occurrence between 60-70 years of age (A. N. Ananthakrishnan, 2015; Burish & Munkholm, 2015). Whilst 7-20% of IBD patients are children, 60-85% are adults, the majority of them being under 40 years of age.

IBD affects with similar proportions males and females. Nevertheless, there is a modest preponderance of CD in females, whereas UC is predominant in males (Russel & Stockbrügger, 1996). On the contrary, in the paediatric population, the distribution of the gender tendency is reversed having more males with CD than females (Auvin et al., 2005).

**4.3 Geographical distribution**

IBD has been characterized as a disease of industrialized nations of the Western world. During the 19th century, countries with higher socioeconomic status experienced a fast shift in agriculture, manufacturing, transportation, diet, urbanization and an increased exposure to hygiene (Gearry, 2016; Kaplan, 2015). These changes lead to chronic immune-mediates diseases, like IBD, to appear. Some studies suggest that IBD is not driven by ethnicity, but rather by the surroundings that fosters us.

IBD occurs in individuals of any ethnic groups, such as African-American, Asia-American or Hispanics whose families have lived in industrialized countries for several generations. Recent studies show that in Asian countries (Japan or Korea), the incidence of IBD is still low, but increasing (Kaplan, 2015; Karlinger, Györke, Makő, Mester, & Tarján, 2000). To highlight this point, the UC incidence in Hong Kong in 1970 was 0.1 per 100,000 and CD was hardly recognized.
In 2000 the incidence of UC was reported from 1.6 to 2.1 per 100,000, and CD caught up almost 1.3 per 100,000 (Zheng et al. 2005; Tan and Goh 2005; Chow et al. 2009). Other new industrialized nations, like Brazil, India, and Turkey, are experiencing similar patterns of increased incidences of UC and CD (Figure 13). This data attributes to a shared environmental factor of origins, together with an improved health care infrastructure to diagnose the disease.

![Map of worldwide prevalence of IBD in 2015](image)

**Figure 13. The worldwide prevalence of IBD in 2015.** Extracted from (Kaplan, 2015)

5. **Etiology**

#### 5.1 Genetic susceptibility

Both distinguished forms of IBD occur in a genetically susceptible individual. High-throughput genotyping strategies, including the meta-analysis of the genome-wide association studies (GWASs), have greatly advanced our understanding and highlight the importance of genetic risk factors in IBD. GWASs have reported 163 genetic risk loci that contribute to the susceptibility of UC or CD (Jostins et al., 2012). Moreover, these studies have also demonstrated a substantial overlap between CD and UC in genetic susceptibility (Budarf, Labbé, David, & Rioux, 2009; Zhernakova et al., 2009). Of these 163 loci, 110 conferred risk to both forms of IBD, whereas 23 loci were unique to UC and 30 loci were unique to CD (McGovern D, Kugathasan S, Cho JH).

New genetic findings from recent GWASs, have identified a variety of pathways implicated in the pathogenesis of IBD, suggesting a diversity of risk-conferring loci. Most of these genes are essential for the maintenance of the intestinal homeostasis including barrier integrity, epithelial restitution, innate and adaptive immune regulation, microbial regulation, reactive oxygen species (ROS), autophagy, endoplasmic reticulum stress (ER-stress) and metabolic pathways crucial for cellular homeostasis (Kohr, Gardet, & Xavier, 2011; Sturm & Dignass, 2008). In 2001, nucleotide-binding oligomerization domain containing 2 (NOD2) was the first susceptibility gene identified for CD associated risk variants. Autophagy-related genes, IRGM and ATG16L1, are genetic variants that confer increased risk to develop CD, highlighting the importance of autophagy and phagocytosis in immune responses in CD pathogenesis. Another pathway that has recently emerged in the pathophysiology of IBD is the unfolded protein response (UPR), induced by ER-stress. ER-stress has been genetically linked with both subtypes of IBD through the candidate gene X-box binding protein 1 splice (XBP1s) (Fritz, Niederreiter, Adolph,
IBD loci genes associated to dys-regulated immune response to microbes and impairing the regulation of the inflammation are **interleukin-23 pathway** (IL-23), **IL-10, STAT** and **JAK2**, involved in both, CD and UC, pathogenesis (Budarf et al., 2009). These loci mutations affect a wide range of biological pathways including autophagy genes, leading to an aberrant elimination of bacteria, and essential pathways genes associated with cellular homeostasis and the regulation of immune system.

**Genome-wide association study (GWAS)**

GWAS is a method that identifies genes involved in human disease. This technique search the genome-wide set for small variations, termed single nucleotide polymorphisms or SNPs, that typically occurs more frequently in people with a certain disease than in people without the disease. At the same time, this study can scan hundred and thousands of SNPs and researchers can highlight genes that may contribute to an individual’s risk of developing a particular disease.

IBD has been reported to be a **polygenic disorder** and population-based studies demonstrated to be **familial** in 5–10% of cases and sporadic in the remainder (Halme et al., 2006). The concordance rate in monozygotic twins is higher in CD (30-40%) than in UC (10-15%), suggesting that non-genetic components, such as the relevant **environmental exposure**, may play a pivotal role in the expression of UC rather than CD (Spehelmann et al., 2008). Furthermore, IBD-disease-associated polymorphisms do not explain the shift in temporal trend analyses of incidence over the past generations (Kaplan, 2015; Kohr et al., 2011). Population-based studies illustrate that IBD susceptibility genes between the Western nations and newly industrialized countries differ. For examples, NOD2 and ATG16L polymorphisms increase the risk of developing CD in European progeny, whereas NOD2 variants in Asia are different than those detected in caucasian and ATG16L1 is not CD-associated. Thus, depending on the interaction gene-environment, individuals with IBD may manifest different phenotypes of UC and CD (Kaplan, 2014; Ng et al., 2012).

### 5.2 *Environmental risk factors*

Although the discovery of genetic variants associated with IBD has progressed rapidly, gene mutation alone is not sufficient to explain the changing epidemiology of CD and UC. Environmental determinants are considered to have a strong role in mediating the risk of IBD, although no single environmental risk has been proven to have a definite causative function (Bernstein 2012). It is believed that those environmental risk factors involved in the onset and/or the course of IBD have an influence on the gut microbiota composition and mucosal immune system including breast-feeding, maternal birth exposure, diet, stress, smoking, antibiotics or vaccination (Danese, Sans, & Fiocchi, 2004; Hrnčířová, Krejsek, Šplíchal, & Hrnčíř, 2014; Sartor, 2010).

**A) Smoking**

Smoking has both positive and negative effects in IBD. Several studies reported that smoking increases the risk of developing CD and exacerbates its clinical course including early surgery and postoperative recurrence. On the other hand, smoking cessation improves prognosis of CD. In
contrast, smoking has a protective effect on UC (J Cosnes, Beaugerie, Carbonnel, & Gendre, 2001; Danese et al., 2004). Over the past decades, Western countries have dropped the prevalence of smoking, for examples in 1980, 40% of the adult population were smokers in UK, whereas in 2013 the number was down to 13%. On the contrary, newly industrialized countries are experimenting a rise on the prevalence of smoking, although the incidence of CD is still low (Giovino et al., 2012). Thus, environmental components of IBD influence population differently depending on the world region. A recent experimental study suggested that 64 polymorphism variants associated with developing IBD were altered by tobacco smoking (Yadav et al., 2017).

**B) Diet**

Given the location of high incidence of IBD, a strong relationship between dietary components and the disease pathophysiology has been considered. The rising rates of IBD in USA and Europe coincided with the expansion of fast food chains, manufactured food and increased used of aluminium foils and antibiotics (Ng, Bernstein, et al., 2013). A French study reported variation in the incidence of CD between the north and south of France, due to difference in dietary components exposure. Dietary patterns in southern regions consist of a high intake of fruits and vegetables, fish, olive oil and wine with a low intake of high-fat animals, alcohol and potatoes. On the contrary, the north of France consumes a “western diet” distinguished by high intake of eggs, potatoes, butter, added-fats and beer (Perrin et al., 2005). Several studies from Europe classify increased sugar intake as the most consistent dietary factor linked to IBD. A Dutch study ranges chocolate and sugary drinks as a likely risk factor for IBD with animal proteins in the third position (Jantchou, Morois, Clavel-Chapelon, Boutron-Ruault, & Carbonnel, 2010; Russel et al., 1998; Sakamoto et al., 1995). The Investigation into Cancer and Nutrition (EPIC) study associated a high intake of linoleic acids (n-6 polyunsaturated fatty acid) with an increased risk to develop UC (Tjonneland et al., 2009). A Japanese study associates the elevated intake of animal protein and dietary fat, specially n-6 fatty acids with less n-3 fatty acid, with the contribution to the development of CD (Shoda, Matsueda, Yamato, & Umeda, 1996).

**C) Medications**

Several medications including oral contraceptives, nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics and postmenopausal hormone therapy have been associated with increased risk of IBD (Ashwin N. Ananthakrishnan et al., 2012; Cornish et al., 2008). Antibiotics are known to alter the gut bacterial composition that contributes to abnormal gut immune response and dysbiosis. The use of antibiotic during the first year of life, known to acquire the commensal microbiota in the new-born, is associated with an elevated risk of developing allergic disease in the future (Farooqi & Hopkin, 1998). Previous studies have analysed the relationship between newly diagnosed IBD patients and antibiotic exposure. CD has been associated with antibiotic exposure in both adult and paediatric patients with IBD (Hildebrand, Malmborg, Askling, Ekbom, & Montgomery, 2008; Shaw, Blanchard, & Bernstein, 2011).

The regular use of NSAIDs, excluding aspirin, seems to be associated with increased incidence of CD and UC (Ashwin N. Ananthakrishnan et al., 2012). A British meta-analysis has associated the use of oral contraceptives agents with risk of CD (Cornish et al., 2008).
D) The hygiene hypothesis

The "hygiene hypothesis", proposed for the first time by Strachan et al. (Strachan, 1989), postulates that due to the industrialization and urbanization of society, children are less exposed to microbes triggering an abnormal host immune response, and leading to autoimmune disease and infections. This "hygiene hypothesis" argues that infections in early childhood protect from developing allergic diseases. Children from westernized countries are protected from lethal infections in early childhood, which in contrast are common in developing countries. This low exposure to severe infection increases the risk of developing allergic diseases (Wills-Karp, Santeliz, and Karp 2001). In a case-control study, Gent et al. have found that CD was more frequent in individuals who had hot-water taps and isolate bathrooms (Gent et al., 1994). Another study from Spain has shown that living in urban areas, high socioeconomic status and high educational levels were risk factors for CD and UC. On the other hand, childhood gastroenteritis and respiratory infections were protective factors (López-Serrano et al., 2010). The new biodiversity hypothesis suggests that decreased contact of people with natural habitats and biodiversity might adversely affect the gut microbiota and its immunomodulatory capacity. This declining biodiversity may contribute to increase the prevalence of allergies and chronic inflammatory disease in urban areas (Hanski et al., 2012).

E) Lifestyle: Stress and exercise

External factors such as psychological stress can trigger gut inflammation through several mechanisms including the autonomic nervous system and the hypothalamus–pituitary–adrenal axis. Both mechanisms lead to the production of pro-inflammatory cytokines, activation of lymphocytes and alteration of gut commensal bacteria and intestinal permeability (Bonaz & Bernstein, 2013). Observational studies have shown a relationship between major life stressors, depression and anxiety with an increased risk of developing IBD. Relapse, surgery, reduced sensitivity to immunosuppressive therapies and hospitalization are factors associated to patients with established IBD and depression or anxiety (Ashwin N Ananthakrishnan et al., 2013; Bonaz & Bernstein, 2013). A German study associates sedentary occupations such as office work, administration and mechanics, with increased risk for developing IBD, whereas manual labour (such as construction, cleaning and maintenance) were linked with a low risk of IBD (Sonnenberg, 1990). Supporting this data, other cohort studies have shown that physical activity is associated with a 44% reduction in risk of CD but no of UC.

F) Appendectomy

Similar to smoking, appendectomy, a surgical operation to remove the appendix, has divergent effects on CD and UC. A case-control study from Sweden reported that patients, younger than 20 years old, who underwent appendectomy, had a lower risk of developing UC. Besides, the same cohort found a long-term elevated risk of CD up to 20 years after the surgery (Kaplan et al., 2008). It is important to highlight that patients who had appendectomy for inflammation and not for non-specific abdominal pain were reported. Studies from Europe and Asia have reported that the appendectomy has a protective effect for the development of UC (Andersson, Olaison, Tysk, & Ekbom, 2001). French and Australian studies investigated that UC patients who had their appendix removed before diagnosis were more unlikely to require immunosuppressive therapy (Radford-Smith et al., 2002) or colectomy (Jacques Cosnes et al., 2002). In an experimental mouse model, appendectomy for appendicitis improved colitis state, although increased the risk of
colorectal neoplasia when appendicitis was not present (Harnoy et al., 2016). However, the association between CD and appendectomy is ambiguous. Several studies have reported an increased risk factor for CD patients who had undergone appendix removal (Andersson, Olaison, Tysk, & Ekbom, 2003; Frisch et al., 2001) although other studies have shown a protective effect or no connection (Reif et al., 2001; Russel et al., 1997). In summary, this apparent contradiction might be explained by the divergent biological mechanism involved in CD and UC.

6. Therapies

Ideally, therapy should be a rapid outcome and have low rate of side effects. The main goals of drug treatment in IBD is to induce maintenance of remission, to achieve mucosal healing, to avoid surgical intervention and to decrease the likelihood of colon cancer as a main consequence of chronic inflammation (Iacucci, De Silva, & Ghosh, 2010). The treatment of IBD includes medical treatment and/or surgical intervention in those patients who do not respond to conservative measures. The primarily therapeutic goal includes normalization of physiological functions and restoring nutritional deficits (water, nutrition and mineral homeostasis), improvement of the quality of life and psychosocial support and protection against aggressive disease. The secondarily therapeutic goal includes stable remission, better prognosis and lower risk of relapse and decrease risk of colon carcinoma (Triantafillidis, Merikas, & Georgopoulos, 2011). As mentioned above, IBD is the result of several combined effects and therefore, each patients displays different clinical picture and different responses to therapy (Schirbel & Fiocchi, 2010).

6.1 Pharmaceutical treatment

Conventional treatments include five major categories including anti-inflammatory drugs, immunosuppressive molecules, antibiotics and probiotics and biologic agents.

A) Anti-inflammatory drugs

Mesalazine or 5-aminosalicylic acid (5-ASA) is considered as a pleiotropic drug since it acts at several steps of the pathological process of IBD. It is used for the treatment of mild to moderate severity of active UC and for maintaining remission. (Baumgart, Vierziger, Sturm, Wiedenmann, & Dignass, 2005). Corticosteroids (CSs) suppress inflammation at the very early onset inhibiting vascular permeability, vasodilatation and neutrophil infiltration (Ito, Chung, & Adcock, 2006). CSs are an effective treatment to induce remission in active CD and UC patients.

B) Immunosuppressives

Immunosuppressives reduce the activation of the immune system by inhibiting proliferation and efficacy of lymphocytes. Immunosuppressant drugs include: Azathioprine and 6-mercaptopurine, Methotrexate, Tacrolimus, Cyclosporin and Infliximab (Triantafillidis et al., 2011).

C) Antibiotics and probiotics

The terminal ileum and the large bowel are the most frequently affected areas in IBD, representing the sites with higher concentration of bacteria. As mentioned above, enteric flora is altered in both
types of IBD (Matsuoka & Kanai, 2015) and therefore the use of antibiotics or probiotics may benefit patients with IBD. Antibiotics are frequently more used in CD than in UC. Several experts have shown that the use of antibiotic as a first-line therapy or in combination with immunosuppressive drugs may be the ideal strategy to treat patients with CD (Greenberg, 2004; Nitzan, Elias, Peretz, & Saliba, 2016).

Probiotics are defined as live non-pathogenic microorganisms which, when administrated to animal or human, improve the microbial balance in the GI tract. They include Saccharomyces boulardii yeast or lactic acid bacteria, like Lactobacillus and Bifidobacterium spp (Federico et al., 2009; Pothoulakis, 2009).

D) Biological agents

TNF-α blockade is a powerful strategy used in both CD and UC. Several anti-TNF-α monoclonal antibodies are available in the United States, including infliximab, adalimumab, and certolizumabpegol. Only the first two are licensed for use in Europe (Triantafillidis et al., 2011). Clinicians recommend anti-TNF-α therapy in patients with moderate to severe CD not responding to conservative treatment and in patients with severe UC refractory to other medical therapies. TNF-α blocking therapy has improved long-term outcomes like symptom management, endoscopic recurrence and mucosal healing and is being recommended for use in the earlier course of the disease.

6.2 Surgery

Anti-TNF-α biologics have revolutionized IBD treatment either as monotherapy or together with immune-modulators. This pharmacological treatment is sufficient to induce remission in patients with moderate-to-severe CD and UC. Despite these new advances, surgery is still necessary in 30-40% of CD patients and 20-30% of UC patients at some point during the course of the disease (Bouguen & Peyrin-Biroulet, 2011; Hancock & Mortensen, 2008). Surgery for severe CD is not curative and patients undergo through resection when conservative treatment are unable to achieve symptomatic control. Unlike CD, surgery in patients with UC is curative and necessary when failure of medical management occurs, severe bleeding and toxic megacolon or perforation.

6.3 Fecal microbiota transplantation

Fecal microbiota transplants (FMT), also termed as a stool transplant, is a therapy to reestablish a normal gut microbiota composition by introducing fecal bacteria from a healthy individual into a diseased patient. The reconstruction of balanced microbiota through FMT has been highlighted as a potential therapeutic strategy for IBD. A meta-analysis from Kassam et al., that used FMT treatment, showed 90% of clinical resolution in patients with recurrent Clostridium difficile infection (CDI) (Kassam, Lee, Yuan, & Hunt, 2013). Another randomized study, conducted by et van Nood al. compared FMT with antibiotics in CDI patients. Cure was achieved in 91% of FMT group, whereas only 31% was observed in the antibiotic group (van Nood et al., 2013). The use of FMT to restore a sustained balance in altered microbiota has proven to be successful in treating recurrent CD. Currently, several phase I trials of FMT for IBD patients are ongoing, expecting to achieve remission and a final treatment for UC and CD (Matsuoka & Kanai, 2015).
Chapter 4

Pathogenesis of IBD

Content at a Glance

Defective physical and biochemical mucosa
- Impaired epithelial junctions
- Impaired Mucus layer
- Impaired AMPs

Altered microbiota

Altered immune response

IBD pathways involved in genetic variants
- NOD2
- Autophagy
- ER-stress
The GI tract is a complex interface that provides physical barrier against luminal environment meanwhile it enables the absorption of dietary nutrients and the exclusion of harmful compounds from the intestinal lumen. The ability to control the passage of molecules throughout the mucosa is defined as the **intestinal barrier function (IBF)**. The first line of defence is the lumen itself where bacteria and antigens are degraded by digestive secretions from the stomach, pancreas and liver (Figure 14). Still within the lumen, **commensal bacteria** also contribute to the defence by producing **antimicrobial substances** and by competing for nutrients (Gerova, 2016). In the intestine, like in many other surfaces, microorganisms are organized as biofilms. Luminal microorganisms are essential to the modulation of the immune host response and their organization in biofilms seems to be important in such effect (Macfarlane, Bahrami, & Macfarlane, 2011). The second line of defence is the **mucus layer**, rich in secreted IgA and AMP, thereby preventing the access of bacteria to the epithelium (Figure 14). The third barrier is composed by the well-sealed monolayer of IECs that avoids toxic molecules and enteric pathogens from entering thought the gut tissue. Finally, the ultimate barrier is the mucosal immune system.

![Figure 14. Structure of the defence lines of the intestinal barrier.](image)

The passage of components from the lumen through the epithelium is achieved by two distinct mechanisms: **paracellular** and **transcellular permeability**. Paracellular passage is controlled by the set of tight junctions and adherens junctions sealing the intestinal monolayer (apical passage of water and small molecules). Large particles such as antigens and microbes use the **transcellular** route to cross the epithelium through enterocytes, dendritic and M cells (Garrett, Gordon, & Glimcher, 2010; Salim & Söderholm, 2011).

Many human diseases including inflammation, metabolic alteration, infection and, neurologic dysfunctions are linked to a deficiency of the IBF. Some of them, as IBD, exhibit alterations on the four IBF’s defense line.

1. **Defective physical and biochemical mucosa**

Dysfunctional gut barrier is a feature of intestinal inflammation. IBD are characterized by an exaggerated immune host response towards commensal microbiota which triggers an
uncontrolled chronic intestinal inflammation. The interactions within the intestinal barrier are altered in IBD context, causing increased intestinal permeability and exposure of luminal content to the immune cells underneath the lamina propria (McGuckin, Eri, Simms, Florin, & Radford-Smith, 2009). Recent genetic studies have demonstrated the relevance of innate intestinal response in IBD aetiology (Coskun, 2014). Defective bacterial and toxic molecules clearance, apical sealing complexes, and innate barrier have been involved in the pathogenesis of IBD (Salim & Söderholm, 2011)

### 1.1 Impaired Epithelial Junctions

It has been demonstrated that patients with both forms of IBD display increased intestinal paracellular permeability which breaks down the physical intestinal barrier and its functions (Michielan & D’Incà, 2015). Several clinical studies reported abnormal distribution and expression of TJs (Lee, 2015; Salim & Söderholm, 2011). A clinical study has shown that patients with CD and UC have a disrupted intestinal barrier function with reduced epithelial resistance (TEER). Occludin, Claudin-5 and -8 were downregulated and re-localized off the tight junction complex, whereas Claudin-2 was strongly upregulated (Zeissig et al., 2007). Another study showed the loss of epithelial JAM-A expression in patients with IBD, concluding that JAM-A is essential for the maintenance of mucosal integrity and permeability (Vetrano et al., 2008). Another study documented that MLCK expression and its enzymatic activity, MLC phosphorylation, are enhanced in IBD with a correlation between increased activity and disease activity. Thus, active IBD patients have a cytoskeletal dysregulation (Blair, Kane, Clayburgh, & Turner, 2006).

Inflammatory cytokines including TNF-α and IFN-γ, which are elevated in the intestine of IBD, are able to modulate the transcription of TJs proteins. Moreover, TNF-α induces apoptosis of enterocytes interfering then with the distribution of TJs that seal the gaps left (Schmitz et al., 1999; Suenaert et al., 2002). IFN-γ or TNF-α might alter paracellular permeability by promoting MLCK and therefore increasing myosin light chain phosphorylation. Inhibition of MLCK reverses increased permeability, indicating an association between inflammatory cytokines and MLCK activation (Zolotarevsky et al., 2002). Neutralization of TNF-α resulted in a marked suppression of gut inflammation and reduced apoptosis of epithelial cells (Marini et al., 2003).

### 1.2 Defective mucus layer

As mentioned above, goblet cells are specialized in the secretion of mucus in the intestine. Reduced number of goblet cells is a pathological feature of UC rather than CD (Figure 15) (Pullan et al, 1994). Despite, most of the hypothesis concerning UC pathophysiology focus on the abnormal immunological response, several papers have investigated the importance of the defective inner mucus layer present in active UC (Johansson, Sjövall, & Hansson, 2013). The thickness of the mucus layers is reduced in patients with UC with increased bacteria adherence in the lining intestinal epithelia and infiltrated bacteria and leukocytes within the mucus of UC biopsies (Swidsinski et al., 2007). Some studies have observed that the lack of MUC2 in mice induces a severe colitis and even cause cancer by altering intestinal crypt morphology and cell maturation and migration (Van der Sluis et al., 2006; Velcich et al., 2002). A study from Chad K. Heazlewood et al. reported that mice with Muc2 mutation present altered Muc2 biosynthesis, decreased stored mucin in goblet cells, increased barrier permeability and enhanced pro-
inflammatory cytokines including IL-1β, TNF-α and IFN-γ in the distal colon. Accumulation of aberrant muc2 biosynthesis triggers ER-stress and UPR in goblet cells. Despite this, inflammation, apoptosis and wound repair are observed in both inflamed and non-inflamed colonic tissues of MC2-deficient mice (Heazlewood et al., 2008). Together, these findings highlight aberrant mucus layer as the main issue in patients with UC rather than hyperactive immune system.

Figure 15. Normal gut vs IBD gut. Healthy gut (left) and impaired mucosal barrier in patients with IBD (right). Intestinal mucosa of IBD is characterized by metaplastic Paneth cells, decreased number of goblet cells, impaired seal between IEC, and deregulation of AMP, decreased mucus layer and therefore, pathogens invasion and chronic inflammation. Adapted from (Michielan & D’Incà, 2015).

1.3 Defective antimicrobial peptides

A particular feature of the pathophysiology of CD is the reduced expression of α-defensins and dysregulated expression of β-defensins (Gersemann, Wehkamp, & Stange, 2012). It has been reported that patients with IBD have metaplastic Paneth cells, normally restricted to the small bowel, re-localized in the colonic mucosa with altered function. Thus, secretion of α-defensins along the colon is found in patients with CD (Cunliffe et al., 2001; Müller, Autenrieth, & Peschel, 2005). In healthy individuals, Paneth cells produce large amounts of HD5 and HD6 (α-defensins) to protect the mucosal epithelium against harmful pathogens. Some research groups have demonstrated that the production of α-defensins in ileal CD is deficient, although it is still controversial whereas the number of Paneth cells is affected (Elphick, Liddell, & Mahida, 2008; Simms et al., 2008). Deficient α-defensins results in an aberrant antimicrobial shield reducing its antibacterial killing capacities and, therefore, being more susceptible to inflammation (table 5)(L.Zhang, R.Gallo. 2016).

Unlike the reduction of α-defensins in patients with CD, β-defensins HBD1–3, secreted by goblet cells in the colon, are found increased in patients with both forms of IBD. Although goblet cells are specialized in the secretion of mucus in the intestine and Paneth cells are the main producers of AMP, goblet cells can also produce antimicrobial factors (Antoni, Nuding, Wehkamp, & Stange, 2014).
Mucosal **HBD1**, normally constitutively expressed, is notably **reduced** in colonic biopsies of CD and UC patients (Jan Wehkamp et al., 2003). On the other hand, **HBD2** and **HBD3** are largely **increased**, especially in **inflamed colonic mucosa** of patients with UC (table 6) (Jan Wehkamp et al., 2002, 2003). **RELMβ** is strongly expressed in the lumen of the ileum mice treated with Dextran sodium sulphate (DSS). Although the molecule does not alter mucosal barrier function its gene deletion reduces the severity of colitis in mice model (McVay et al., 2006). **REGIII** is also found overexpressed in colitis model initiated by DSS (Ogawa et al., 2003). REG family is found increased in gastric and colorectal cancers (Zheng et al., 2011).

The AMP **cathelicidins** (hCAP18/LL-37) are found significantly increased in both inflamed and non-inflamed colonic tissue of UC patients but not CD (Schauber et al., 2006) whereas serine proteases inhibitors including **Elafin** and **secretory leukocyte protease inhibitor** (SLPI), which also act as AMPs, are enhanced strongly only in inflamed colon of UC (Schmid et al., 2007).

**TFF3**, secreted together with MUC2 by goblet cells, is involved in the viscosity of the mucus. Increased presence of TFF3 in the mucus leads to enhanced viscosity of the mucus, suggesting an association of TFF3-mucin to regulate the mucus gel (Kanai, Mullen, & Podolsky, 1998). Although TFF3-lacking mice do not develop spontaneous colitis, they demonstrated an increased susceptibility to DSS-induced colitis. These findings suggest that mucus quality is important to protect against colitis (Mashimo, Wu, Podolsky, & Fishman, 1996; McGuckin et al., 2009).

**Dextran Sodium sulphate (DSS)**

Dextran Sodium sulphate is widely use to induce colitis in mouse model to mimic UC and CD pathophysiology. DSS is administrated in the drinking water and after 7 days the animal develops an acute or chronic inflammation in the intestine. Although the mechanism of action of DSS is not yet clear, it seems that the detergent damages the mucosal epithelium of the large intestine enhancing the intestinal permeability and allowing the dissemination of luminal content into the inner body (Sambasivarao, 2013).

Taking all this into account, IBD patients exhibit a depressed intestinal barrier associating a disruption of the IEC barrier integrity and a continuous leakage from the lumen of the intestine to the inner body, which leads to a permanent inflammation of the gut.

**Human Antimicrobial Factors**

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Intestinal location</th>
<th>Cellular location</th>
<th>Changes in IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD5</td>
<td>Ileum</td>
<td>Paneth cells</td>
<td>Reduced in ileal CD</td>
</tr>
<tr>
<td>HD6</td>
<td>Ileum</td>
<td>Paneth cells</td>
<td>Reduced in ileal CD</td>
</tr>
<tr>
<td>hBD1</td>
<td>Colon</td>
<td>Epithelial and inflammatory cells</td>
<td>Reduced in CD and UC</td>
</tr>
<tr>
<td>hBD2</td>
<td>Colon</td>
<td>Epithelial and inflammatory cells</td>
<td>Increased in UC</td>
</tr>
<tr>
<td>hBD3</td>
<td>Colon</td>
<td>Epithelial and inflammatory cells</td>
<td>Increased in UC</td>
</tr>
<tr>
<td>RELMβ</td>
<td>Colon</td>
<td>Goblet cells</td>
<td>Increased in DSS model</td>
</tr>
<tr>
<td>REGIII</td>
<td>Paneth cells</td>
<td></td>
<td>Increased in DSS model</td>
</tr>
</tbody>
</table>
Elafin
Colon
Epithelial and inflammatory cells
Increased in inflammatory cells
Decreased in epithelium

SLP1
Colon
Epithelial and inflammatory cells
Increased in UC

Cathelicidins
Colon
Epithelial and inflammatory cells
Increased in UC

Table 5. AMP involved in IBD. Adapted from (Gersemann et al., 2012)

2. Immunological Factors

Available evidences confirm that the aberrant innate and adaptive immune pathways contribute to the intestinal inflammation in IBD patients. The colonic epithelium lies close to a high density niche of diverse microbes with a continuous network of interactions. Intestinal immune system controls against harmful pathogens at the same time that it allows immune tolerance to resident commensal microbiota. Perturbation of this balance leads to intestinal inflammation and seems to predispose humans to develop IBD (Abraham & Medzhitov, 2014). Immunological studies have focused on the innate immune mucosal response including the epithelial barrier integrity, innate bacterial sensing, autophagy and UPR (Y. Z. Zhang & Li, 2014). Experimental evidences from in vitro, in vivo and human studies suggest that several pathways may influence with inflammatory cascades in patients with IBD.

IBD patients exhibit a disturbed gut innate immune mechanism. The innate immune response is not specific to a particular pathogen and allows the host to mount a quick response to aggression, within minutes or hours. This type of immunity responds to recognition of microbial antigens mediated by TLRs and NOD-like receptors (Meeting et al., 2000). In healthy conditions, PRRs in colonic mucosa are low but constitutively expressed, located basolaterally, to prevent the interaction with luminal microbes (Yamamoto-Furusho & Podolsky, 2007). Genetic studies have evidenced that several innate immune genes are involved in the pathogenesis of IBD, including NOD2 and TLR2 and -4 (Hausmann et al., 2002; Hugot et al., 2001).

A decade ago, Franchimont et al. reported that TLR4 Asp299Gly polymorphism was associated with both UC and CD (Franchimont et al., 2004). TLR4- and myD88-deficient mice had altered mucosal healing and disturbed barrier function after DSS administration (Rakoff-Nahoum, Hao, & Medzhitov, 2006), suggesting that TLR4 signalling pathway is involved in the initiation of intestinal inflammation. A British study shows that mRNA levels of TLR4 are higher in inflamed colonic mucosa than in non-inflamed controls in active disease of both IBD types (Hausmann et al., 2002; Levin & Shibolet, 2008). In healthy colonic epithelium TLR4 expression is found in small amounts on IEC, maintaining a basal state of activation. Upregulation of TLR4 and its accessory molecules, CD14, LBP, and MD-2, leads to increased inflammatory cytokines such as IFN-γ and TNF-α both known to be present in active IBD (Abreu et al., 2002; Nagai et al., 2002).

To date, TLR2 is the only TLR able to directly modulate the complex network of epithelial TJ in the intestine. Several studies conducted by E. Cario et al. have shown that stimulation of TLR2 protects TJ integrity and enhances TEER of IECs through apical redistribution of ZO-1. In TLR2-deficient cells, inflammatory stress stimulus, induced TJ-loss and decreased integrity of the barrier (Ey, Eyking, Gerken, Podolsky, & Cario, 2009). In vivo studies demonstrated that TJ disruption together with anti-apoptosis failure was observed in TLR2- and MyD88-KO mice under inflammatory stress. Stimulation of TLR2 through its ligand PCSK, protects IECs barrier integrity.
and decreases intestinal permeability, improving the clinical signs in acute DSS colonic inflammation in mice (E. Cario, Gerken, & Podolsky, 2007; Elke Cario, 2010).

In contrast, other studies demonstrated that TLR-2 and/or -4 ligands are able to increase paracellular permeability and expression of inflammatory cytokines including TNF-alpha, IL-1β and IL-8, thereby leading to chronic inflammation in mice (Hedl, Li, Cho, & Abraham, 2007). However, MDP (Nod2 agonist) stimulation reduced TLRs agonist effects, inducing intestinal permeability normalization, reduced cytokines expression and weakening of colonic inflammation. Nod2 mutation or deletion prevented the protective effect of MDP. This protective effect was described to be achieved by blocking the nuclear translocation of NF-kB induced by TLRs pathways (Barreau et al., 2010; T Watanabe, Kitani, Murray, & Strober, 2004).

3. The role of microbiota

3.1 Commensal microbiota

Microorganisms inhabit in our skins, noses, mouths, gastrointestinal tracts and genital tracts and build a magnificent symbiotic ecosystem within our body. In humans, over $10^{12}$ colony-forming units (CFU) of commensal microorganisms populate our GI tract. The three domains of life including Arche, Bacteria, and Eukarya and viruses are present within the GI microbiota (Donaldson, Lee, & Mazmanian, 2015). About 1-2 Kg of microbes constitutes the most complex ecosystem in our body. This microbiota not only helps nutrient and drug metabolism but also plays a pivotal role in preventing pathogenic colonization and intestinal homeostasis (Rajilić-Stojanović, 2013).

Among the four predominant bacteria phyla, Firmicutes, Bacteroides, Proteobacteria and Actinobacteria present in the healthy human large intestine, the Gram-positive Firmicutes and Gram-negative Bacteroidetes are the most abundant (Maria Gloria Dominguez-Bello, Blaser, Ley, & Knight, 2011). The interplay between gut commensal flora, IECs and innate and adaptive immune cells play a crucial role in maintaining the homeostasis of the intestine preventing inflammation or immune-mediated disease (Maynard, Elson, Hatton, & Weaver, 2012).

Along the GI tract, the diversity in number and phyla of microbes is due to the acidic pH coming from the gastric, the bile acid and the level of oxygen (O’Hara & Shanahan, 2006). Stomach and small intestine possess an acid habitat along with high levels of oxygen and a wide range of antimicrobial factors that roughly shape the composition of the microflora. Moreover, the bacteria in the small intestine get washed down very quickly, in contrast with the colon, where transit is slower and bacteria can adhere to the mucus. Therefore, the gut flora in the small gut is lower than in the colon and it is composed by facultative anaerobes bacteria that tolerate the acidity in addition of the ability to compete with other bacteria (Figure 16) (Donaldson et al., 2015). The microbial microbiota is shaped differently every day depending on the diet, occasional infections, or the uptake of drugs such as antibiotics (Tremaroli & Bäckhed, 2012).

The neonate gut is sterile and the colonization begins right after birth, influenced by the manner of delivery, diet and hygiene (Grölund, Lehtonen, Eerola, & Kero, 1999). C-section or natural birth influence the microbiota’s development, which might contribute to modifications on the normal gut physiology. Intestinal bacteria are essential to establish a mutualistic relationship within the gut and influence the energy balance of the body, drug metabolism, selection of pathogenic strains and maturation of the immune system (M. G. Dominguez-Bello et al., 2010). Beneficial
microbiota plays a crucial role on the development and function of a tolerant immune system and avoids overgrowth of opportunistic pathogens. Commensal microbiota has the potential role to induce pro- and anti-inflammatory responses shaping the composition of the bacterial communities and modulating the proper function of the immune system. One of the other ways the gut immune system uses to verify the overgrowth of pathogenic population is by producing immunoglobulins (Ig) such as IgA. Some bacteria, especially Gram-negative, activate resident DC which, in turn, activate plasma cells to secrete IgA in the intestinal mucosa (He et al., 2007). The gut immunomodulation is then perfectly orchestrated with both the innate and adaptive immune system. The immunomodulatory components that participate in the intestinal protection are; the GALT, regulatory and effector T cells, secretory plasma cells IgA and macrophages and dendritic cells residents in the lamina propria.

Figure 16. Composition of the intestinal microbiota. The environment becomes gradually anaerobic along the intestine and up to 99% of the flora in the colon is anaerobic. Extracted from (Donaldson et al., 2015).

The intestinal microbiota is involved in many important functions in the GI tract. The normal commensal flora produces essential nutrients for the host. For example, bacteria are involved in numerous metabolic activities, such as fermentation of carbohydrates, use of nitrogenous substance and biotransformation of bile acid and other steroids, which provide an important energy source for the host (Bäckhed et al., 2012; Macfarlane, Macfarlane, & Durand, 2003; Rajilić-Stojanović, 2013). Short chain fatty acids (SCFA), such as butyrate or acetate, are the result of intestinal fermentation, which are absorbed by the enterocyte and used as energy. Moreover, as mentioned above, bacteria synthesize essential vitamins such as vitamin K and B, which cannot be produced by the host. The intestinal microflora is also involved in lipid and protein metabolisms. Recent studies have also shown the ability of intestinal flora to metabolize xenobiotics and drugs (Clayton, Baker, Lindon, Everett, & Nicholson, 2009).
3.2 Microbiota in IBD

Dysbiosis is defined by an abnormal composition of the microbiota mainly described to be related to an abnormal ratio between beneficial and harmful bacteria. Dysbiosis breaks down the host-bacteria mutualism and is likely one of the defining issues in the development of IBD (Hold et al., 2014). Recent metagenomics studies suggest that not only the quantity of microbiota is reduced but also the quality of microbial composition is modified, with a reduction of Firmicutes and Bacteroidetes (Comito & Romano, 2012; Scaldaferri & Fiocchi, 2007). Among the phylum Firmicutes, the reduced presence of the *Fecalibacterium prausnitzii* species has been well reported in patients with CD in contrast to healthy controls. This bacteria might be essential to maintain colonic homeostasis since its reduction decreases the protection of the gut mucosa (Sokol et al., 2009).

Bacteria that belongs to Proteobacteria are known to be the most pathogenic phylum in humans. They are found increased in patients with IBD. This interesting shift might be a key factor involved in the pathophysiology of the disease, suggesting that Proteobacteria play a harmful role in the initiation and/or the maintaining states of chronic inflammation in IBD patients (Mukhopadhyya, Hansen, El-Omar, & Hold, 2012). It is well documented that patients with IBD have increased levels of *Escherichia coli*, especially the pathogenic variant **Adherent/Invasive E. coli** (AIEC) attached at the surface of the ileal epithelium (Darfeuille-Michaud et al., 2004). In addition, quantities of the molecular **enterotoxin B. fragilis** expressed by *Bacteroides fragilis* has been found altered in patients with active IBD (Prindiville et al., 2000). There is also an alteration of microbiota composition between inflamed areas and non-inflamed mucosa associated with a deficit in function and production of AMP such as defensins (Matricon, Barnich, & Ardid, 2010).

The disturbance of commensal microbiota in the pathophysiology of IBD also affects the production of bacteria metabolites. CD and UC patients display a decreased production of SCFA, which is mainly produced in the lumen of the colon. Besides being a major source of energy for IECs, SCFA are known to exert anti-inflammatory effects (Thibault et al., 2010).

**Biofilms** are composed by a group of microorganism, attached to a surface, embedded together with an extracellular matrix named extracellular polymeric substances (EPS). Biofilms are largely found in the lower GI tract, playing an important role digesting substances (Macfarlane et al., 2011). Biofilms are formatted by two phases: the **adhesion phase**, where bacteria adhere to a substrate surface, and the **maturation phase** where bacteria proliferate and differentiate. Depending on the genetics of each microorganism specie, the biofilms display the ability to disseminate or increase biofilm formation. Moreover some biofilms-derived form might get resistant to environmental stress such as antibiotic treatment, leading to chronic infections (Srivastava, Gupta, Kumar, & Kumar, 2017). Parket et al. shown in 2003 the thigh relationship between pathogenic biofilm formation and inflammatory disease such as IBD (Parsek & Singh, 2003). Some studies focus on bactericidal and **antibiofilm** therapies to treat IBD.

4. Genetic polymorphism involved in IBD pathophysiology

As mentioned in chapter 3 section 5.A, genetic predisposition and environment factors are the two main imputs favouring the development of IBD. Studies of the genetic loci implicated in IBD reported that different pathways, essential for the maintenance of intestinal homeostasis, are
disrupted. This section focuses on the three main pathways involved in IBD including NOD2, autophagy and ER-stress (Liu & Stappenbeck, 2016).

4.1 NOD2

A) NOD2 mechanism of action

In healthy conditions, NOD2, encoded by the CARD15 gene, recognizes muramyl dipeptide (MDP)-mentioned in chapter 2 section 3.2 B-, a fragment of peptidoglycan (PGN) found in the cell walls of both Gram-positive and negative bacteria. Upon ligand sensing, NOD2 undergoes auto or hetero-oligomerization with Nod1 and/or other Nod2 receptors, which, in turn, activate the downstream adaptor molecule RIP2 (also termed RICK). This NOD2-RIP2 interaction seems to be essential for the induction of cytokines, since mice carrying RICK mutation do not respond to MDP stimulation (Henckaerts & Vermeire, 2007; Park et al., 2007). Activation of RIP2 recruits and activates TAK1 complex (TAK1-TAB2-TAB3), which leads to activation of \( \text{IkB kinase (I\kappa k)} \) complex. I\( \kappa k \) phosphorlylates and degrades NF-\( \kappa B \) inhibitor (I\( \kappa B \alpha \)) which activates NF-\( \kappa B \) (Philpott, Sorbara, Robertson, Croitoru, & Girardin, 2014). On the other hand, NOD2 activates also MAPK signalling pathway thought the regulation of ERK1, ERK2, JNK and p38 (W Strober & Watanabe, 2011). Both signalling pathways NF-\( \kappa B \) and MAPK trigger pro-inflammatory signaling cascade including the expression of TNF-\( \alpha \), IL-6, IL-8 and some defensins (Fritz et al., 2005; Rubino, Selvanantham, Girardin, & Philpott, 2012).

PGN is also recognized by TLR-2, and like NOD2, TLR2 activation results in NF-\( \kappa B \) activation and therefore induction of pro-inflammatory cytokines (Borm, van Bodegraven, Mulder, Kraal, & Bouma, 2008). Because TLR2 and NOD2 are activated by PGN targeting NF-KB, both signalling pathways might coordinate pathogen responses. Watanabe et al have shown that in physiological conditions, NOD2 inhibits TLR-2-driven activation of NF-\( \kappa B \), to control colonic inflammation induced by commensal microbiota (NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 response). Cells depleted in TLR2 do not respond to PGN stimulation leading to a failure of PGN-induced cytokine production. Macrophages lacking NOD2 stimulated with PGN produced more IL-12 than NOD2-sufficient macrophages. To evidence a possible cross-regulation between TLR2 and NOD2, APC cells with and without NOD2 were co-stimulated with PGN and MDP. NOD2-sufficient cells reduced the production of IL-12, meaning that NOD2 might regulate negatively PGN-induction (Tomohiro Watanabe, Kitani, Murray, & Strober, 2004). Although it has not been corroborated yet, speculation is that in the absence of NOD2, PGN induces the activation of NF-\( \kappa B \) producing large amounts of pro-inflammatory cytokines. In contrast, MDP downregulates PGN-mediated signaling through TLR2, regulating inflammation against microbiota (Warren Strober, Murray, Kitani, & Watanabe, 2006).

Besides the regulatory role of NF-\( \kappa B \) induction, NOD2 is able to act as a bacterial killer, it induces cytokines and stimulates and maturates antigen-presenting cells (APC), thereby regulating the adaptive immune response (Philpott & Viala, 2004).

B) NOD2 in CD

In genome-wide-associated studies from the Human Genome Project, three NOD2 polymorphisms Arg702Trp, Gly908Arg, and Leu1007fsInsC, were found associated to a genetic
risk factor for CD. These three mutations are located in the LRR domain affecting the recognition and the fixation of MDP (Figure 17) (Hugot et al., 2001; Ogura et al., 2001).

**Figure 17. NOD2 protein.** Within LRR region, triangles indicate an amino acid change due to a CD associated polymorphism. Adapted from (Philpott et al., 2014).

CD is a consequence of an excessive response to commensal organisms leading to mucosal immune system reactivity and increasing Th1-cell mediated inflammation. Although the mechanism by which NOD2 mutation influences the development of CD is poorly understood, CD-associated NOD2 polymorphisms lead to a loss of function of MDP-downstream pathways, promoting gut inflammation and compromising the ability to control gut microflora (Chamaillard et al., 2003; Mondot et al., 2012).

Two different hypotheses have been suggested to explain the possible association of NOD2 with CD susceptibility. The first model is that in the absence of functional NOD2 protein, TLR2 and/or TLR4 are not negatively regulated and this leads to an excessive NF-κB response and production of inflammatory cytokines by innate and adaptive immune cells, which in turn, drives gut inflammation (Becker et al., 2003; Macpherson & Harris, 2004). Besides, IECs NOD2 polymorphisms have been associated with a reduced capacity to produce AMPs, altering this line of host defence. Moreover, CD patients displayed a reduction of α-defensin-5 and -6 in the ileal mucosa, especially in those patients with NOD2 polymorphisms (J. Wehkamp et al., 2004). It has been shown that Paneth cells deficient for NOD2 have a decreased mRNA expression of α-defensin. Moreover both WT and NOD2−/− mice challenged with *L. monocytogenes* are susceptible to liver infection, although knock-in NOD2 mice exhibit severe bacterial infection (Kobayashi et al., 2005b). Nonetheless, these data are contradicted by some other research articles showing that deficient α-defensin production is not associated with NOD2. For instance, J. Robertson et al. demonstrated that NOD-2-deficient mice did not exhibit any modification in bacterial communities or the expression of immune signs (Robertson et al., 2013). However, another study contradicted the previous one and showed that *Nod2*-mutated mice, mimicking CD mutation in NOD2, present a gain-of-function that leads to increased IL-1β (Maeda et al., 2005). However these results cannot be reproduced neither in human studies using macrophages or peripheral blood mononuclear cell (PBMC), nor in animal studies (Inohara et al., 2003; J. Li et al., 2004).

### 4.2 Autophagy

**A) Basal autophagy**

Autophagy is a self-degradative process important for cell survival and that regulates the source of energy at critical moments in response to nutrient starvation. It also plays a role in removing misfolded proteins, clearing damaged organelles, it breaks down intracellular pathogens and processes them for APC (Glick, Barth, & Macleod, 2010). A basal level of autophagy is needed for the cells to adapt to their environment, and to exert surveillance in order to maintain tissue...
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**homeostasis** (Mizushima, Levine, Cuervo, & Klionsky, 2008). Autophagy is a crucial feature in the maintenance of gut homeostasis. The intestine is continually challenged by physical and chemical stressors. While maintaining an accurate balance between host tolerance and microbial loads, autophagy induced by internal and external inputs, provides control and adaptation to cells under cellular stress. Autophagy can be divided in three different types, all of them leading to intracellular proteolytic degradation: macro-autophagy, micro-autophagy and chaperone-mediated autophagy.

**Macroautophagy** refers to non-selective intracellular degradation and it consists in four different steps including: initiation, nucleation, elongation and fusion. A double membrane-bound vesicle engulfs random cargo followed by elongation and maturation of the vesicle into autophagosome. Orchestrated by several number of autophagy genes (ATGs), autophagosome merges with the lysosome driving the cargo to degradation (Figure 18) (Glick et al., 2010). The autophagy gene **ATG16L1** (autophagy-related 16-like 1) plays a crucial role in the stabilization of the autophagosome. Together with ATG12-ATG, the complex associates with the outer member of the autophagosome generating from associated protein 1 light chain 3 (LC3)-I to LC3-II and thereby supporting the elongation and maturation of autophagosome (Figure 18) (Fujita et al., 2008; Mizushima et al., 2001).

Unlike regular autophagy, **micro-autophagy** is a non-selective lysosomal degradation by which cytosolic cargo is taken up straight by the lysosome via invagination of its own membrane (W. W. Li, Li, & Bao, 2012). In **chaperone-mediated autophagy** (CMA), substrate proteins are carefully translocated into the lysosomal lumen through a translocation complex of chaperones located in the lysosomal membrane. CMA involves a selective timed degradation of specific proteins with a regulatory cell function (Cuervo & Wong, 2014).

**Figure 18. Autophagy mechanism.** Autophagy is a multi-step process which includes initiation, formation of autophagosomes, a double membrane-bound vesicle that engulfs and delivers cytoplasmic material to lysosomes, for digestion, maturation, and degradation. Adapted from (Tomoya Iida, Kei Onodera, 2000)

**B) Autophagy Variants as risks factor for IBD**

Genome-wide association studies have identified variants in autophagy genes **ATG16L1** and **IRGM** (immunity-related GTPase family M) as genetic risk factors for CD, involving autophagy pathway in the course of the disease (Lassen et al., 2014; Parkes et al., 2007).
GWA approaches have identified a threonine-to-alanine substitution (T300A) in the autophagy gene \textit{ATG16L1} associated with increased risk of developing CD but not UC. To date, 58.1\% of CD patients carry this variant, particularly patients with ileal CD (Wellcome trust consortium Case, and Consortium 2007; Prescott et al. 2007). CD patients, homozygous for ATG16L1/Ala300 variant, present “loss-of-function” autophagy due to the impairment of autophagosome formation which, in turn, displays an altered capacity to handle and clear cytoplasmic content such as bacteria (Muzes, Tulassay, & Sipos, 2013). Human epithelial cells coding ATG16L1 variant show aberrant engulfment and degradation of internalized \textit{Salmonella} within the autophagosome (Kuballa, Huett, Rioux, Daly, & Xavier, 2008). Another study shows that ATG16L1 mutant intestinal cells exhibit a deficiency for pathogenic adherent-invasive \textit{Escherichia coli} (AIEC) clearance. This pathogen colonizes ileal lesions of CD patients, compared to control cells which are able to control the replication of the pathogenic bacteria (Bedran-Russo, Karol, Pashley, & Viana, 2013). Moreover, hypomorphic mice ATG16L1/T300A risk allele (ATG16L1\textsuperscript{HM} mice), Paneth cells exhibited abnormalities in their granules of secretion containing AMPs. Its aberrant phenotype includes, fewer and disorganized granules with a disrupted granule exocytosis, decreased number of lysozyme and increased transcription of inflammatory mediators (Cadwell et al., 2008). The same study reports that patients with CD homozygotes for the ATG16L1 risk variant display similar impaired structure in Paneth cells than those observed in ATG16L1\textsuperscript{KO} mice. Also, impaired autophagy is associated with nucleation and elongation of autophagosome in goblet cells, driving a deficiency in mucus secretion (Patel et al., 2013). IRGM is the second autophagy-related gene identified in a genome-wide association study associated with CD. A single nucleotide polymorphism exhibits a decreased expression of IRGM and decreased capacity of autophagy to clear up intracellular bacteria (Mccarroll et al., 2009). Brest et al. have demonstrated that mutated IRGM alters autophagy efficiency compromising the intracellular control of bacteria invasion, leading to infection in CD patients (Brest et al., 2011).

C) NOD2 and the link with autophagy

NOD2 has also been described to play a pivotal role in the induction of autophagy to eliminate intracellular pathogens (xenophagy). NOD2 is known to interact with ATG16L1, a molecular key within the autophagy network (T Watanabe et al., 2004). Previous studies showed that NOD2 recruits ATG16L1 and co-localize at the plasma membrane to facilitate the initiation of the autophagosome (Balzola, Bernstein, Ho, & Lees, 2010). However, cells with \textit{NOD2} mutation failed to recruit ATGL16, impairing the autophagosome formation (Balzola et al., 2010). Thus, malfunction of NOD2 impacts not only the killing and handling of pathogenic microbes but also its presentation by the major histocompatibility complex class II (MHC-II) to induce immune response (Cooney et al., 2010; Hold et al., 2014).

4.3 Endoplasmic reticulum Stress

A) Endoplasmic reticulum stress and cellular homeostasis

The ER is an essential subcellular organelle responsible for the synthesis and maturation of proteins that traffic through the secretory pathway. It confers an optimal environment to assemble and maturate proteins. The “milieu” of the ER contains molecular chaperones and folding factors essential for the quality control of the folding protein (Hetz, Martinon, Rodriguez, & Glimcher, 2011). Within ER, take place the co-translational and post-translational modifications of proteins including formation of disulfide bonds, signal-peptide cleavage, N-
linked glycosylation and glycosphatidylinositol (GPI)-anchor (Ellgaard & Helenius, 2003). Failure of protein folding is detected before it reaches the final folded state and is degraded through the **ER-associated degradation** (ERAD) pathway.

Aggregation of unfolded or misfolded proteins due to changes in the environment such as modification of Ca$^{2+}$ levels, oxidation-reduction conditions, or inflammation leads to a dysfunction within ER, termed then **ER stress** (Wang & Kaufman, 2014). ER-stress triggers UPR in order to adapt cells to harmful environment and to solve ER disturbances (A. Kaser & Blumberg, 2010). In order to bring back the folding capacity of the ER, UPR signaling pathway decreases the folding demand by downregulating the transcription of gene set and increasing its abilities to clear the misfolded proteins via ERAD. UPR also upregulates the transcription of genes involved in quality and control of folding proteins. The three main sensors of UPR are ER transmembrane proteins including inositol-requiring enzyme-1α and β (**IRE1α** and **β**), protein kinase-like ER kinase (**PERK**), and activating transcription factor 6 (**ATF6**) (Figure 19) (Schröder & Kaufman, 2005; Walter & Ron, 2012). If those mechanisms fail in adapting the cell to new conditions and recovering ER homeostasis, the cell undergo cell death by apoptosis.

In homeostatic conditions, ER chaperone binding immunoglobulin protein (BiP), also named glucose-regulated protein 78 (**GRP78**), maintain UPR sensors inactive by interacting with their luminal domain (Figure 19). Aggregation of unfolded or misfolded proteins disassociates BiP from the luminal domains of the ER-stress sensors, thereby initiating UPR downstream signalling pathway (Cao, 2015).

- **IRE1**
  IRE1 (type I transmembrane kinase) displays two isoforms, IRE1α expressed ubiquitously and IREβ mainly expressed by the epithelial cells of the gut and respiratory tract (Figure 19). Once BiP is unbound from the N-terminal domain of IRE1, the protein dimerizes and auto-phosphorylates in order to become active. Activated IRE1 splices 26-base intron from **XBP1** mRNA (Yoshida et al., 2003). This new spliced form, **XBP1s**, is a unique transcription factor that regulates genes responsible for ERAD and a number of subsets of ER chaperones (A. Kaser, Lee, Franke, & Glickman, 2008; Mimura et al., 2016).

- **PERK**
  Similar to IRE1, **PERK** (type I transmembrane kinase) undergoes auto-phosphorylation and oligomerization upon ER-stress. Activated PERK suppresses global protein translation by phosphorylating the α-subunit of **eukaryotic translation initiation factor 2** (eIF2α) (Figure 19). Besides reduced mRNA translation, PERK activation selectively translates a subset of mRNA such as the transcription factor **ATF4**, which regulates the gene expression involved in antioxidant response, amino acid biosynthesis and cell survival promotion (Harding et al., 2000; Wang & Kaufman, 2014). ATF4 also induces the transcription of CCAAT/enhancer-binding protein homologous protein (**CHOP**), which is required for ER stress-mediated apoptosis and the transcription of DNA damage-inducible protein 34 (**GADD34**), to arrest cell growth (Cao, 2015). Prolonged ER-stress, due to misfolded proteins or oxidative stress or altered Ca$^{2+}$ homeostasis, drives the cell to apoptosis through upregulation of CHOP and therefore activation of caspase-8 signaling cascade (Min Lu, David A. Lawrence, Scot Marsters, Diego Acosta-Alvear, Philipp Kimmig, Aaron S. Mendez, Adrienne W. Paton, James C. Paton, 2014).
• **ATF6**

Upon aggregation of misfolded proteins, ATF6 (type II transmembrane protein) dissociates from BiP and traffics to the Golgi apparatus, where it is processed in its luminal domain by site-1-protease (S1P) and in its transmembrane region by S2P (Figure 19). Cleaved ATF6 regulates the transcription of UPR-targeted genes including, ER chaperone genes, components of ERAD, and it promotes protein folding, maturation and secretion as well as the expression of XBP1 (Yamamoto et al., 2007; Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001). ATF6 is a crucial transcriptional regulator of the mammalian UPR and its absence might result in cell death due prolonged ER stress.

In addition to regulating UPR target genes, IRE1, PERK and ATF6 target the apoptotic downstream signaling pathway NF-κB and JNK. Activation of NF-κB under stress conditions drives the cell to modulate apoptotic pathways. In response to ER stress, IRE1α binds to the Iκκ complex which in turn, activates NF-κB (Figure 20)(Hu, Han, Couvillon, Kaufman, & Exton, 2006).

**B) Endoplasmic Reticulum Stress in intestinal inflammation and IBD**

Recently, IBD genetic studies have identified susceptible risk alleles, such as XBP1 and ARG2 (Anterior Gradient 2), both involved in ER-stress and UPR signalling cascade, associated with the pathogenesis of IBD. Previous studies showed that genetic deletion of components involved in UPR are linked with spontaneous intestinal inflammation and/or enhanced sensitivity to DSS to induce colitis (a Kaser and Blumberg 2009)

Animal studies reported that induction of ER-stress in mice with IECs-specific deletion of XBP1 exhibit an spontaneous development of intestinal inflammation carrying the hallmarks of human IBD including loss of Paneth cells, mononuclear and polymorphonuclear cells infiltration and severe ulcerations in the small bowel (A. Kaser et al., 2008). IECs of the small intestine of Xbp1KO mice present an increased GRP78, leading to enhanced apoptosis, a reduced number of Paneth cells and the absence of its secretory granules, together with decreased release of AMPs. Thus, dys-regulation of ER-stress contributes to spontaneous inflammation in the small intestine, and compromises host defence against enteropathogens (Hosomi, Kaser, & Blumberg, 2015). Similar to mice-deficient for Nod2 or ATG16L1 which are associated with handling and cleaning pathogens defects, hypomorphic XBP1 mice also show a defective intracellular bacteria sensing. Aberrant XBP1 provokes an accumulation of unfolded and misfolded proteins, not letting important proteins such as AMPs translate and secret correctly. This means that ER-stress pathway is important for the AMPs activity in the epithelium to control bacterial invasion (Cadwell et al., 2008; Kobayashi et al., 2005a). Paneth cell–specific deletion for Xbp1 promotes also spontaneous ileal inflammation, as observed in IEC Xbp1-deficient mice (Adolph et al., 2013).

XBP1-mediated signalling is the most conserved branch of UPR, which prevents constitutive ER-stress and plays a key role in inflammatory and immune processes (Bettigole & Glimcher, 2015; Kaufman & Cao, 2010). However, mechanisms by which XBP1-deficiency leads to intestinal inflammation are not well defined yet. Basal levels of ER-stress are always present in the intestine, but when cells are not able to properly manage the stress, the intestine undergoes through spontaneous inflammation and epithelial cells are more sensitive to environmental factors triggering gut inflammation. Mice with Xbp1-deficiency promote massive activation of IRE1 in ileal mucosa, leading to the activation of JNK and NF-κB pathways, which, in turn, increase the production of inflammatory mediators in the gut such as TNF-α, IL-1 and monocyte chemoattractant protein 1 (MCP1) (Mahdi, Rizvi, & Parveen, 2016). Previous studies have
demonstrated that colonic epithelial cells from patients with UC have a dys-regulated activation of eIF2a leading to changes in protein translation, including anti-oxidative enzymes, junctional proteins and secretory pathways, and altered colonic mucosa barrier function (Tréton et al., 2011). Ravindran et al have demonstrated PERK protein upregulation in inflamed colonic tissue of UC and CD patients, compared to healthy controls (Ravindran et al., 2016).

Another study showed that IRE1β−/− mice exhibited increased ER stress and early exacerbated inflammation upon DSS colitis (Bertolotti, 2001). In 2013, Tsuru et al. reported that IRE1β is involved in the translation and secretion of mucin. IRE1 β-deletion mice accumulates aberrant MUC2 in the lumen of ER of goblet cells increasing the ER stress signal such as ER distention and increased XBP1 mRNA splicing form. These results suggest that IRE1β promotes the folding and secretion of mucins in Goblet cells (Tsuru, 2013). The development of spontaneous colitis and rectal bleeding is observed in IRE1α-deficient mice along with diminished number of goblet cells and damaged intestinal barrier function. ER-stress signals showed downregulation of Xbp1s mRNA and upregulation of CHOP (H.-S. Zhang et al., 2015).

Another study observed that CHOP exacerbates the development of colitis by contributing to apoptosis of colonocytes. CHOP-deficient mice show an improvement upon colitis and a decreased number of apoptotic cells compared to wild-type mice. In conclusion this study reported that down-regulation of CHOP might contribute to ameliorate colitis (Namba et al., 2009).

Anterior Gradient 2 (Agr2) is a protein disulfide isomerase that plays an important role regulating intestinal homeostasis. Agr2−/− mice display abnormal location of Paneth cells into the villi of the ileum impairing the secretion of its proteins. These mice also have a decreased number of goblet cells, Mucin2 expression and a constitutive induction of ER stress in intestinal mucosa. These results suggest a correlation between Agr2 in intestinal homeostasis, ER stress and the etiology of IBD (Zhao et al., 2010)
Figure 19. The three major sensors of UPR. Three families of signal transducer, ATF6, IRE1 and PERK, sense the accumulation of misfolded proteins in the lumen of ER, and transfer the signal to the nucleus to regulate the transcription of UPR target genes. The transcriptional response increases the capacity of protein folding at the same time that downregulate the translation of proteins and increases degradation through ERAD. Extracted from (Kadowaki & Nishitoh, 2013).

Figure 20. Apoptosis signalling under ER stress. Extended ER-stress or dysfunction of the UPR, induced apoptosis signalling through IRE1 and PERK pathways. Active PERK increases levels of CHOP which, in turn, activates the transcription of GADD34. GADD34 dephosphorylates eIF2a, increasing protein load into the ER. On the other hand, activated IRE1 promotes activation of NF-κB, JNK and p38, involved in apoptotic-induced response. Adapted from (Kadowaki & Nishitoh, 2013).
Chapter 5

Proteases in the gut and IBD

Content at a Glance

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1. Proteases in the gut

Proteases, also known as proteinases and peptidases, are degradative enzymes for **protein catabolism** that hydrolyse a peptide bond to generate amino acids (López-Otin and Bond 2008). Proteases play a crucial role in controlling various **biological processes**, both intra- and extracellularly. They regulate and generate new bioactive molecules, modulate protein-protein interaction and process crucial cellular information. Thus, in the gut, proteases influence stem cell mobilization, blood coagulation, **inflammation**, autophagy, apoptosis and other multiple vital cellular functions (Antalis et al. 2007).

Proteases consist of 2% of the mammalian genome and they can be distributed in **five groups** depending on the mechanism of hydrolytic cleavage: serine-, metallo-, cysteine-, aspartate- and threonine proteases. Serine, Metallo and Cysteine proteases are the most important proteases regulating biological functions (Figure 21).

![Protease wheel](image)

**Figure 21. Protease wheel.** Scheme of the phylogenetic tree of human and mouse proteases. Proteases are classified in five catalytic classes and 63 different families. Metalloproteases are the biggest class of enzymes in both organisms. Adapted from (Puente et al. 2003)

The proteolytic activity needs to be **tightly regulated** since exaggerated proteolysis might lead to **tissue damage**. As it will be discussed further, dysregulation in **protease balance** has been reported in gastrointestinal diseases such as IBD or Irritable bowel syndrome (IBS) (Nathalie Vergnolle 2016). The **proteolytic activity** is regulated by various mechanisms in order to keep the ideal balance. First, proteases are synthesized as inactive **zymogen**e that becomes activated through proteolytic cleavage by upstream protease, pH shift or dimerization. Another manner to regulate its activity is by natural endogenous **inhibitors** (Van Spaendonk et al. 2017; Edgington-Mitchell 2016). The **protease inhibitors** present in the GI tract come from either circulation (distant production from other digestive organs such as liver), or locally produced by **IECs** or **infiltrated inflammatory cells** (Nathalie Vergnolle and Chignard 2006).

The **GI tract** is the organ containing the **highest** amount of proteases, both **endogenous** and **exogenous**. The microbial community, including bacteria yeast and helminths, are also an important source of proteases (Carroll and Maharshak 2013). Besides complex cellular signalling pathways, proteases in the gut are critical for digestion and maintenance of protein homeostasis (Edgington-Mitchell 2016). IECs themselves express a wide spectrum of proteases crucial for the
regulation of their environment, such as matrix metalloproteases that help in the turnover of the extracellular matrix (ECM) (Naito and Yoshikawa 2005). Proteases influence the regulation of intestinal permeability, either by targeting directly the tight junction proteins that seals the IEC monolayer or indirectly through the activation of protease-activated receptors (PARs) - Described in chapter 5 section 3- (Nathalie Vergnolle and Chignard 2006).

2. Proteases and their inhibitors

2.1 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases known to degrade and remodel components of the ECM. They are secreted as inactive zymogens by different cell types including mesenchymal cells, T cells, polymorphonuclear leukocytes, keratinocytes, tumor cells and enterocytes (Pender and MacDonald 2004). Depending on their substrate specificities, MMPs are subdivided in six groups: Collagenases, gelatinases, stromelysins, matrilysins, membrane-types MMPs and non-classified MMPs.

Besides ECM turnover, MMPs are involved in other tissue maintenance functions such as wound healing and regulation of a broad range of molecules such as chemokines, cytokines, growth factors, cytoskeleton and junctional proteins (Rodríguez, Morrison, and Overall 2010). Dysregulation of MMP activity leads to the development of several pathologies including chronic inflammatory diseases as IBD and cancer (Louis et al. 2000).

Endogenous inhibitors of MMPs, known as tissue inhibitors of metalloproteinases (TIMPs) are classified in four groups: TIMP-1-4 (Brew and Nagase 2011). Dys-function or altered expression of TIMPs is associated with inflammation and tissue damage. TIMPs are tissue-specific, constitutively or inductively expressed and their transcription depends on some cytokines and growth factors (Murphy 2011).

2.2 Serine proteases

Serine proteases are enzymes that hydrolyze peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the active site. Serine proteases are the most abundant group of proteases including over 26,000 serine peptidases classified into 13 clans and 40 families. Serine proteases are widely distributed in nature and present in the three domains of life (archaea, bacteria, and eukaryote) and even in viral genomes (Page and Di Cera 2008). Widespread throughout the human body, serine protease are usually endopeptidases which hydrolyse the peptide bond in the middle of a polypeptide chain. However, exopeptidases, that cleave only terminal amino acid residue, have been also found involved in the digestion. They act at a neutral pH and are involved in many diverse biological processes such as digestion, blood coagulation, apoptosis and fight infections (Cera 2009). The mechanism of action of serine proteases is described to attack the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate.

Mammalian serine proteases comprise plasminogen activators, chymotrypsin, trypsin and proteolytic enzymes produced by polymorphonuclear cells, such as cathepsin G and neutrophil elastase. Bacteria from commensal microbiota are also an important source of proteases present in the GI tract.
As mentioned above, a broad spectrum of proteases inhibitors is crucial to regulate the enzymatic activity. It exists two families of endogenous serine proteases inhibitors, Serpins and Chelonianins. Both inhibitors bind to the target protease disrupting its active site in an irreversible manner. Serpins are the largest and most widely distributed superfamily of protease inhibitors (Law et al. 2006). They circulate throughout the GI tract regulating serine proteases including trypsin, chymase, tryptase, elastases, and cathepsin G (Nathalie Vergnolle 2016). Another family of extracellular serine protease inhibitor, chelonianin, includes secretory leukocyte proteinase inhibitor (SLPI) and elafin (Zani et al. 2009). Chelonianin family inhibits the proteolytic activity of neutrophil serine proteases (NSPs) such as elastase, proteinase 3, and cathepsin G.

- **Trypsin-like serine protease**

Trypsin-like belong to the group of serine endopeptidase, produced and secreted as inactive zymogen precursors for the most part in the pancreas. The inactive zymogen (or trypsinogen) becomes active by enterokinase cleavage in the duodenum, where they act as major digestive enzymes (Rinderknecht 1986). Besides digestive properties, active trypsin forms are involved in proteolytic cascade by activating other proteases. Trypsinogens cleaves a peptide bond on the carboxyl-terminal side of arginine or lysine residues except when these residues are directly associated with a proline residue (Siepen et al. 2007).

Human pancreatic juice produces three different isoforms of trypsinogen: Trypsinogen-1 (cationic), trypsinogen-2 (anionic) and trypsinogen-3 or mesotrypsin. They are expressed not only in the pancreas, but also in other tissues, such as in various epithelial cells tissues, human brain and tumors (Koshikawa et al. 1997). Trypsinogens are encoded by the PRSS (protease serine) gene: PRSS1 (trypsinogen-1), PRSS2 (trypsinogen-2), found at proximal loci on chromosome 7q35, and PRSS3 (trypsinogen-3 or mesotrypsin), located on chromosome 9q13 (Itkonen 2010; Chen and Ferec 2000). While cationic and anionic trypsins share 96% of homology, trypsin-3 shares 87.8% and 88.7% identity with trypsin-1 and trypsin-2 respectively. Trypsin-1 and trypsin-2 are the most abundant isoforms secreted by the pancreas, accounting for 13% and 6% of pancreatic juice respectively. Both enzymes are responsible for protein degradation associated with physiological digestion and are inhibited by serpins.

On the other hand, human trypsin-3, the latest trypsinogen to be discovered, is found in a lower proportion secreted by pancreas and it is known to be resistant to inhibition by proteases inhibitors (Rinderknecht, Renner, and Carmack 1979). No endogenous inhibitor of trypsin-3 has been identified so far. Hence, trypsin-3 possesses the catalytic ability to cleave the reactive sites of canonical trypsin inhibitors including soybean trypsin inhibitor (SBTI) or human pancreatic secretory trypsin inhibitor (SPINK1) (Sahin-Tóth 2005; Gangaraju Vamsi K. Lin HaifanRichárd Szmola, Zoltán Kukor 2009). Besides trypsin-3, exist at least two other differentially-spliced forms of PRSS3, trypsinogen-4 and -5, encoded by alternative promotors and expressed differently depending on the tissues. Trypsinogen-4 is highly expressed in human brain and at lower levels in other tissues and tumors. Trypsinogen-5 expression is localized specially in the brain, gut, uterus and keratinocytes (Rowen et al. 2005; Salameh and Radisky 2013).
Recently, it has been discovered the implication of tryps-3, produced by epithelial cells, in some disease such as IBS (Kerckhoffs et al. 2008; Rolland-Fourcade et al. 2017). This section will be mentioned below.

2.3 Cysteine proteases

Most of the cysteine proteases are found intracellularly. Besides their fundamental functions of catabolism and protein processing, cysteine proteases mediate other signalling pathways involved in programmed cell death and inflammation. Cysteine group comprises caspas, autophagins, calpains and deubiquitinases intracellularly and cathepsins B, K and L extracellularly (Nathalie Vergnolle 2016). Caspases are a well-known family of cysteine proteases that play a role in cell death. Caspase 8 is involved in apoptosis and inflammation, both activating NFkB signalling pathway leading to pro-apoptotic gene transcription (Man, Kanneganti, and Jude 2016). It has been reported that increased cysteine proteases affect the intestinal mucosa integrity targeting inflammation and cell death signalling pathways (Ruemmele, Seidman, and Lentze 2002). Cathepsins play an important role in degradin g intracellular proteins, and maintaining the intestinal epithelium turnover and homeostasis (Tamhane et al. 2016). Altered activity of caspases or cathepsins is associated with IBD (Menzel et al. 2006).

To avoid unwanted protein degradation, like other proteases, cysteine proteases are synthesized as inactive precursors. The zymogen form blocks the active site containing the cysteine to substrate entry. Cysteine protease zymogen becomes active by the help of accessory molecules and by trans-activation from other enzymes or by auto-activation under the influence of acidic pH (Verma, Dixit, and Pandey 2016).


Protease-activated receptors (PARs), are seven-transmembrane domain receptors, and constitute a family of four G-protein-coupled receptors (GPCRs) with a unique mechanism of activation by proteolysis. To date, four different PARs have been discovered: PAR1, -2, -3 and -4 (Soh et al. 2010). PARs are widely expressed in human tissues, predominantly in vascular, immune cells, IECs, and nervous systems. Proteases signal to a multiple variety of cells to regulate a set of crucial biological processes, involved in physiology regulation and diseases (Figure 22) (N Vergnolle et al. 2001; Hyun et al. 2008). These receptors are activated by serine-, cysteine-proteases and MMPs (Nathalie Vergnolle 2008).

Activation of PARs is an irreversible proteolytic cleavage. After proteolytic activation, the receptor cannot be reactivated and undergoes through endocytosis via a clathrin-mediated process, where most of them are ultimately degraded into lysosomes (Hoxie et al. 1993). Proteases bind to and cleave at a specific site of the extracellular N-terminal domain of PARs to unmask a new N-terminus tethered ligand. The newly generated N-terminal domain binds to the second extracellular loop and triggers intracellular signalling (Figure 22) (Amadesi and Bunnett 2004). The tethered ligand sequence is different for each PARs. Synthetic peptides, also termed activating peptides (APs), mimic the tethered ligand of each different PARs and are capable of activating the receptor even without proteolytic cleavage. However, the analogues of the PAR3 tethered ligand has not been identified yet (N Vergnolle 2000). In addition to activating PARs by
generating the new tethered ligand, proteolytic cleavage can also silence their activation by a process called “disarming”. Proteolysis cleaves the amino acid downstream from the PAR tethered ligand sequence, therefore the receptor is prevented from inducing a signal (Figure 22 C) (Elste and Petersen 2010; Nathalie Vergnolle and Chignard 2006).

GPCRs, including PARs, consists of an α-subunit (Gα) linked with αβγ dimer that binds directly with the intracellular tail of the receptor. PARs signal transduction pathway activates multiple signalling pathways which regulate many biological functions. PAR-initiated signalling activates phospholipase C, which produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) which mobilize Ca2⁺ from the endoplasmic reticulum to the cytosol. Besides, PAR downstream effectors include activation of MAPK and NF-κB pathway. These pathways are linked with cellular proliferation and the activation of pro-inflammatory mediator genes, respectively (Adams et al. 2011; Ossovskaya 2004).

In the gut, PARs are ubiquitously expressed, including intestinal epithelial cells, neurons, inflammatory cells, etc. These receptors are present in both sides of the polarized IECs, apically and basolaterally, suggesting that luminal, circulating and secreted proteases can activate them (Nathalie Vergnolle 2008). Given that GI tract is a rich source of proteases, particularly produced during disease, PARs might play a crucial role regulating multiple processes. Proteases coming from coagulation cascade, inflammatory cells, microbiota and intestinal epithelial cells are able to cleave and trigger PAR signalling to maintain the gut homeostasis, ion exchange, motility, permeability, inflammation, visceral hypersensitivity and healing mechanisms (Figure 22) (Amadesi and Bunnell 2004; Nathalie Vergnolle 2016; N. Vergnolle 2005).
4. Inflammatory Bowel Diseases meet proteases

Proteases involved in disease possess multiple mechanisms of action to participate in the pathogenesis course (Figure 23). Proteolytic cleavage increases the bioactivity of pro-inflammatory mediators such as CXCL8 (also termed IL-8) and CXCL5, cleaved by proteinase-3 and cathepsin G, respectively. Proteases also contribute in the activation of programmed cell death such as caspases and autophagins (Chin et al. 2003) and also through the initial activation of PARs. Some proteases from probiotic bacteria are capable of digesting the glycans of the mucus, thereby modifying mucus structure (Subramani et al. 2010). Finally, proteolytic activity also cleaves IgG present at the intestinal mucus surface, thereby altering the composition and function of intestinal immune response (Brezski et al. 2009).

Figure 23. Scheme of the mechanism of action of proteases in the GI. Extracted from (Nathalie Vergnolle 2016).

Excessive concentrations of proteolytic activity have been found in the stools of patients with UC and CD, as well as supernatants of biopsies from these patients (Cenac et al. 2007; Carroll and Maharshak 2013). Several studies have reported the important role of proteases in maintaining chronic gut inflammation. During inflammation, secretion and activity of proteases are increased, mostly produced by infiltrated and resident cells including intestinal epithelial cells, smooth muscle, lamina propria and leukocytes (Nathalie Vergnolle and Chignard 2006; Nathalie Vergnolle 2008). In addition, increased fecal proteases might result from both commensal and pathogenic gut bacteria, which can secrete serine, cysteine and MMPs (G. T. MacFarlane et al. 1988; Steck et al. 2013). To date, genetic studies have found 75 genes coding for proteases and 7 genes coding for protease inhibitors involved in CD pathology and 14 proteases and 4 protease inhibitors genes identified in UC patients. The most relevant proteases genes found in IBD include cylindromatosis/turban tumor syndrome gene (CYLD), acylaminoacyl-peptidase (APEH), dystroglycan (DAG1), macrophage-stimulating protein (MST1) and ubiquitin-specific peptidase 4 (USP4) (Cleynen et al. 2011).
4.1 Epithelial MMPs and their inhibitors in IBD

Beside their role in ECM turnover, MMPs exert their proteolytic action on a wide range of molecules, including cytokines, growth factors, surface receptors, cytoskeleton proteins and junctional proteins, leading to their regulation (Rodríguez, Morrison, and Overall 2010). UC patients display an excessive production of MMP-7 related to severe inflammation levels (table 7) (Rodríguez, Morrison, and Overall 2010; Matsuno et al. 2003). IBD patients also have increased levels of MMP-9, which in healthy mucosa is usually undetectable. Deficient-MMP-9 mice exhibit resistance to experimental gut inflammation (Castaneda et al. 2005). In addition, a disruption of the balance between MMPs and TIMPs (MMPs inhibitors) is linked with inflammation and tissue damage (Mäkitalo et al. 2010). Intestinal mucosa protein extracts from IBD patients exhibit increased expression of MMP-1, -2, -3, -12, -13 and -14 compared to healthy gut. TIMP-1, a MMPs endogenous tissue inhibitors, was increased in UC biopsies, whereas TIMP-2 mRNA expression remains unaltered (von Lampe 2000; Mäkitalo et al. 2010).

4.2 Epithelial serine proteases and their inhibitors in IBD

Matriptase-1, membrane-type serine protease-1, is an integral transmembrane trypsin-like protease widely expressed in the epithelial cells covering the colon and the GI tract (Bugge, List, and Szabo 2007). Previous studies reported that matriptase-1 is essential in the formation and integrity of intestinal barrier. In Caco-2 experiments, the loss of matriptase-1 leads to leakage of intestinal barrier along with increased TEER and over-expression of claudin-2 (Buzza et al. 2010). Likewise, in vivo model have shown that Matriptase-hypomorphic mice develop severe colitis to DSS. Moreover, matriptase-1 expression in colonic epithelium of CD and UC patients is downregulated (Netzel-Arnett et al. 2012).

Most of the studies that have investigated proteases levels in IBD patients did not take into consideration the proteolytic activity but only their mRNA levels or protein expression. This is a limitation of the models because to identify the function of proteases it is essential to investigate the whole net activity of proteases and their inhibitors (Nathalie Vergnolle 2016). Only few studies have analysed the proteolytic activity in IBD. Colonic tissue from UC and CD patients display increased levels of active trypsin, chymotrypsin, and elastase (Cenac et al. 2007; Kjeldsen et al. 1998; Bustos et al. 1998).

Jean-Paul Motta et al. have demonstrated that inflamed and non-inflamed colonic mucosa of CD and UC patients have upregulated elastolytic activity. By in situ zymography the origin of proteolytic activity is detected in intestinal epithelial cells (J. P. Motta et al. 2012). mRNA levels of Elafin, an elastase inhibitor, and Secretory leukocyte protease inhibitor (SLPI) are increased in UC colonic biopsies but not in CD (Lawrance, Fiocchi, and Chakravarti 2001; Flach et al. 2006), while the study of Motta et al. reported a decreased expression of mRNA Elafin in intestinal epithelial cells of both UC and CD patients, using an in situ hybridization technique (Motta et al. 2012). In IBD mouse model, the intestinal expression of elafin displayed a strong anti-inflammatory effect against inflammatory parameters (J. Motta et al. 2011; Schmid et al. 2007).

Moreover, Neutrophil elastase (NE), proteinase3 and cathepsin G, a chymotrypsin subfamily, are increased in IBD. These proteases are secreted along with other pro-inflammatory molecules due to inflammatory stimuli (Table 6) (Pham 2006). NE induces inflammation upregulating CXCL8 via TLR-4 downstream signalling pathway, (Devaney et al. 2003). Cathepsin G, present during inflammation within the submucosa, is able to activate PAR4, although such signalling in the gut
and its potential pathophysiological consequences have never been studied. UC patients display increased colonic levels of cathepsin G and PAR4, both are related to increase paracellular permeability (Dabek et al. 2009).

**Tryptase** and **chymase** are released by mast cells, which constitute another source of proteases contributing to IBD pathogenesis (Groschwitz et al. 2009; Raithel et al. 2001). **Kallikreins** and **Granzymes** are also upregulated in IBD (Moreau et al. 2005). **Granzymes** promote cell death by activating several **apoptosis mediators** such as caspase-3, -8 and IBD (Table 7). **PAR1-signalling** is also involved in epithelial barrier dysfunction. In vivo and in vitro experiments have shown that PAR1 agonist such as **thrombin**, increases paracellular permeability through the induction of apoptosis and ZO-1 disruption (Chin et al. 2003; Trapani and Smyth 2002; N. Vergnolle 2005).

Most of the proteases cleaving and activating PARs belong to the **serine clan** of proteases. Serine proteases activate PARs through **paracrine** and **endocrine** signalling, and participate to vital or pathologic processes such as IBD, IBS or colorectal cancer (Rothmeier and Ruf 2012; Darmoul, Gratio, Devaud, Peiretti, et al. 2004; Nathalie Vergnolle 2016). Activation of PARs present in epithelial cells leads to changes in **paracellular permeability**. Cenac et al. (2002; Darmoul, Gratio, Devaud, and Laburthe 2004) have shown that in mouse model, PAR2 activation by trypsin, tryptase and chymase, all from serine proteases family, promotes an increase in colonic permeability displaying inflammation and disruption on the intestinal barrier integrity. These results have been supported by other studies which have shown an alteration on the intestinal barrier function and increased permeability using PAR2 agonists (Coelho et al. 2002; Darmoul, Gratio, Devaud, and Laburthe 2004). Investigation into the mechanism of action exhibited the involvement of **calmodulin** and **MLCK** in the PAR2-mediated modification of intestinal permeability. **PAR2 agonist** and **calmodulin** increase **MLC phosphorylation** which leads to epithelial cell cytoskeleton contraction and enhanced mucosal permeability. ML-7, an MLCK inhibitor, abolished disruption of TJ composition and function (Turner et al. 1997; Cenac et al. 2003). Another study revealed that activation of **ERK1/2** by trypsin in cultured colonocytes also phosphorlates MLCK, leading to epithelial cells disruption (Cenac et al. 2004).

### 4.3 Epithelial cysteine proteases

Inappropriate production (up-regulation) of **caspases** or **autophagins** have been detected especially in UC patients, leading to activation of autophagy and apoptosis (Seidelin and Nielsen 2006). **Caspase-1** and **5** form the inflammasome complex, promoting IL1-β and IL-18 maturation, both pro-inflammatory cytokines involved in the pathogenesis of IBD (Siegmund 2002; Nathalie Vergnolle and Chignard 2006). **Caspase-8** is involved in both apoptotic and inflammatory signalling pathway, activating respectively pro-apoptotic proteins and NF-kB (Table 7) (Man and Kanneganti 2016). Mice deficient in IECs for caspase - 8, a protease involved in apoptosis, necrosis and cell death, have shown increased cell death of epithelial cells in general and Paneth cells in particular, developing spontaneous inflammation in the terminal ileum and high susceptibility to colitis (Günther et al. 2011). Also caspase-4 and -5 have been found associated with IBD with increased risk of evolution towards colorectal cancer(Flood et al. 2015). Sina et al. have shown that **cathepsin K**-deficient mice treated with DSS develop severe colitis and that they improve by adding recombinant cathepsin K. They demonstrated that cathepsin K is highly secreted by goblet cells in the intestinal mucus, acting as an **antimicrobial peptide** shaping bacterial communities and **anti-inflammatory** properties(Sina et al. 2013). **Cathepsin L** has been found increased in colorectal adenocarcinoma accelerating cell proliferation (Tamhane et al. 2016). Another study
observed increased cathepsin D, B and L in intestinal macrophages from inflamed mucosa of IBD patients associated with tissue degradation. Inhibition of these cysteine proteases improved colitis (Menzel et al. 2006).

### Proteases involved in IBD

<table>
<thead>
<tr>
<th>Proteases</th>
<th>source</th>
<th>expression in IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase</td>
<td>Neutrophil/Mast cell/Monocyte/Eosino</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>Neutrophil/Monocytes</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>Neutrophil/Monocytes</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Chymase</td>
<td>Mast cell/Basophils</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Tryptase</td>
<td>Mast cell/Basophils</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Hepatocyte</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Factor V and VIII</td>
<td>Various (hepatocytes, placenta, leukocytes...)</td>
<td>↑ in UC</td>
</tr>
<tr>
<td>Plasminogen activator</td>
<td>Various (small intestine, colon, myocytes...)</td>
<td>↓ in CD/UC</td>
</tr>
<tr>
<td>Protein C</td>
<td>Hepatocyte</td>
<td>↓ in CD/UC</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Various (pancreas/epithelial cells/endothelial cells/neuronal cells..)</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Kallikreins</td>
<td>Various (pancreas/salivary glands/prostate/leukocytes...)</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>Granzymes</td>
<td>Leukocytes (CD8+/CD4+)</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Various (leukocyte/fibroblast/muscle cells/epithelial cells..)</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>MMP-2</td>
<td>various (muscle cells/adipocytes/lung/liver)</td>
<td>↑ in UC</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Ubiquitous (largely by smooth muscle cells)</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Pancreas/Skin/B cells/salivary gland/lung</td>
<td>↑ in CD</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Ubiquitous</td>
<td>↑ in CD/UC</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Ubiquitous (largely by myocytes)</td>
<td>↑ in UC</td>
</tr>
<tr>
<td>MMP-28</td>
<td>Ubiquitous (largely by lung)</td>
<td>= in CD/UC</td>
</tr>
<tr>
<td>TACE/ADAM17</td>
<td>Ubiquitous</td>
<td>= in CD/UC</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>LMPC/macrophages/epithelial cells</td>
<td>↑ in UC</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Ubiquitous</td>
<td>↓ in memory T cells CD</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>Ubiquitous</td>
<td>↑ in UC</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Ubiquitous</td>
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</tr>
<tr>
<td>Caspase-14</td>
<td>keratinocytes/placenta</td>
<td>= in UC</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>various (thyroid/liver/kidney/leukocytes)</td>
<td>↑</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>Various (placenta/intestine/pancreas/macrophages)</td>
<td>↑</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>lamina propria mononuclear cells/macrophages</td>
<td>↑</td>
</tr>
</tbody>
</table>
Table 6. Proteases involved in IBD. Origin and expression. Adapted from (Nathalie Vergnolle and Chignard 2006).

5. Further treatments to target IBD

Taken together, data reviewed in this chapter have shown the importance of proteolytic balance on GI diseases. In healthy conditions, the proteolytic activity is tightly regulated by proteases inhibitors, although this equilibrium is disrupted in organic and functional IBD pathology. A high number of proteases are up-regulated in IBD promoting inflammation, but others have a contradictory effect, decreasing some aspects of the acute inflammatory response. Thus, protease inhibitors have become a hot topic as possible therapeutic treatments, although characterization of the proteases involved in inflammation and hypersensitivity is needed. It is essential to avoid large spectrum of proteases inhibitors and focus on therapies targeting specific proteases.
Chapter 6

Our intestinal *in vitro* models

Content at a Glance

- Epithelial cell models
- Caco-2 cell line
- HT29mtx
1. Epithelial cell models

As mentioned in chapter 2 section 1, intestinal epithelial monolayers consist on a physical barrier that allows an efficient absorption of nutrients and that secretes a wide range of biochemical components as AMP, mucus, cytokines or chemokines to protect the epithelium. The single cell-layer intestinal mucosa is selectively permeable allowing bacteria metabolites, digested nutrients and soluble molecules (Lee 2015). Depending on the physicochemical properties of the compound, its size, its molecular weight and its charge distribution the mechanism of transepithelial transport differs. In addition, molecule interaction, mucus layer solubility and intestinal motility are crucial factors influencing the transport process. In vitro, intestinal motility is hardly reproduced especially in models of epithelial cell cultures. Therefore, study of absorption is a limited in in vitro models. However, in vitro culture of epithelial cells is considered a good model to study barrier function and transport mechanism (Verhoeckx et al. 2015; Berkes et al. 2003).

During the last past years the most widely used and best characterized system, which closely mimics the intestine conditions in vivo, has been epithelial cell lines including Caco-2 and HT29. While Caco-2 cultures are a mixed cell type from human colonic carcinoma, HT29 are considered as mucus-secreting cells which differentiate into mature goblet cells (Lesuffleur et al. 1990).

**Measurement of Transepithelial Electrical Resistance (TEER)**

Transepithelial electrical resistance (TEER), is a well-used value that reflects physical structure of epithelial monolayer and evaluates its paracellular permeability and integrity of TJ established between polarized cells. Thus, TEER is applied for assessing the epithelial barrier function (Figure 24) (Cereijido et al. 2008).

![Illustration of TEER measurement](image)

**Figure 24. Illustration of TEER measurement.** To the left, a monolayer of differentiated Caco-2 cells cultured in a transwell system and an electrode placed. The short electrode tip must be touch the cell monolayer, while the longer tip must be in contact with the outer compartment. Adapted from (Verhoeckx et al. 2015)

2. Caco-2 cells model

Caco-2 is a heterogeneous epithelial cell line established from a human colorectal carcinoma developed by Jorgen Fogh at Sloan-Kettering Institute for Cancer Research (Fogh, Fogh, and Orfeo 1977).
Although the tumor cell line was derived from a colon carcinoma, when cultured, Caco-2 spontaneous starts to differentiate into colonoocytes. Caco-2 display many characteristic of small bowel as they form a polarized cell layer expressing a brush border on their apical surface and intracellular TJ complexes. Besides, these cells express a wide number of enzymes and transporter proteins characteristic of enterocytes. Thus, this cell line has been used to study epithelial barrier function. However, a limitation is that caco-2 model is restricted to enterocytes, whereas the intestinal mucosa is formed by a conglomerate of absorptive cells, goblet cells, Paneth cells, endocrine cells and M cells among others (Hilgendorf et al. 2000; Verhoeckx et al. 2015).

To better simulate the in vivo parameters present in the intestine, Caco-2 cells are cultured on permeable supports to help enterocyte differentiation after reaching the confluence state. Cultures of these cells on filter inserts, for 20 days, let the cell differentiate into a polarized form achieving a particular morphology and functionality including sucrase isomaltase, phosphatase alkalin enzymes or tight junction proteins (Figure 25 and 26).

**Figure 25. Differentiation of Caco-2 cells on a filter support.** Cultured caco-2 cells (A) start to proliferate until confluence (B). Then they start to differentiate (C) until they become polarized with apical microvilli and enterocytes features after a 20 days of culture. Adapted from (Verhoeckx et al. 2015)

**Figure 26. Transwell system used during our experiments.** From Corning vendor, 12 mm Transwell with 3.0 μm pore polyester membrane insert. Adapted from corning website.

3. **HT29-mtx cell model**

HT29 is a human colorectal adenocarcinoma cell line with mature intestinal cell features such as the production of mucus. The other cell variant HT29-mtx is given to HT29 cells resistant to methotrexate (MTX), resulting into a cell transformation mucus-secreting differentiated cells (Maoret et al. 1989; Lesuffleur et al. 1990)This cell line was established in 1964 by Fogh and Trempe from a primary tumor of a 44 years old Caucasian female. HT29-mtx are mucus-secreting goblet cells characterized by the production of mucus (mucins) and the formation of TJ complexes
that lead to the development of confluent monolayers. In contrast with Caco-2 cells, HT29 can produce very high amounts of mucin. These cell types are mainly used to study food compounds or bacteria, which may influence mucus secreting properties in the gut.
Aims and hypothesis
Aims of the thesis and hypothesis

Crohn’s disease (CD) and Ulcerative colitis (UC) are chronic relapsing inflammatory bowel diseases (IBD) with mucosal ulcerations characterized pathologically by intestinal inflammation and epithelial injury. Although IBD is an idiopathic disease, over the ten past years, genetic studies have identified more than 170 loci of susceptibility for developing IBD. The strongest associations have highlighted fourth main pathways altered in IBD: bacterial sensing (NOD2 in CD), autophagy (ATG16L1 and IRGM in CD), Th17 Profile (IL23 receptor CD) and ER-Stress (XBP1 UC) pathways. Environmental factors including host microbiota play also an important role in promoting CD and UC.

About half of the patients with CD carry at least one polymorphism on NOD2 gene. NOD2 is a bacterial sensor expressed both in immune and epithelial cells, which specifically recognizes muramyl dipeptide (MDP), a fragment of the peptidoglycan from bacterial wall. Many studies have demonstrated the role of NOD2 in CD, but mechanisms by which NOD2 mutations promote CD are not yet understood. Many studies support the fact that a loss of function linked with NOD2 mutations participates to the development of CD.

Autophagy pathway is an intracellular catabolic process used to degrade and recycle cytoplasmic components, to maintain homeostatic cellular processes. In the context of CD, two variants genes of autophagy have been identified including ATG16L1 and IRGM. These polymorphisms reduce selective autophagy but not constitutive basal autophagy.

Accumulation of unfolded or misfolded proteins lead to a dysfunction within Endoplasmic Reticulum (ER), termed ER-stress. ER-stress triggers Unfolded Protein Response (UPR) in order to adapt cells to harmful environment and to solve ER disturbances. CD and UC patients display an aberrant ER-Stress response due to a polymorphism on its downstream pathway, including AGR2 or XBP1. Altered ER-Stress has been found in both inflamed and non-inflamed colonic mucosa of IBD patients. Mice deficient for XBP1 in intestinal epithelial cells including enterocytes, goblet and Paneth cells develop enteritis, this epithelial deficiency is associated to a reduced capacity of the intestinal mucosa to control inflammation.

The intestinal epithelium is involved in host defence by secreting antimicrobial peptides (AMPs) and mucus. The pathogenesis of IBD is characterized with increased intestinal permeability and altered expression of mucins and AMPs. A reduction of AMPs production by Paneth cells is described in the ileum of CD patients. An early depletion of goblet cells and the presence of emptied goblet cells are seen in the colon of UC patients. Recent data have described high trypsin proteolytic activity present in IBD tissues and stools but its origin (microbiota, immune and/or epithelial cells) is still debated. Recent work from the laboratory suggests that the intestinal epithelium is a major source of proteases and in particular trypsin activity, but the conditions associated with the expression and release of trypsin activity need to be better defined. Cleavage and activation by proteases of protease-activated receptors (PARs) has also been found associated with intestinal pathologies such as IBD or IBS. Trypsin proteolysis is able to cleave and activate PAR2 and -4, both receptors highly expressed in human intestinal mucosa. Moreover, the expression of PAR1, -2 and -4 is ubiquitous in the gut (epithelium, neurons, macrophages, mast cells, fibroblasts, infiltrated immune cells, etc) and PAR2 and -4 is up-regulated in the colonic mucosa of IBD patients. Inhibition of each of these receptors is a protective element against the development of colitis in animal models of IBD. However, little is known about the source of active
proteases in the gut, about their roles, and their mechanisms of action in the course of IBD, and which proteases could be present in the gut and be responsible for the activation of PARs.

Although alterations of the proteolytic activity and abnormal induction of the autophagy process, ER stress and NOD2 pathways have been independently associated with IBD pathophysiology, no study has investigated the link between the trypsin proteolytic activity of intestinal mucosa and the three main pathways involved in IBD.

The general hypothesis of this thesis is that in intestinal epithelial cells, the pathways that have been associated with IBD (NOD2, autophagy or ER-stress) are responsible for an increased secretion of proteolytic activity in general, and trypsin proteolytic activity in particular. Further, we hypothesized that protease activity released by activation of NOD2, autophagy or ER stress is responsible for epithelial dysfunctions associated with such IBD susceptibility pathways.

✓ Our first aim was to study the three IBD-related pathways and the effect of their activation on the secretion of trypsin proteolytic activity by intestinal epithelial cells.

✓ Our second aim was to identify the type of trypsin-like enzyme(s) enhanced in IBD patients and in our in vitro model of colonic epithelium.

✓ Our third aim was to analyse the role of trypsin-3 in the altered caco-2 monolayer homeostasis induced by ER-stress.

✓ As a fourth aim, we study whether a crosstalk exists between ER-stress and trypsin proteolytic activity.
Results

Content at a Glance

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Project 2. Autophagy
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  Paper
  Extra Caco-2 results
  HT29-mtx
Project 1.
The relationship between activation of NOD2 and trypsin-like proteolytic activity secretion in intestinal epithelial cells.

Introduction

IBD is characterized by severe inflammation of the small intestine and the colon leading to diarrhea and abdominal pain. The two main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). The NOD-like receptors NOD2, expressed in the epithelium, sense muramyl dipeptide (MDP) from Gram-positive and -negative bacteria. Three different mutations of NOD2 have been reported in ileal CD pathogenesis including Arg702Trp, Gly908Arg and leu1007fsinsC. In healthy conditions NOD2 tightly regulates the nuclear factor NF-κB ensuring ileal expression of α-defensins and secretion of chemokines and cytokines (Peyrin-Biroulet and Chamaillard 2007). In patients carrying NOD2 polymorphisms, NF-κB activation is abnormal, facilitating bacteria translocation and triggering inflammation by TLR2 and -4 stimulation (Hedl et al. 2007). It has been observed that trypsin proteases are associated with UC and CD, although the source and their implications have to be better defined in the context of IBD.

We hypothesized that NOD2 was able to modulate the secretion of trypsin activity by intestinal epithelial. Human enterocyte-like, Caco-2, and goblet cell lines HT29-mtx were stimulated with MDP (20µg/mL), a NOD2 inducer.

Methodology

Cell culture and reagents. Described in the paper. NOD2 was stimulated with 20µg/mL of MDP (sigma).

Trypsin like activity. Described in the paper

Statistical Analysis. Described in the paper

Results

Trypsin activity remained unchanged upon NOD2 stimulation

2, 4 and 6 hours after MDP stimulation, trypsin-like proteolytic activity was quantified in the supernatant of both cell lines. As shown in the figure 1, proteolytic activity remained unchanged upon MDP stimulation, in both cell lines, compared to control conditions (vehicle).

Discussion and conclusion

One of the reasons why MDP stimulation does not modify trypsin proteolytic activity could be explained by the low levels of NOD2 expression in caco-2 cells. Barnich et al., reported the expression of NOD2 in HT29 cells although Caco-2 exhibited low level of endogenous NOD2 (Barnich et al. 2005). Despite, Caco-2 cell line is a model to study NOD2 response
Rosentiel et al. shown that although basal levels of NOD2 are low in Caco-2, stimulation of TNF-α/IFN-γ in caco-2 cells upregulates the expression of NOD2 (Rosenstiel et al. 2003) This limitation could be overcome by using transfected caco-2 cells overexpressing NOD2 and verifying NOD2 activation through NF-κB activity. Regarding HT29 cell line, although they express the receptor, it seems that NOD2 does not have any impact on trypsin activity. Altogether, our findings suggest that no link exists between NOD2 and trypsin-like activity.

Figures

A. Caco-2

B. HT29-mtx

Figure 1. Trypsin like activity quantification after MDP stimulation. A-B. Caco-2 (A) or HT29-mtx (B) cells monolayer where stimulated with MDP. At 2, 4 and 6 hours supernatant was collected to measure trypsin-like activity. At least n=15 well /condition. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test).
Project 2. Autophagy signaling pathway and the release of trypsin-like proteases in intestinal epithelial cells.

Introduction

In mammalian cells autophagy was initially described as a mechanism to breakdown damaged macromolecules and organelles, allowing the recycling of amino acids via the fusion of the autophagosome with the lysosome (Christian de Duve and Wattiaux 1966; C De Duve 1966). Different extracellular signals trigger autophagy including cellular stress, amino acid deficiency, hypoxia or nutrient starvation. To date, new functions of autophagy have been described such as its involvement in innate and adaptive immune response.

In the context of CD, two variants genes in autophagy have been identified, ATG16L1 and IRGM (Rioux et al. 2007; Salem et al. 2015). These polymorphisms reduce selective autophagy but not its basal levels. Patients carrying autophagy genes mutation display an abnormal morphology of Paneth cells with reduced size and lower number of antimicrobial peptides being secreted. However, a recent study has shown that pediatric CD patients never treated, exhibited increased autophagy in Paneth cells. It is suggested that disorganization of the secretion granules observed in Paneth cells of CD is due to an overactivation of autophagy named crynophagy (Thachil et al., gastro 2012).

To address the question whether autophagy signaling pathway is able to modulate the secretion of trypsin in IBD pathophysiology, intestinal epithelial cells including Caco-2 and HT29-mtx, were treated with two main autophagy stressors: rapamycin and Nutrient starvation. Mammalian target of rapamycin signaling pathway (mTOR), is a protein kinase that regulates cell growth, proliferation, and survival (Ballou and Lin 2008). Rapamycin is a specific inhibitor of mTOR, and as such mimics cellular starvation by blocking signals required for cell growth and proliferation, thereby inducing autophagy. Cell responds to change in nutrition availability to maintain metabolism homeostasis. Nutrient starvation (NS) induces autophagy, providing cells with needed nutrient supplies. In cell culture Earle's Balanced Salt Solution (EBSS) medium is used to mimic nutrient depletion.

Methodology

Cell culture and reagents. Described in the paper. Autophagy was induced by culture medium Earle's Balanced Salt Solution (EBSS, sigma) or Rapamycin (0.5µM, sigma) in the culture medium. Cysteine proteases inhibitor E64 was used at 50µM, Sigma.

Trypsin like activity. Described in the paper.

Cysteine like activity. Cysteine-like activity was measured in supernatant from 6 well plates with the substrate Z-Phe-Arg-AMC (200µM, sigma) in Potassium phosphate buffer 100mM (pH6), 10mM EDTA and 1mM DTT. Hydrolysis rate was measured by the change in fluorescence (360/460 nm excitation/emission wavelengths) every 30 seconds for 15 minutes at 37°C on a microplate reader 96-well plate NOVOstar. Activity was
standardized to the rate generated by papain of known concentration from papaya latex (Sigma).

**Real-time PCR analysis.** Described in the paper. Sense and antisense specific primers are shown in table 1.

**Measures of paracellular Permeability.** Described in the paper

**Measurement of cell cytotoxicity with Lactate dehydrogenase (LDH).** Described in the paper

**Statistical Analysis.** Described in the paper

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense 5’ – 3’</th>
<th>Antisense 3’- 5’</th>
</tr>
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<tbody>
<tr>
<td>PRSS1</td>
<td>CCACCCCAATAACGACAGGAAG</td>
<td>GCGCCAGAGCTGCAGT</td>
</tr>
<tr>
<td>PRSS2</td>
<td>CCAAATACAACAGCCGG</td>
<td>AGTCGGCACCAGAACTCAGA</td>
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<td>ST14</td>
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<td>Elafin</td>
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<td>Elastase 2A</td>
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<td>TTAGTTATTGCAATCAGGAAG</td>
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**Results**

**Autophagy stimulation decreased trypsin activity and mRNA relative expression of serine proteases in Caco-2 cell line**

Caco-2 cells monolayers were stimulated with rapamycin (0.5µM) or incubated with EBSS medium to induce autophagy. Results show that trypsin-like activity was decreased under autophagy in Caco-2 cells (Figure 2). Mucus-secreting cell line, HT29-mtx, exhibited a general low secretion of trypsin-like activity and its levels remained unchanged by autophagy induction (Figure 2). Next, we analyzed the mRNA relative expression of a wild-spectrum of serine proteases, including PRSS-1, PRSS-2, PRSS-3, Matriptase II and Elafin, an elastase inhibitor, and we observed a general downregulation of the expression of these proteases and of the Elafin protease inhibitor, by autophagy induction (Figure 3).

**Nutrient starvation induces cysteine-like proteolytic activity**

Our data showed that when autophagy is induced by nutrients starvation in both cell lines, a great increase of cysteine-like activity is observed (Figure 4).
Autophagy inducers do not induce necrosis

To exclude the possible occurrence of cell death in our autophagy model, lactate dehydrogenase (LDH) cytotoxic assay was performed in Caco-2 cells (Figure 5). Our data demonstrate that our autophagy model, either nutrient starvation or rapamycin, does not induce cell death.

Alteration of intestinal barrier upon Nutrient starvation

We investigate whether autophagy induction modified intestinal barrier. Incubation of caco-2 cells with nutrient starvation increased paracellular permeability at 6 hours (figure 6A). This increase was inhibited when cysteine protease activity was blocked by E64 inhibitor (Figure 6A). Moreover, mRNA expression of TFF3 was upregulated (N=7 wells/condition. Rapamycin=2wells/condition) (Figure 6B).

Discussion and conclusion

Autophagy signaling pathway (confirmed by LC3a/b, IRGM and ATG16L1 gene expression (data not shown)) decreased the release of trypsin proteolytic activity by intestinal epithelial cells. Besides, mRNA levels of a large spectrum of serine proteases are also downregulated. These results clearly disproved the hypothesis originally proposed that autophagy could lead to an increased trypsin-like activity released by intestinal epithelial cells. A link between autophagy and the increased serine protease activity found in the stools and biopsies of IBD patients is therefore very unlikely.

However, increased cysteine proteolytic activity was observed in NS conditions, and it was associated with increased permeability and TFF3 mRNA upregulation. The differences observed between rapamycin and NS effects might be explain by the fact that rapamycin induces autophagy by inhibiting mTOR, while NS senses several autophagy signaling pathways. Caspases belong to the cysteine protease group playing crucial roles in cell death and inflammation. To eliminate a possible link between the drop of trypsin activity, the increased cysteine activity and cell death, LDH assay was performed. The cytotoxic assay shown no cell death. Thus, we excluded programmed cell death in our model although caspases mRNA expression should be verify. Cysteine inhibitor E64 normalized paracellular permeability under NS condition. This results suggested that increased cysteine activity might be the responsible for permeability increase. Cathepsins, a cysteine protease, play an important role in degrading intracellular proteins, and keeping intestinal mucosa turnover and homeostasis (Tamhane et al. 2016). Altered activity of cathepsin D, -B and -L is associated with IBD (Menzel et al. 2006). Therefore, further experiments should verify the expression of cathepsins in NS model and to investigate their impact on intestinal biology.

Also, NS condition upregulated TFF3 mRNA expression, a peptide involved in epithelial restitution and integrity of the mucus. TFF3 has been associated with cell junction modulation upregulating claudin-1 and decreasing claudin-2 expression (Meyer zum Büschenfelde, Tauber, and Huber 2006). We hypothesis that through TFF3, autophagy repairs epithelial damage.
Results

We concluded that downregulation of proteolytic activity might be a protective mechanism for the cells to avoid losing essential components for the cell maintenance. On the other hand, NS increases cysteine activity which alters intestinal barrier. Further experiments are needed to verify the role of cysteine activity on intestinal barrier.

Figures

A. Caco-2

B. HT29-mtx

Figure 2. Trypsin-like activity after autophagy induction. Monolayer of (A) Caco-2 or (B) HT29mtx were stimulated with autophagy stressors such as rapamycin or Nutrient starvation. Trypsin-like activity released was quantified at 2, 4 and 6 hours after induction. At least n=15 well /condition. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01 vs control group.
Figure 3. **mRNA relative expression of serine proteases.** Monolayers of differentiated Caco-2 cells were cultivated in Transwell system, and were stimulated with rapamycin or nutrient starvation for 6 hours. mRNA expression of PRSS-1, PRSS-2, PRSS-3, Matriptase I and Elafin were monitored. At least n=8 well /NS condition and n=2 well / rapamycin. Data expressed as mean±SEM was compared using one-way non-parametric anova (Bonferroni test). **p<0.01 vs control group.

![Graph](image)

Figure 4. **Cysteine-like activity.** Monolayers of differentiated Caco-2 and HT29-mtx cells were stimulated with rapamycin or nutrient starvation. Cysteine activity was measured at 2, 4 and 6 hours. At least n=10 well /condition. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test). **p<0.01 and ***p<0.001 vs control group.

![Graph](image)

Figure 5. **LDH cytotoxic activity.** Monolayer of Caco-2 cells were treated with nutrient starvation or rapamycin. LDH activity was measured at 2, 4 and 6 hours after autophagy induction. At least n=5 well /condition. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test).
Figure 6. **Autophagy induction modulates intestinal barrier alteration.** Monolayers of differentiated Caco-2 cells were cultivated in Transwell system and were treated with nutrient starvation or rapamycin. (A) Paracellular permeability was monitored by measuring the apical-to-basolateral flux of dextran 4kDa-FITC after 6 hours with nutrient starvation with or without E64, a cysteine protease inhibitor. (B) Relative mRNA expressions of TFF3 after 6 hours stimulation. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test). **p<0.01 and ***p<0.001 vs control group. ϕp<0.05 vs. NS group.
Project 3.
Endoplasmic reticulum stress boosts trypsin activity and release by enterocytes and alters barrier function.

Although IBD is an idiopathic disease, over the past ten years, genetic studies have identified more than 170 loci of susceptibility for the development of IBD. These genes affect in particular unfolded protein response (UPR)-related genes like AGR2 and X-box binding protein 1 (XBP1). Environmental factors and host microbiota play also an important role on promoting CD and UC (A. Kaser, Martínez-Naves, and Blumberg 2010).

Accumulation of unfolded or misfolded proteins lead to a dysfunction within Endoplasmic Reticulum (ER), termed ER-stress. ER-stress triggers UPR which facilitates the folding, the exportation and the degradation of proteins through three main proteins including Inositol Requiring Enzyme -1 alpha and beta (IRE1αβ), pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6) (Cao 2015). IRE1 is the initiator protein signaling pathway of the UPR. During ER stress, IRE1 contributes to a non-conventional splicing of the X-box binding protein-1 (XBP1) mRNA, activating the transcription of the UPR genes. Recently, ER stress has been described to play a key role in IBD. Mice deficient for Xbp-1 in intestinal epithelial cells (IECs), including enterocytes, goblet and Paneth cells, develop enteritis, linked to reduced capacity of IECs to control the inflammatory signals (A. Kaser et al. 2008). Mice deficient for IRE1β, exhibit hypersensitivity to DSS-induced colitis. Finally, some polymorphisms affecting the XBP1 gene are associated with IBD susceptibility (Bertolotti 2001). In UC patients, in both inflamed and non-inflamed colonic tissue, ER-stress is dysregulated leading to inflammation and cell dead in intestinal epithelium.

Endogenous serine proteases in intestinal epithelium are required to create and maintain the barrier (Ronaghan et al. 2016). However, recent data demonstrated that high amounts of serine proteases such as trypsin, chymotrypsin or elastase in IBD tissues and stools (Nathalie Vergnolle 2016; Giuffrida, Biancheri, and MacDonald 2014; J. Motta et al. 2011). Although the mechanism, the role and the source of serine proteases in the pathogenesis of IBD is not well understood, sustained increase of proteolytic activity drives decreased TEER and dysfunction on intestinal biology (Ronaghan et al. 2016).

Protease-activated receptors (PARs) are activated by the cleavage of proteinases including thrombin or trypsin. They are expressed apically and basally in intestinal epithelium and thus they respond to luminal or mucosal proteases. Four different PARs (PAR1-4) have been cloned (Soreide 2008). These receptors are involved in many biological functions such as gut epithelial transport, proliferation, pain and inflammation. PAR-2, which is activated by trypsin, plays an important role in intestinal permeability (Cenac et al. 2003; Róka et al. 2007; Hyun et al. 2008).

Therefore, the aim of this project was to analyze whether aberrant ER-stress in IECs modifies the secretion of trypsin proteolytic activity, its implication in intestinal homeostasis and the aim was also to study the molecular mechanisms associated with ER stress-induced biological functions.
Ulcerative colitis-associated endoplasmic reticulum stress induces trypsin activity affecting epithelial functions

Núria Solà Tapias¹, Alexandre Denadai-Souza¹, Claire Rolland-Fourcade¹, Catherine Blanpied¹, Anissa Edir¹, Corinne Rolland¹, Delphine Bonnet², Laurent Alric², Guillaume Portier², Sylvain Kirzin², Gilles Dietrich¹, Emmanuel Mas¹,², Céline Deraison¹, Chrystelle Bonnart¹, Nathalie Vergnolle¹,³*, Frédérick Barreau¹*

¹IRSD, Université de Toulouse, INSERM, INRA, ENVT, UPS, Toulouse, France
²Pole Digestif, CHU, Toulouse, France
³Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada
* Equal contribution, co-senior authors

Short title: Endoplasmic reticulum stress and proteases in inflammatory bowel disease

*Address for correspondance:

Dr Nathalie Vergnolle,
Institut de Recherche en Santé Digestive (IRSD)
INSERM UMR-1220
Purpan Hospital, CS60039,
31024 Toulouse cedex 03 France
Phone : +33 5 62 74 45 00; Fax : +33 5 62 74 45 58
e-mail : nathalie.vergnolle@inserm.fr
Abstract (<250 words)
Colonic intestinal epithelial cells (IECs) from inflammatory bowel disease (IBD) patients exhibited an excessive induction of endoplasmic reticulum stress (ER-Stress) linked to an altered intestinal barrier function and inflammation. Colonic luminal content of IBD patients is also characterized by increased serine protease activity, known for its contribution to mouse gut inflammation. However, the interplay between ER-Stress and serine proteases in the destabilization of intestinal barrier function associated with inflammation is unknown. Colonic biopsies from Ulcerative colitis patients exhibited increased Trypsin-like activity and in particular Trypsin-3 expression is associated with elevated ER-Stress. Stimulation of ER-stress in human intestinal Caco-2 cell line cultured in a transwell displayed enhanced trypsin-like activity and enhanced Trypsin-3 expression in the apical compartment. Increased trypsin activity destabilized intestinal barrier function by increasing intestinal permeability and inducing inflammatory signs. The deleterious impact of ER stress-associated trypsin activity depends on the activation of Protease-Activated Receptors 2 and 4. In conclusion, excessive ER-Stress in IECs increased the release of trypsin activity which, in turn, alters intestinal barrier function then favoring the development of inflammatory process. Trypsin-3 could be responsible for this increased activity and ER stress-associated epithelial dysfunctions.

**Key words:** Inflammatory Bowel Disease, Intestinal epithelial cells, Endoplasmic Reticulum Stress, Mesotrypsin/PRSS-3, Permeability.
**Results**

**Significance of this study:**

**What is already known on this subject?**
- Excessive Trypsin activity is measured in the colon of IBD patients.
- Colonic mucosa of IBD patients exhibited enhanced endoplasmic reticulum stress.
- Endoplasmic reticulum stress is a component of colonic inflammation.

**What are the new findings?**
- IBD-associated excessive Trypsin activity in colonic tissues (both Crohn’s and UC patients) originates mostly from the epithelium.
- Colonic epithelial cells released a large amount of Trypsin activity in the luminal compartment upon induction of ER stress.
- ER stress-induced altered barrier function is mediated by Trypsin activity, PAR2 and PAR4 activation in human intestinal monolayers.
- Colonic epithelial cells from UC patients but not CD patients exhibited an increased expression of Trypsin-3, concomitant to the presence of ER stress marker.
- Activation of ER-stress induces a specific release of Trypsin-3 at apical side of human epithelial monolayers.
- Trypsin activity secreted in response to ER-stress does not modify the induction ER-stress.
- Trypsin-3 is unable to induce ER-stress.

**How might it impact on clinical practice in the foreseeable future?**
- Trypsin activity in patient biopsies could be used as a marker of epithelial dysfunction.
- Trypsin-3 protein level could be proposed as a marker associating epithelial ER stress and increased permeability in UC patients.
- Specific inhibitors for Trypsin-3 could be developed as new therapeutic options for the treatment of UC.
Introduction.

Inflammatory Bowel Diseases (IBD) include Crohn’s disease (CD) and ulcerative colitis (UC). Although considered as idiopathic pathologies, consensus for the mechanisms involved in IBD proposed that in genetically predisposed individuals, excessive immune response to luminal content leads to the development of chronic inflammatory disorders of the gut (1). Over the past ten years, genetic studies have identified more than 170 loci of susceptibility involved in the development of IBD. Among those genes, some are linked to the unfolded protein response (UPR), with genes coding for X-box binding protein 1 (XBP1)(2).

Accumulation of unfolded or misfolded proteins in the lumen of the Endoplasmic Reticulum (ER) leads to cellular dysfunction termed ER stress (3, 4). ER stress triggers UPR gene expression in order for cells to adapt to harmful environment and to solve ER disturbances. Three ER-localized proteins, inositol-requiring kinase/endonuclease 1 (IRE1), pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6) were defined as UPR main proteins. Under ER stress, activated IRE1 removes 26-bp nucleotide intron from mRNA encoding for XBP1 (5). XBP1 spliced form then regulates gene expression that boosts folding protein regulation, ER-accumulated protein degradation, protein quality control, and phospholipid synthesis (3, 5, 6).

Previous studies have shown that ER stress is linked to intestinal inflammation (7-11). Genetic deletion of Xbp1 in intestinal epithelial cells (IECs) caused spontaneous enteritis in mice (10). Besides, deletion of IRE1β led to higher susceptibility to dextran sulfate sodium (DSS)-induced colitis (7), particularly when the gene was specifically deleted in intestinal epithelial cells (12). A new mouse model of UC has been characterized by an abnormal induction of ER-stress in goblet cells leading to colitis (13, 14). In human, recent studies have shown that colonic mucosa of IBD patients exhibited a dysregulation of ER stress (11, 12, 16). Glucose-regulated protein 78-kDa (GRP78) and XBP1 splicing genes were increased in inflamed gut of CD patients (10, 11, 15). Similarly, UC patients, have increased GRP78 expression in inflamed colon (9-11). Additional studies have reported that uninflamed colonic mucosa from UC patients displayed increased expression of spliced XBP1, GRP78, glucose-regulated protein 94-kDa (GRP94) and ATF6 (16). While it is clear now that ER stress in intestinal epithelial cells is associated with an inflammatory phenotype and by in large with IBD, the mechanisms by which ER stress could contribute to inflammation is less clear. What
mediators released upon ER stress by intestinal epithelial cells participate to inflammatory signs? We have made the hypothesis that active epithelial proteases could be released upon ER stress induction and thereby participate to generate inflammatory features in the context of IBD.

Genetic studies have evidenced an association between increased levels of proteases and IBD (17). Elevated metalloproteinase and trypsin activity in colonic tissues and stools from CD and UC patients have been demonstrated (18, 19). However, the cellular origin of this activity and the nature of proteases released are unclear. Recent studies have identified the colonic epithelium as an important source of proteolytic activity. Both elastolytic and trypsin-like activity were associated with human colon epithelium (20-22), where the activation of Protease-Activated Receptors (PARs) modulates a number of cellular signals, including ion transport, barrier function or inflammatory mediator release (23-27). Here, we identified that trypsin-like activity in tissues from IBD patients is associated with the epithelium. We investigated whether ER stress in human intestinal epithelial cells can induce the release of proteolytic activity and whether this could contribute to the inflammatory phenotype associated with ER stress in intestinal epithelium. We have further identified Trypsin-3 as an epithelial protease associated with ER stress and IBD.
Results

Trypsin activity in IBD colon tissues is associated with the epithelium

As previously reported, trypsin-like activity was released by human colonic biopsies incubated for 1h in culture media (Figure 1A). This released activity was significantly increased in supernatants of biopsies harvested from IBD patients (both CD and UC) (Figure 1A). In situ zymography using substrate for trypsin-like activity was performed in human colonic tissues and confirmed a significant increase in trypsin-like activity in tissues from CD and UC, compared to controls (Figure 1B right panel). Photomicrographs of in situ zymography also revealed that most of the trypsin-like activity detected in colonic tissues from CD and UC patients was associated with the epithelium (Figure 1B left panel), suggesting that intestinal epithelial cells were a major source of trypsin-like activity.

ER stress in human intestinal epithelium provokes the apical release of trypsin-like activity

We investigated whether the induction of ER stress in human intestinal epithelial cells could provoke the release of trypsin-like activity. In monolayers of differentiated Caco-2 cells, Tunicamycin or Thapsigargin, two ER stress inducers, triggered the release of trypsin-like activity compared to controls (Figure 1C). This release of ER stress-induced trypsin-like activity was specific of the apical compartment (Figure 1C), but was not observed in the basal compartment (Figure 1D). The ER stress-induced release of trypsin-like activity in the apical compartment was inhibited by AEBSF incubation (not shown) and was significant at 2, 4, and 6h post stimulation (Figure 1C), but was inhibited by a pre-treatment with the ER stress inhibitor 4-PBA, at 6h post thapsigargin exposure (Figure 1E). We monitored that Tunicamycin and Thapsigargin treatments duly induced ER stress, by observing a significant increase of the ER stress markers XBP1s, CHOP, ATF4 and ATF6, compared to unstimulated controls (Supplementary Figure 1A). We also demonstrated that Tunicamycin and Thapsigargin treatment did not induce cell death, as no change in lactate deshydrogenase (LDH) activity was observed in controls or stimulated cell cultures (Supplementary Figure 1B). The ER stress-associated apical release of trypsin-like activity was also observed in HT29-MTX cells, another human intestinal epithelial cell line, at 2, 4 and 6h after Thapsigargin exposure (Supplementary Figure 2). These data demonstrated a polarized secretion of trypsin activity by intestinal epithelial cells upon ER stress induction.
Identification of protease(s) responsible for ER stress-associated trypsin activity in intestinal epithelial cells

Trypsin-specific activity-based probe (ABP) was used to further characterize the ER stress-induced trypsin activity released by intestinal epithelial cells. When incubated in the presence of the biotin-PK-DPP ABP, trypsin-like activity in apical supernatants from thapsigargin-treated cells was completely inhibited (Figure 2A). This demonstrates that the ABP employed was able to retain the protease(s) responsible for ER stress-related trypsin-like activity. The ABP was then used to establish the profile of active proteases present in intestinal epithelial cell supernatants upon stimulation or not with the ER stress inducer Thapsigargin. Bands corresponding to active proteases were discriminated from non-specific labelling by pre-incubation with the large spectrum protease inhibitor AEBSF. Supernatants from stressed cells showed a strong signal for a band at 33-kDa, compared to a very weak signal for the same band in control cells. The signal for this ER stress-associated 33-kDa band disappeared in the presence of AEBSF (Figure 2B), indicating that this band corresponds to an active protease. This 33-kDa band signal was increased by 10-fold, compared to unstimulated cells supernatant (Figure 2C).

Unbiased mass spectrometry analysis revealed the presence of seven proteins corresponding to proteolytic enzymes in ABP-treated samples of cell supernatants from control or thapsigargin-treated cells (Table 1). Only one type of protease signal was significantly increased (ratio thapsigargin-treatment/control >2) in ER stress-treated cells compared to controls. This signal corresponded to putative Trypsin-6 precursor, encoded by the PRSS3 Pseudogene-2. As a pseudogene, the expression of its predicted protein has never been described. The results of the mass spectrometry analysis did not therefore permit the clear identification of a known protease. Among all forms of known trypsins, only the molecular weight of Trypsin-3 (encoded by PRSS3 gene) corresponded to the 33-kDa band that was observed in the ABP profiling gel (Figure 2B). Because no specific antibodies exist to discriminate between Trypsin-1 and -2, only a specific anti-trypsin-3 antibody was available, we decided to further study the expression of trypsin-3 in ER stress conditions.

Trypsin-3 expression in ER stress conditions and in IBD patients

The presence of Trypsin-3 in supernatants of ER stress-stimulated intestinal epithelial cells was investigated. At 6 and 24 hours after ER stress induction, western blot performed in culture supernatants from apical compartment showed a strong band at 33-
Results

kDa, corresponding to Trypsin-3, not present in control conditions (Figure 3A). The densitometry analysis revealed that Trypsin-3 expression was increased by 3-fold and 25-fold at 6 and 24 hours respectively (Figure 3A). This increased Trypsin-3 protein expression was not observed in basal lateral compartment of ER stress-stimulated Caco2 cells (not shown). In cell extracts, Trypsin-3 protein expression was not modified between control and ER stress-stimulated conditions both at 6 or 24 hours (Supplementary Figure 3A). In Caco2 cells, ER stress did not induce changes in mRNA expression of PRSS-3 (gene of Trypsin-3) at 6-h, but significantly increased this expression at 24-h (Supplementary Figure 3B). In HT29-MTX cells, increased PRSS-3 mRNA expression was observed as soon as 6h after ER stress induction (Supplementary Figure 3C). This increased expression of PRSS3 mRNA seemed to be inhibited in the presence of the ER stress inhibitor PBA (not shown). Taken together, these results strongly evidenced that ER-stress induced the specific release of Trypsin-3 by intestinal epithelial cells in the apical compartment, and successively induced the transcription of its gene.

In human colonic tissues, the presence of Trypsin-3 was mostly associated with the epithelium as demonstrated by the co-staining with the epithelial cell marker Ep-CAM (Figure 3B left panel). Trypsin-3 expression was significantly stronger in tissues from UC patients, compared to controls or to CD patients (Figure 3B). ER stress marker XBP1 was only up-regulated in tissues from UC patients compared to controls or CD patient tissues (Figure 3C). Taken together, these data reinforced the link between induction of ER-stress and increased release of Trypsin-3 by intestinal epithelial cells, this seems particularly relevant in UC patients.

**ER stress modifies intestinal barrier function by a mechanism involving the apical release of trypsin-like activity**

We investigated whether ER stress induction and its associated release of proteolytic activity were able to disrupt barrier function of differentiated Caco-2 monolayers. Thapsigargin-induced ER stress altered paracellular permeability, mRNA expression of antimicrobial peptides and mucins as well as the secretion of CxCL8 (Supplementary Figures 4 A-C and Figure 4). All these epithelial dysfunctions were clearly associated to the induction of ER stress, and they were all inhibited by the ER stress inhibitor PBA (Supplementary Figure 4). Apical addition of protease inhibitors (AEBSF or Leupeptin) inhibited ER stress-induced increased permeability in Caco2 cells
(Figure 4A), suggesting that proteolytic activity released apically by ER stress disrupted epithelial barrier integrity.

ER-Stress did not affect mRNA expression of human β-defensin 1 (HB1), but increased mRNA expression of β-defensin2 (HBD2), Trefoil factor 3 (TFF3), mucin 2 (MUC2) and CXCL8 (Figure 4B). Apical addition of the protease inhibitor AEBSF blocked ER stress-induced TFF3, CXCL8 and HBD2 increased mRNA levels, suggesting that ER stress-associated proteolytic activity controls secretory barrier function (Figure 4B).

CXCL8 protein release was increased in supernatants of thapsigargin-treated cells compared to non-stimulated cells (Figure 4C). The addition of AEBSF or Leupeptin to thapsigargin-treated cells exacerbated CXCL8 protein release (Figure 4C).

In HT29-MTX cells, Thapsigargin-induced increased mRNA expression of MUC5 and HBD2 were inhibited by apical addition of AEBSF (Figure 5A). CXCL8 protein was increased in supernatants of Thapsigargin-treated HT29-MTX cells compared to controls and this increase was exacerbated by AEBSF apical treatment (Figure 5B). These data demonstrate that many of the effects of ER stress on intestinal epithelial cells are mediated by the release of trypsin-like activity on the apical compartment.

**ER stress-associated trypsin activity alters barrier function of Caco-2 monolayers by activating PAR2 and PAR4 receptors.**

The mechanisms by which trypsin-like activity was involved in ER stress-associated epithelial changes were investigated by studying the involvement of the two trypsin receptors PAR2 and PAR4. Thapsigargin treatment of Caco-2 cells increased mRNA expression of PAR2 and PAR4 at 6 and 24 hours (Figure 6A). Pre-incubation of Caco-2 cells with PAR2 antagonist (GB83) and/or PAR4 antagonist (ML354) blocked ER stress-induced increased paracellular permeablility both at 6 and 24h (Figure 6B, only 6h shown). ER stress induced the release of CXCL8 both in the apical and basal compartment of stimulated Caco2 cells (Figure 4C and 6C). Pre-incubation with PAR2 and/or PAR4 antagonists further enhanced ER stress-induced CXCL8 release (Figure 6C), similar to the effects of the trypsin inhibitor AEBSF (Figure 4C).

**Apical trypsin activity or trypsin-3 do not modify ER stress processes**

ER stress markers XBP1s, CHOP, ATF4 and ATF6 were increased by Thapsigargin treatment after 6h in Caco2 cells, and the apical addition of the protease
inhibitor AEBSF did not modify the expression of these markers (Supplementary Figure 5A). Similar results were obtained in HT29-MTX cells (not shown). These results suggest that ER stress-associated proteolytic activity did not affect the induction of ER stress. Apical addition of Trypsin-3 to Caco-2 cells did not modify the expression of XBP1s, CHOP, ATF4 and ATF6 (Supplementary Figure 5B), and did not induce the release of CXCL8 (Supplementary Figure 5C).
Discussion

Abnormal induction of ER stress and increased serine proteases activity in colonic tissues have been established in IBD patients (16, 19), as well as in mouse colitis models (7, 9, 13, 20, 21). Here, we confirmed that excessive trypsin protease activity was released by colonic tissues from IBD patients, compared to healthy controls (19). We also evidenced that the IBD patient tissue-associated trypsin activity was detected mostly in intestinal epithelial cells. The induction of ER stress enhanced apically-released trypsin activity by intestinal epithelial cells. We showed that this trypsin activity was responsible for many of the ER stress-induced alterations of epithelial homeostasis, through a mechanism involving the activation of PAR2 and PAR4. Finally, we identified that Trypsin-3 could be an enzyme responsible for ER-stress induced epithelial disturbances. Indeed, Trypsin-3 has been detected in apical supernatants of ER stress-stimulated epithelium. Trypsin-3 is known to activate both PAR2 and PAR4 (28) and to induce increased epithelial permeability (22). Finally, Trypsin-3 expression was increased in tissues from UC patients, concomitantly to an increased expression of ER stress markers, while both expression were unchanged in tissues from CD patients.

Experimental and clinical studies have reported that colonic tissues and luminal contents in inflammatory mouse models and IBD patients displayed excessive serine protease activity (19, 21, 24). While the cellular source of this activity has been discussed, no study had yet identified where this activity could come from. The role of proteases from infiltrated immune cells has been suggested, as well as a possible pancreatic source or even a microbiota source. In this study, we demonstrate that colonic mucosa from IBD patients releases higher trypsin proteolytic activity than colonic mucosa of healthy controls, and this activity in colonic tissues is associated with the epithelium. Interestingly, in a recent study, similar increased trypsin-like activity was detected in human intestinal epithelial cells from irritable bowel syndrome patients (22). In that study, this increased trypsin-like activity was associated with an increased Trypsin-3 expression and secretion, and could be up-regulated by stress hormones or bacterial motifs, such as epinephrine and LPS respectively (22). From our results, it appears that ER stress is another regulator of Trypsin-3 expression and release by human intestinal epithelial cells. Further, our results suggest that Trypsin-3 up-regulation in IBD is specific of UC, as it was not observed in CD. While Trypsin-3 appears as a good candidate responsible of increased proteolytic activity in UC patient tissues, it seems that other
proteases could be responsible for trypsin-like activity in colonic tissues from CD patients.

Mass spectrometry analysis definitively pointed towards members of the trypsin family as epithelial enzymes responsible for ER stress-induced proteolytic activity. However, with this approach, we were not able to clearly identify a known protease. Indeed, mass spectrometry identified peptides related to the putative trypsin-6 protein. No study has yet reported the existence of this protein, coded by the PRSS3 Pseudogene-2. Putative Trypsin-6 has peptides in common with Trypsin-1 and Trypsin-2, which could then also be considered as possible enzymes responsible for ER stress-induced trypsin-like activity. No selective inhibitor has been reported to discriminate between the activity of the 3 known forms of trypsin: Trypsin-1, Trypsin-2 and Trypsin-3. Likewise, no specific antibody discriminates between Trypsin-1 and Trypsin-2 proteins. Only a Trypsin-3 antibody has been described that does not cross-react with Trypsin-1 or Trypsin-2. This lack of specific tools has hampered our ability to determine which enzyme is specifically responsible for ER stress-induced proteolytic activity. We have tried to apply an shRNA approach targeting Ttrypsin-3 expression in Caco-2 cells, but all PRSS gene expression (PRSS1, PRSS2 and PRSS3) were affected by this approach, preventing a selective inhibition. Although Trypsin-3 expression reflects ER stress-induced trypsin activity, we cannot rule out that Trypsin-1, Trypsin-2 or even the possible existence of Trypsin-6 might be responsible, at least in part, for the ER stress-induced proteolytic activity.

Abnormal induction of ER stress in intestinal epithelial cells is also associated with IBD pathophysiology(16). Previous studies have linked aberrant induction of ER stress in colonic mucosa of UC patients (9, 10). Likewise, our results reported that colonic biopsies of UC, but not CD, carry an abnormal induction of ER-stress by upregulation of XBP1s. Our data correlate atypical ER stress induction and excessive secretion of Trypsin-3 in colonic mucosa of UC patients, pointing out Trypsin-3 as an interesting molecular motif to target ER stress pathways, and potentially for UC treatments. We verified that the induction of ER stress was not modified by the presence of proteolytic activity, and that Trypsin-3 did not induce ER stress. We revealed that the release of trypsin activity by intestinal epithelial cells is truly a consequence and not a cause of ER stress. Indeed, our results clearly demonstrated that most of the disruptive effects of ER stress on intestinal epithelial cell barrier were mediated by the apical release of trypsin
activity. This constitutes a breakthrough in understanding ER stress pathways and effectors. We demonstrated that the up-regulation of β-defensin-2, Trefoil factor 3, Mucin-2, and CXCL8 mRNA expression, as well as the increased permeability induced by ER stress are all caused by the apical release of trypsin-like activity.

Interestingly, inhibition of trypsin-like proteolytic activity induced by ER stress further enhanced the detection of CXCL-8 protein in both apical and basolateral compartments of enterocytes and muco-secreting monolayers, while CXCL8 mRNA transcripts were inhibited by protease inhibitors. These results point to a differential role for proteases at the transcriptional level and the protein levels. It could be hypothesized that ER stress-released proteases cleave the CXCL8 proform thereby releasing more proteins. However, the fact that PAR2 antagonist further enhanced this CXCL8 protein release points to a receptor-mediated effect rather than a maturation process. Interestingly, our data suggest that ER stress-associated trypsin-like activity inhibits the acute release of CXCL8, but favors the subsequent chronic CXCL8 mRNA up-regulation. This is also in favor of a disruptive role for trypsin-like proteolytic activity in epithelial biology, and a potential benificial role for the inhibition of trypsin activity.

Proteases use different regulatory mechanisms of action for cell signaling, including proteolytic cleavage of molecules or receptors to induce a large spectrum of intracellular signals. Proteolytic processing by proteases is required to mature antimicrobial peptides like the human defensin-5 or to increase the chemotactic activity of CXCL-8 (proteinase-3) or CXCL-5 (cathepsin G)(29, 30). In contrast, proteases like cathepsin G, proteinase-3 or elastase 2 can also degrade other cytokines including IL-6 or TNF-α as well as some proteins involved in cellular contacts (31-34). In addition to these roles, proteases are also able to activate, by proteolytic cleavage of their extracellular N-terminal domain, the PARs (35, 36). PARs cleavage activates a broad range of cellular effects in the intestine, including ion exchange, motility, nociception, permeability, secretion (22, 25, 37-39). In agreement with PARs involvement in epithelial physiology, our data demonstrated that pre-treatment with PAR2 and/or PAR4 antagonists inhibited the effects of ER stress on altered epithelial monolayer. Previous studies have demonstrated that PAR2 and PAR4 are both activated by trypsin-like enzymes (40), including Trypsin-3 (28). In addition, PAR2 activation is known to induce increased epithelial permeability (38). Trypsin-3 also induces increased permeability through a PAR2-dependent mechanism (22). Taken together, these studies are in
agreement with our finding reporting the involvement of PARs in increased permeability. Our study though brings to knowledge the fact that ER stress induction might be among the triggers to induce increased epithelial permeability in IBD, and in UC in particular, through a protease and PAR-dependent mechanism. Indeed, the involvement of PARs and PAR2 in particular in the development of colitis in animal models has been well documented (41).

Overall, this study deciphers the mechanisms by which ER stress participates to epithelial dysfunction in intestinal epithelial cells. This study points out the role of trypsin-like activity, and to Trypsin-3 as a potential molecular target for the treatment of intestinal inflammation. These results appear to be particularly relevant in a UC context, where both ER stress and trypsin-3 are induced.
Material and Methods

**Patients.** Human colonic tissues were obtained from individuals treated at the Centre Hospitalier de Toulouse (France) (Table S1). Biopsies were collected during colonoscopy procedures aimed at clinically evaluating the disease of established and well-characterized CD and UC patients or done in individuals undergoing colon cancer screening who were otherwise healthy (healthy controls). Written and verbal informed consent was obtained before enrollment in the study, and the Ethics Committee approved the human research protocol (Comité d’Ethique sur les Recherches Non Interventionnelles) (Identifier: NCT01990716). Isolated biopsy specimens were embedded in optimal cutting temperature (OCT) compound (Dako) at −186°C and stored for in situ trypsin activity.

**Cell culture and reagents.** Caco-2 cells were purchased from the DSMZ collection (Braunschweig, Germany). Caco-2 cells were routinely grown at 37°C in a 5% CO₂ water-saturated atmosphere in GlutaMAX DMEM (Gibco, Saint Aubin, France) supplemented with 10% of heat inactivated fetal bovine serum (Biowest, Nuaillé, France), 1% nonessential amino acids, and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin (Gibco) as previously described (42). Cultured medium was changed three times per week and cells passed once a week. Cells were grown to confluence as a monolayer in Transwell inserts, 12-well plate of 12-mm, polyester membrane, 0.3-µm pore size, (Costar, Paris, France) for 20 days. Endoplasmic reticulum stress (ER Stress) was induced by adding Thapsigargin or Tunicamycin (10µg/mL, sigma) in the culture medium as previously described (13). Before stimulation, cells were washed 2 times with Ca²⁺/Mg²⁺- free PBS (Sigma, Saint Quentin Fallavier, France). Trypsin-like activity was inhibited by adding in the culture medium, AEBSF (200µM) or Leupeptin (50µM), two serine inhibitors. Caco-2 cells were pre-treated 45 minutes with PARs antagonists, PAR2, (ML354; 10µM) and PAR-4 (0.5µM) Tocris, (Bioscience, Lille, France), before thapsigargin stimulation.

**In situ zymography of colonic biopsies.** Frozen OCT sections of colonic tissues from patients (8-µm thickness) were permeabilized with PBS2% Tween-20, rinsed with washing solution (PBS) and incubated at 37°C overnight with N-p-Tosyl-GPR-amino-4-methylcoumarin hydrochloride (50µg/mL, Sigma, Saint Quentin Fallavier, France) together with 0.3% low melting agarose as previously described (22). All sections were
visualized with the LSM710 microscope (Carl Zeiss France) and analyzed by observers blinded of patients subgroup, with Zen 2009 software (Carl Zeiss)

**Trypsin like activity.** Trypsin-like activity was measured in basal and apical supernatants as previously described (43) with the substrate N-p-Tosyl-GPR-amino-4-methylcoumarin hydrochloride (0.1mM, sigma) in 50 mM Tris, 10mM CaCl\(_2\) buffer (pH 8). Hydrolysis rate was measured by the change in fluorescence (360/460 nm excitation/emission wavelengths) every 30 seconds for 15 minutes at 37°C on a microplate reader 96-well plate NOVOstar. Activity was standardized to the rate generated by trypsin of known concentration from porcine pancreas (Sigma). Control and IBD patients biopsies with OCT were cryostat sectioned (8 μm thickness) and washed with PBS, 2% Tween-20. Samples treated with the substrate N-p-Tosyl-GPR-amino-4-methylcoumarin hydrochloride (0.1 mM, Sigma) in 0.3% low melting agarose were incubated overnight at 37°C as previously described (22). Nuclei were stained with Topro3 (Invitrogen). Images were analyzed with ImageJ software.

**Activity-based probe.** The Biotin-PK-DPP serine protease activity-based probe was obtained from the laboratory of Dr. Nigel W. Bunnett (Columbia University, USA), with the participation of Dr. Laura Edgington-Mitchell (Monash University, Australia) and synthetized as previously described (44). Supernatant were treated with Pefabloc SC plus kit (Roche. Mannhein, Germany) with or without AEBSF (4 mM for 15 min at 37°C under stirring (1000 rpm). Then, the ABP biotin-PK-DPP was added to each reaction to a final concentration of 1 μM, and samples were incubated for 60 min at 37°C under stirring (1000 rpm). Next, protein from supernatant was precipitated with 15% trichloroacetic acid/acetone and separated by SDS–polyacrylamide gel electrophoresis (4-15%). Membrane was block with 1% BSA and incubated for 1 hour at room temperature with 1/1000 streptavidin-HRP (LifeTechnologies). Immunoblots were visualized with chemiluminescence. (Chemidoc XRS Bio-Rad)

**Western blot.** Caco-2 cells proteins were extracted with the kit Nucleospin RNA/Protein Kit (Macherey-Nagel) as per manufacturer’s instructions. Proteins from supernatant were concentrated with trichloroacetic acid/acetone, separated by SDS–polyacrylamide gel electrophoresis (4-15%) and transferred onto a nitrocellulose membrane (Life Science). Membranes were blocked with 1% milk, 1% BSA, incubated with anti-PRSS3 antibody (ab107430-Abcam) (1/100) overnight at 4°C and with
secondary antibody conjugated with HRP (1/3000, w4018-Promega). Immunoblots were visualized with chemiluminescence (Chemidoc XRS Bio-Rad).

**Immunofluorescence in biopsies.** Colonic samples were cut (5-μm thickness) with cryostat (Leica Microsystems, GmbH) as previously described (22). Sections were incubated with antibodies against trypsin-3 (1/500 #ab107430-Abcam) and the epithelial cell maker EpCAM/CD326 (1/500, #2929, Cell Signalling) followed by secondary antibody conjugated to AlexaFluor 488 donkey anti-rabbit IgG (1/500, #A21206, Molecular Probes) and 568 donkey anti-mouse IgG (1/500, #A10037, Molecular Probes). Slides were mounted with Prolong Gold Antifading Reagent with 4’,6’-diamidino-2-phenylindole (DAPI) (#P36935, Molecular Probes), to counterstain cell nuclei. The fluorescence intensity mean corresponds to trypsin-3-immunoreactivity in epithelial cells, as determined by EpCAM co-staining, and it was quantified with ImageJ software.

**Real-time PCR analysis.** Total RNA was extracted with the NucleoSpin RNA/Protein Kit (Macherey-Nagel, Hoerdt, France) and converted to complementary DNA using the Maxima First Strand cDNA Synthesis Kit for reverse transcription-quantitative PCR (RT-qPCR) (Thermo Scientific). Polymerase chain reaction was performed using SYBR Green Master I Kit (Roche), sense and antisense specific primers (see Table 2) in a LightCycler 480 Instrument (Roche). After amplification, the relative expression of mRNA was determined with method 2-DDCt by using hGAPDH as reference gene.

**Measures of paracellular Permeability.** Paracellular permeability of differentiated Caco-2 monolayer cells was monitored as previously described (45). Briefly, dextran-Fluorescein IsoThioCyanate (FITC) (4000 kDa, Sigma) was added into the apical compartment of a transwell, together with the corresponding reagent. Paracellular permeability was monitored by the passage of dextran from the apical to the basolateral medium, as previously described(42).

**Measure of CXCL8 protein expression.** Supernatant from cells were stocked at -80°C. Concentration of CXCL8 was measured by enzyme-linked immunosorbent assay (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions (46).

**Measurement of cell cytotoxicity with Lactate dehydrogenase (LDH).** Supernatant from treated cells or not treated cells were collected at 2, 4 and 6 hours. Cell
Results

cytotoxicity was measured following the protocol of Pierce™ LDH Cytotoxicity Assay Kit.

**Statistical Analysis.** Results are expressed as mean± SEM except for, immunostaining and *in situ* zymography quantification of patient biopsies, where each dot represents one patient. Statistical analyses were performed using GraphPad Prism 5.00 (GraphPad software, San Diego, CA) software package for PC. Multigroup comparisons were performed using a 1-way analysis of variance followed by a Bonferroni correction for multiple tests. Two-group comparisons were performed using an unpaired t test don’t assuming the Gaussian distribution. The Gaussian distribution was tested by Kolmogorov-Smirnov test. A value of P <0.05 was considered statistically significant. All P values were 2-sided.
Results

Table 1. Main active trypsin proteases secreted from Caco-2 cells. This list shows the active ABP-labelled proteases identified by LC-MS/MS analysis of pooled supernatant from caco-2 cells control and stimulated with thapsigargin. The table shows the respective gene symbol, protein name, predicted molecular weight and the ratio thapsigargin/control.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Protein Name</th>
<th>Predicted MW (in kDa)</th>
<th>Ratio Thaps/Control</th>
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<td>PRSS3P2</td>
<td>Putative Trypsin-6</td>
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<td>PSA</td>
<td>Kallikrein- Q8NHM43</td>
<td>103.3</td>
<td>1.83</td>
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<td>1.22</td>
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<td>BLMH</td>
<td>Bleomycin Hydrolase</td>
<td>52.6</td>
<td>0.89</td>
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<td>Cathepsin D</td>
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<td>0.81</td>
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<td>PSMA5</td>
<td>Proteasome Subunit Alpha 5</td>
<td>26.4</td>
<td>0.53</td>
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<tr>
<td>CASP14</td>
<td>Caspase 14</td>
<td>27.7</td>
<td>0.29</td>
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Table S1. Characteristics and outcomes of patients from which biopsies were collected. N, number. F, female. M, male.

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<th>CD</th>
<th>UC</th>
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<tr>
<td>Number (F/M)</td>
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<td>28 (14/14)</td>
<td>10 (6/4)</td>
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<tr>
<td>Age: median (years, range)</td>
<td>50.2 (18-77)</td>
<td>38.7 (23-69)</td>
<td>42.2 (26-62)</td>
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<tr>
<td>Without treatment</td>
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Table S2. Sequence of oligonucleotides used for RT-qPCR experiments. GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; PRSS3, protease serine 3 (mesotrypsin); PAR-2, Protease-activated receptor 2; PAR-4, Protease-activated receptor 4; HD1, human beta-defensin 1; HD2, human beta-defensin 2; MUC2, Mucin 2; TFF3, Trefoil Factor 3; CHOP, CCAAT-enhancer-binding protein homologous protein; XBP1s, X-Box Binding Protein 1 spliced; ATF4, Activating Transcription Factor 4; ATF6, Activating Transcription Factor 6, CXCL8, C-X-C Motif Chemokine Ligand 8.

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<th>Sense 5’ – 3’</th>
<th>Antisense 3’- 5’</th>
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**Acknowledgements.**

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**Conflict of Interest**

Authors have no conflict of interest to declare
References


**Figures legend.**

**Figure 1. Trypsin-like activity in ER stress-stimulated intestinal epithelial cells and in the intestinal mucosa of IBD patients.**

Biopsies from Healthy control (HC, n=14), Crohn’s Disease (CD, n=28) and Ucerative colitis (UC, n=10) were incubated for 1 hour in HBSS medium and Trypsin activity released by biopsies in culture supernatants was monitored. Data are expressed as mean ±SEM and were compared using Student's t-test. *P<0.05 vs. Healthy control group (A).

Human colonic tissues were obtained from individuals treated at the Centre Hospitalier de Toulouse (France) (Table S1). Biopsies from Healthy control (HC, n=14), Crohn Disease (CD, n=28 and Ucerative colitis (UC, n=10) were embedded in optimal cutting temperature (OCT) for in situ trypsin activity measures. Representative confocal photomicrographs of in situ zymography assays performed in colonic tissue slices (scale bar: 50μm). Graph representation of mean fluorescence intensity quantified from 6 to 12 patients per group. Data are expressed as mean±SEM and were compared using Student's t-test. *P<0.05 vs. Healthy control group. (B) Monolayers of differentiated Caco-2 cells cultured on Transwell system were stimulated with Tunicamycin (10μg/mL) or Thapsigargin (10μg/mL), two classic ER-Stress inducers and/or pre-treated with 1mM 4PBA, a chemical chaperon that inhibits ER-stress activation. (C-E) Trypsin-like activity was measured in (C) apical and (D) basal supernatants recovered from control or ER stress-treated Caco-2 cells and in (E) apical supernatant of ER stress-treated Caco-2 cells incubated with PBA for 6 hours. Assembled data from at least four independent experiments with 5 wells per set. Data are expressed as mean±SEM and were compared using Student's t-test. *p<0.05 and **p<0.01 and ***p<0.001 vs. control group.

**Figure 2. Inhibition and activity profile of active proteases present in supernatants from intestinal epithelial cells in response to ER-Stress.**

(A-C) Monolayers of differentiated Caco-2 cells cultured on Transwell system were stimulated with Thapsigargin (10μg/mL). (A, B) Incubation of apical supernatant of 6 hours ER-Stressed and control Caco-2 cells, with a Trypsin-specific activity-based probe (ABP), biotin-PK-DPP. (A) Trypsin-like activity was measured in supernatants after ABP binding. (B) Gel electrophoresis was performed to detect the molecular weight of active trypsin-like proteases. Samples from each condition were pre-incubated with serine
proteases inhibitor (AEBSF) to confirm the specificity of the serine protease band. (C) Relative ABP-33kDa protein quantification (D) Mass spectroscopy analysis by Student’s t-test. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

**Figure 3. Trypsin-3 expression in ER stress-stimulated intestinal epithelial cell supernatants and in colonic mucosa of UC and CD patients.**

Monolayers of differentiated Caco-2 cells cultured on Transwell system were stimulated with Thapsigargin (10µg/mL). Relative Trypsin-3 protein quantification by Western blot analysis from apical supernatant protein extracts of control and ER-stressed at 6 (left) and 24 hours (right) (A) Human colonic tissues were obtained from individuals treated at the Centre Hospitalier de Toulouse (France) (B) Biopsies from Healthy control (Ctrl), n=19), Crohn Disease (CD, n=16) and Ulcerative colitis (UC, n=11) were embedded in optimal cutting temperature (OCT) for immunostaining with anti-Trypsin-3 antibody and an epithelial cell maker EpCAM/CD326. Representative confocal photomicrographs of immunostaining performed in colonic tissue slices (scale bar: 50µm). Graph representation of mean fluorescence intensity quantified from 6 to 12 patients per group. Data are expressed as mean±SEM and were compared using Student's t-test. *P<0.05 vs. Healthy control group. (D) Relative mRNA expressions of XBP1-spliced form in colonic biopsies from healthy control (Ctrl), Crohn’s disease (CD) and Ulcerative colitis patients. Data are expressed as mean±SEM and were analysis by Student’s t-test. *p<0.05, **p<0.01 and ***p<0.001 vs control/healthy control group.

**Figure 4. Effects of protease inhibitors on ER stress-induced epithelial dysfunction in enterocytes**

(A-C) Monolayers of differentiated Caco-2 cells were cultivated in Transwell system, and were stimulated with Thapsigargin (10µg/mL) for 6 hours in presence or not of Trypsin inhibitors (AEBSF (200µM) or Leupeptin (50µM)). (A) Paracellular permeability was monitored by measuring the apical-to-basolateral flux of dextran 4kDa–FITC. (B) Relative mRNA expressions of β-Defensin-1, β-Defensin-2, Trefoiled factor-3, mucin-2 and CXCL8 after 6 hours of ER-Stress stimulation. (C) Levels of CxCL-8 monitored by Elisa, and released by Caco-2 cells in the apical and basolateral compartments of Transwell system. Data expressed as mean±SEM were compared using
one way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01, ***p<0.001, 
****p<0.0001 vs control group; ϕp<0.05, ϕϕp<0.01, ϕϕϕp<0.001 vs. thapsigargin group.

**Figure 5. Effects of protease inhibitors on ER stress-induced epithelial dysfunction in muco-secreting epithelial cells**

(A, B) Monolayers of HT29MTX cells were cultivated in Transwell system, and were stimulated with Thapsigargin (10µg/mL) for 6 hours in presence or not with Trypsin inhibitor (AEBSF (200µM)). (A) Relative mRNA expressions of β-Defensin-2, mucin-5 and mucin-2 after 6 hours of ER-Stress stimulation. (B) Levels of CxCL-8 monitored by Elisa, released by HT29MTX cells in the apical and basolateral compartments of Transwell system. Data expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). **p<0.01 and ***p<0.001 vs control group; ϕϕp<0.01 vs. thapsigargin group.

**Figure 6. Protease-Activated Receptor -2 and -4 expression and function in caco-2 cells treated or not with ER-stress inducer.**

Differentiated Caco-2 cells cultured in a transwell system were stimulated with Thapsigargin (10µg/mL) (A) Relative gene expression of PAR2 and -4 was quantified at 6 and 24 hours after stimulation. (B, C) Caco-2 cell monolayers were stimulated with Thapsigargin (10µg/mL) and pre-treated with antagonist PAR2 (GB83, 10µM) and/or PAR4 (ML354, 0.5µM). (B) paracellular permeability was measured at 6 and 24 hours and (C) CXCL8 was quantified in the apical and basal supernatant at 24 hours. Data expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01, ***p<0.001 vs control group; ϕϕp<0.01, ϕϕϕϕp<0.0001 vs. thapsigargin group.
Supplementary figures

Figure S1. Thapsigargin- and Tunicamycin-induced ER Stress markers and cell death.

(A, B) Monolayers of differentiated Caco-2 cells cultured in a transwell system were stimulated with Tunicamycin (10µg/mL) or Thapsigargin (10µg/mL) for 6 hours. (A) Relative gene expression of ER-stress markers, (XBP1s, ATF6, ATF4, CHOP) were measured. (B) Percentage of LDH (Lactate dehydrogenase) activity was measured in the culture media, 2, 4 and 6 hours after exposure to Tunicamycin or Thapsigargin. Data are expressed as mean±SEM and were analysis by Student’s t-test. **p<0.01 and ***p<0.001 vs. control group.

Figure S2. Trypsin-like activity in supernatants of human ER stress-stimulated muco-secreting cells.

(A) Monolayers of HT29MTX cells cultured in a Transwell system were stimulated with Thapsigargin (10µg/mL). (A) Trypsin-like activity was measured in apical and basal supernatants recovered from control or ER stress-treated HT29MTX cells for 2, 4 and 6 hours. Data are expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). *p<0.05 vs control group.

Figure S3. Trypsin-3 levels cellular expression upon ER-stress stimulation.

(A, B) Caco-2 cell cultured in transwells were stimulated with thapsigargin (10µg/mL). (A) Protein levels of trypsin-3 were measured by Western Blot at 6 and 24 hours. B-actin was used as a control. (B) Gene expression of PRSS-3, trypsin-3 precursor, was quantified at 6 hours and 24 hours after stimulation. (C) Monolayers of HT29MTX cells cultured in a Transwell system were stimulated with Thapsigargin (10µg/mL). Relative gene expression of PRSS3 was measured at 6 hours after ER-stress induction. Data are expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01 vs control group.

Figure S4. Effects of ER stress inhibitor PBA on ER-stress-induced epithelial dysfunctions.

(A-C) Monolayer of differentiated Caco-2 cells cultured in a transwell system were pre-treated with PBA (1mM) and followed by Thapsigargin (10µg/mL) stimulation. (A)
Paracellular permeability was measured after 6 hours stimulation. (B) Relative gene expression of Human β-Defensin-2, Trefoil factor-3, mucin-2 and CXCL8 was analyzed after 6 hours stimulation (C) CXCL8 from 6-wells supernatants was quantified by Elisa. Data are expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). **p<0.01, ***p<0.001 vs control group; ϕp<0.05, ϕϕp<0.01 vs. thapsigargin group.

Figure S5. Effects of protease inhibitor and of Trypsin-3 on ER-Stress induction.
(A) Caco-2 cells were stimulated with Thapsigargin in the presence or not of AEBSF. ER-stress markers including XBP1s, CHOP, ATF4 and ATF6 were measured. (B-C) Caco-2 cells were stimulated with Thapsigargin and with Trypsin-3 (1nM and 10 nM). (B) ER-stress markers (XBP1s, CHOP, ATF4 and ATF6) were measured. (C) CXCL8 from 6-wells supernatants was quantified by Elisa. Data are expressed as mean±SEM and were analysis by Student’s t-test. *p<0.05, **p<0.01, ***p<0.001 vs. control group. ϕϕp<0.01 vs. thapsigargin group.
Results

Figure 1

A

B

C

D

E

Figure 1

A

B

C

D

E
**Figure 2**

A) Trypsin activity (mU/mL)

B) ABP quantification

C) Fold change

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**Control**

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**Tg**

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**Results**

**Figure 3**

A

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Relative quantity of Trypsin-3 (arbitrary units)

Control | Thapsigargin
0 | 10 | 20 | 30 | 40 | 50 | **** |

B

Nucleus | Trypsin-3 | Epithelial cells | Merge

Control | ![Image] |
CD | ![Image] |
UC | ![Image] |

Intensity of epithelial expression (arbitrary units)

Control | Crohn disease | Ulcerative colitis
* | ** |

C

XBP1s relative expression

Ctrl | CD | UC
0 | 2 | 4 | 6 | 8 | *** | *** | *** | *** |

Ctrl | CD | UC
0 | 2 | 4 | 6 | 8 | *** | *** | *** | *** |
Figure 4

A 6 hours

B 6 Hours

C

Apical

Basolateral
Figure 5

A

6 Hours

B

CXCL8 (pg/mL)
Figure 6

A

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Fold increase (2^ΔΔct)

Control  Tg  Control  Tg  Control  Tg  Control  Tg

0  2  4  6

PAR2  PAR2/4

6 hours  24 hours

***  ***  *


B

6 Hours

Percent of dextran 4kDa-FITC

Control  Tg  Tg + Par-2  Tg + Par-4  Tg + Par-2/4

0  1  2  3  4  5

***  ***


C

24 Hours

Apical  Basolateral

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CXCL8 (pg/mL)

Control  Tg  Tg + Par-2  Tg + Par-4  Tg + Par-2/4

***  ***

ΦΦΦ  ΦΦΦ  ΦΦ ΦΦ

Apical  Basolateral
Supplementary Figure S1.

A

6 Hours

Fold increase (2^{ΔΔct})

Control Tunicamycin Thapsigargin

XBP1s

***

****

CHOP

***

***

ATF4

***

****

ATF6

B

% of LDH activity

Control Tunicamycin Thapsigargin

2 hours 4 hours 6 hours
Supplementary Figure S2.

**A**

**apical**

Trypsin activity (mmU/mL)

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**basal**

Trypsin activity (mmU/mL)

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Supplementary Figure S3.

A

6 Hours

Control | Thapsigargin

![Image of gel blots for Control and Thapsigargin at 6 Hours]

24 Hours

Control | Thapsigargin

![Image of gel blots for Control and Thapsigargin at 24 Hours]

B

PRSS-3

6 Hours

![Bar graph showing fold change in PRSS-3 expression at 6 Hours]

24 Hours

![Bar graph showing fold change in PRSS-3 expression at 24 Hours]

C

PRSS-3

![Bar graph showing fold change in PRSS-3 expression at 24 Hours]
Supplementary Figure S4.

A

6 Hours

B

MUC2

HBD2

CXCL8

MUC2

HBD2

CXCL8
Supplementary Figure S5.

A 6 Hours

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B 6 Hours

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C 6 Hours

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Caco-2 cells results not included in the paper

**Methodology**

**Cell culture and reagents.** Described in the paper. Before ER-stress induction, cells were pre-stimulated with antagonist PAR1 10µM (SCH79797, Tocris) or ML-7 20µg/mL (SIGMA) for 45 minutes.

**Real-time PCR analysis.** Described above on the paper. Primer sequence Table 1.

**Measures of paracellular Permeability.** Described in the paper

**Measure of CXCL8 protein expression.** Described in the paper

**Measurement of cell cytotoxicity with Lactate dehydrogenase (LDH).** Described in the paper

**Measurement of PAR2 cleavage.** CHO cells co-expressing luciferase PAR2 were obtained by Morley Hollenberg and Rithwick Ramachandran, Calgary, Canada). CHO-lucPAR2 cells were routinely grown at 37°C in a 5% CO2 water-saturated atmosphere in Ham F12 GlutaMAX (Gibco) supplemented with 10% of heat inactivated fetal bovine serum (Biowest, Nuailé). Cultured medium was changed three times per week and cells passed once a week. 15000 cells were seeded in 96 well-plates (Thermos Fisher) for 2 days. Before stimulation cells were washed 2 times with ca2+/Mg2+-free PBS (Sigma). Then, cells were incubated with 100µL of cell supernatant from non-stimulated cells or thapsigargin-stimulated caco-2, followed by 15 min of incubation at RT. Supernatant was collected and transferred to a new 96 well plate (sigma). The plate was centrifuged 5 min at 1200 rtm. Next, 80 µL of supernatant was transfer to a white 96 well plate (Corning). Luciferase substrate was added (nanoglo luciferase assay promega #N1110) and the plate was imminently read in the dark on a microplate reader 96-well plate NOVOstar at. Several dilutions of trypsin from porcine pancreas (sigma) (1U/mL) were used as a positive control and thrombin from human plasma (sigma) (1U/mL) as a negative control.

**Results**

**mRNA expression of PRSS-1 and -2 increased after 6 hours of ER-stress induction**

As shown previously, ER-stress induces increased trypsin-like activity in the apical compartment of intestinal epithelial cells. Trypsin-like substrate is cleaved by the three main trypsin forms including trypsin-1, trypsin-2 and trypsin-3, (PRSS-1, -2 and -3 trypsinogen genes respectively), and also by tryptase. Previous data from the laboratory have demonstrated that intestinal epithelial cells did not express tryptase (Rolland-Fourcade et al. Gut 2017). Thus, 6 hours after ER-stress induction, mRNA expression of these three main trypsinogen genes present in intestinal epithelial cells, PRSS-1, PRSS-2 and PRSS-3 were quantified (Figure 7A). At this time-point, only PRSS1 and PRSS2 but not PRSS3 mRNA expression were significantly up-regulated. In addition, mRNA levels of matriptase I, elastase-2A and elafin (an elastase inhibitor) were analyzed, showing that Elastase-2A was strongly up-regulated, while its inhibitor Elafin was significantly down-regulated (Figure 7B). The expression of Matryptase-1 was not modified by exposure to ER stress inducers (Figure 7B).
Trypsin activity alters barrier function of Caco-2 monolayers by activating PAR2 and -4 receptors but not PAR1

ER stress-associated increased paracellular permeability was due to trypsin proteolytic activity and was blocked by pre-treating caco-2 cells with PAR2 antagonist (GB83) and PAR4 antagonist (ML354). Pre-treatment with the PAR1 antagonist (SCH79797) aggravated the increase in paracellular permeability associated with ER stress (Figure 8). Combination of PAR1 and PAR2 antagonist and combination of PAR1 and PAR4 antagonist pre-treatments have no additional effect, both normalized permeability. Similar results were observed at 6 (Figure 8A) and at 24 hours (Figure 8B).

PAR1 antagonist decreased ER stress-associated CXCL8 secretion

The release of CXCL8 in apical and basal compartment of ER stress-stimulated cells was normalized to control levels by pre-incubation with PAR1 antagonist. The addition of PAR2 and -4 antagonists on the contrary enhanced the release of CXCL8 both in the apical and basal compartments (Figure 9). These data suggest that each PAR signaling pathway play a different role protecting or harming CXCL8 release.

Trypsin proteolytic activity from stressed caco-2 cells cleave PAR2

Next, we have examined the ability of supernatants from ER stress-stimulated cells to cleave PAR2 receptors. Stable cell line carrying the firefly luciferase PAR2 was exposed to supernatants of ER-stress-induced cells and control. Supernatants from stressed cells strongly cleaved PAR2 compared to non-stimulated cell supernatant (Figure 10).

Increased permeability is MLCK-dependent

Activation of PAR2 leads to phosphorylation of MLC via myosin light chain kinase which, in turn, induces cytoskeleton contraction. To study whether PAR2-MLCK was the mechanism used to increase permeability, ML-7, a MLCK inhibitor, was used. 6 and 24 hours after ML7 pre-treatment, stressed cells exhibited normalized paracellular permeability (Figure 11).

Discussion and conclusions

See below in General discussion and conclusions.
**Figures**

**A**

**PRSS1**

PRSS1

0.0
0.5
1.0
1.5
2.0
2.5
Fold increase (2^△△ct)

**PRSS2**

PRSS2

0.0
0.5
1.0
1.5
2.0
2.5
Fold increase (2^△△ct)

**PRSS3**

PRSS3

0.0
0.5
1.0
1.5
Fold increase (2^△△ct)

**Thapsigargin**

Control

**B**

**Matriptase I**

Matriptase I

0.0
0.5
1.0
1.5
Fold increase (2^△△ct)

**Elafin**

Elafin

0.0
0.5
1.0
1.5
Fold increase (2^△△ct)

**Elastase 2A**

Elastase 2A

0
2
4
6
Fold increase (2^△△ct)

---

**Figure 7.** mRNA expression of other serine proteases or inhibitors. (A-B) Monolayers of Caco-2 cells were stimulated with thapsigargin for 6 hours. Relative mRNA expressions of PRSS-1, PRSS-2 and PRSS-3 (A), matriptase 2, Elafin and Elastase 2a (B) were quantified. At least n=8 well/condition. Data expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01 vs control group.

**Figure 8.** The impact of PAR1 antagonist on intestinal barrier homeostasis. (A-B) Monolayer of caco-2 cells cultured in a transwell stimulated with thapsigargin and were pre-treated with antagonist PAR1, -2 and -4. (A) Paracellular permeability was monitored by measuring the apical-to-basolateral flux of dextran 4kDa–FITC after 6 hours stimulation (A) and 24 hours (B). Data expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test).
***p<0.001 vs control group; ϕp<0.05, ϕϕp<0.01, ϕϕϕp<0.001, ϕϕϕϕp<0.0001 vs. thapsigargin group.

Figure 9. **PAR1 antagonist decrease CXCL8 release.** Monolayer of caco-2 cells cultured in a transwell stimulated with thapsigargin and were pre-treated with antagonist PAR1, -2 and -4. Levels of CXCL8 were monitored by Elisa in apical and basal supernatant of the transwell system. Data expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). ***p<0.001 vs control group; ϕp<0.05, ϕϕp<0.01, ϕϕϕp<0.001, ϕϕϕϕ p<0.0001 vs. thapsigargin group.

Figure 10. **PAR2 cleavage.** Monolayer of caco-2 cells cultured in a transwell were stimulated with thapsigargin for 6 hours. Supernatant was collected. Stable cell line carrying the firefly luciferase PAR2 were incubated with supernatant of stressed cells. PAR2 cleavage was measured. *p<0.05 vs control group
**Figure 11. The impact of MLCK on permeability.** Monolayer of caco-2 cells cultured in a transwell stimulated with thapsigargin were pre-treated with ML-7, a MLCK inhibitor. Paracellular permeability was monitored by measuring the apical-to-basolateral flux of dextran 4kDa–FITC after 6 and 24 hours after thapsigargin and ML-7 treatment. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test). ****p<0.0001 vs control group. ϕϕp<0.01, ϕϕϕp<0.001 vs. Thapsigargin group.
Results

HT29-mtx results

Methodology

Cell culture and reagents. Described in the paper.

Real-time PCR analysis. Described in the paper. Primer sequence Table 1.

Measure of CXCL8 protein expression. Described in the paper

Results

ER-stress increased the three trypsinogen genes in HT29mtx cells

Similar to what was observed in caco-2 cells, stressed HT29-mtx cells showed upregulation of PRSS-1, PRSS-2 after 6 hours of induction. However, stressed mucus-secreting cells also showed upregulated mRNA levels of PRSS-3 mRNA (Figure 12). This result, together with trypsin like activity, confirmed that ER-stress induces trypsin-like proteolytic activity at the apical side of IECs, and an increased expression of trypsin-3.

Trypsin activity alters barrier homeostasis

Stressed HT29mtx with or without serine protease inhibitors displayed upregulated mRNA levels of HBD2 and MUC2 (Figure 13A). Moreover, apical release of CXCL8 quantified by ELISA increased after 24 hours stimulation in stressed cells. A small increase of CXCL8 was observed in the presence of serine proteases inhibitors (Figure 13B). Although more experiments should be done to confirm mRNA results (n=3 samples/condition), these data showed, like in Caco2, that ER-stress activation strongly alters intestinal barrier function, but in contrast to Caco2, HT29-MTX did not seem to use the release of trypsin-like activity to control HBD2 expression. Like in Caco2 cells however, trypsin activity released by thapsigargin-treated HT29-MTX was not responsible for the increased release of CXCL8 in the basal or apical compartment of monolayers, nor for the overexpression of MUC2 mRNA.

mRNA levels of PAR2 and -4 increased under ER-stress

Stressed HT29-mtx cells showed upregulation of mRNA levels of PAR2 and -4, while inhibition of trypsin proteolytic activity, by AEBSF, normalized PAR4 mRNA levels (Figure 14). These data together with Caco-2 results presented in the article, evidenced an association between ER-stress and the up-regulation of PARs expression. However, the number of samples should be increased to confirm these results.

Discussion and conclusion

Taken together, these data suggest that mucus-secreting cell line mostly behaves like enterocytes under ER-stress induction. Both cell lines increased trypsin proteolytic activity at the apical side of the monolayers, and several biological changes associated with ER stress induction are mediated by this increased trypsin-like activity. In HT29-MTX, PRSS-3 mRNA expression was increased as early as 6 hours after ER stress stimulation, suggesting that trypsin-3 could also be the main candidate to explain the increased trypsin-like activity in monolayer supernatants. PAR2
and -4, are also upregulated in HT29MTX cells under ER-stress. Although the effects of PAR antagonists were not tested in HT29MTX cells, the fact that PAR2 and PAR4 are up-regulated could suggest a similar mechanisms as in Caco2, involving these two receptors. In conclusion, these data suggest that ER-stress on mucus secreting cells increases trypsin proteolysis and induces inflammatory signs disrupting intestinal barrier likewise stressed caco-2.

**Figures**

**Figure 12.** mRNA relative expression of the three trypsinogen genes. Monolayers of HT29MTX cells were stimulated with Thapsigargin for 6 hour. mRNA levels of PRSS-1, PRSS-2 and PRSS-3 were quantified. At least n=8 wells/ condition. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01 vs control group.

**Figure 13.** ER-stress alters intestinal homeostasis. Monolayers of HT29MTX cells cultivated in Transwells, were stimulated with Thapsigargin for 6 hours in presence or not of serine protease inhibitor. (A) Relative mRNA expressions of β-Defensin-2 and mucin-2 were quantified. (B) Levels of CXCL8 monitored by Elisa, at the apical and basolateral compartments of a Transwell system. Data expressed as mean±SEM were compared using one way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01 and ***p<0.001 vs control group. ϕp<0.05 vs. thapsigargin group.
Figure 14. Up-regulation of PAR2 and -4 in HT29-mtx cells treated with ER-stress inducer. Differentiated Caco-2 cells cultured in a transwell system were stimulated with Thapsigargin (10µg/mL) in the presence or not of AEBSF (200µM). Relative gene expression of PAR2 and -4 was quantified at 6 hours after stimulation. At least 3 samples/condition. Data expressed as mean±SEM were compared using one-way non-parametric anova (Bonferroni test). *p<0.05, **p<0.01 vs control group.
General discussion and conclusions
Discussion

In our study, we demonstrated that epithelial colonic mucosa constitutes for a large part the origin of increased trypsin proteolytic activity in the lumen of CD and UC patients compared to healthy controls. Most of earlier studies have focused on infiltrated immune cells and microbiota to explain the origin of upregulated expression of proteinases (Nathalie Vergnolle 2016). However, N. Vergnolle’s team suggested in 2012 that intestinal epithelial cells could be a potential source of proteases during gastrointestinal pathologies (J. P. Motta et al. 2012). Affected and unaffected tissue of UC and CD showed increased elastolytic and trypsin activity detected on mucosal tissue (J. P. Motta et al. 2012). Moreover, patients with IBS exhibited increased levels of a set of serine proteases including tryptase, elastase, trypsin or cathepsin G in the stools compared to healthy controls (Nathalie Vergnolle 2016; Schmid et al. 2007; J. P. Motta et al. 2012). Our result reinforces the previous data, strengthening the concept that intestinal epithelial cells are major producers of proteases in gastrointestinal pathologies.

**Autophagy** stimulation increased cysteine protease activity but decreased trypsin-like proteolytic activity. Moreover, mRNA expression of specific serine proteases such as PRSS1, -2, -3, matriptase-1 and the serine protease inhibitor, elafin, were downregulated. Autophagy alters intestinal homeostasis as evidenced by increased paracellular permeability or mRNA levels of TFF3. Increased permeability was normalized when cysteine proteases inhibitor was added to the culture. These data demonstrate that active autophagy downregulates trypsin proteases expression, while it induces a strong release of cysteine proteolytic activity which, in turn, alters intestinal barrier. Increased permeability is associated with the mRNA downregulation of matriptase-1, which is a membrane proteases known to keep TJ homeostasis. On the contrary, upregulation of TFF3 suggest that the cell is working to repair the causes of damage. Two different papers have demonstrated the association between TFF3 and cell-cell adhesion. TFF3 upregulates the expression of claudin-1 and ZO-1, two crucial TJ, while decreased claudin-2 expression. Moreover, TFF3 facilitates cell migration in damage mucosa (Meyer zum Büschenfelde, Tauber, and Huber 2006; Xu et al. 2012). Necrosis is associated with increased levels of caspases, a group of cysteine proteases (Negroni, Cucchiara, and Stronati 2015). Since our results showed increased levels of cysteine protease activity, it was important to assess cell death in our experimental conditions. LDH activity was quantified as a marker of cell death by necrosis, which releases freely into the medium intracellular enzymes such as LDH. Indeed, LDH activity was not increased suggesting that necrosis is not associated with the experimental induction of autophagy. However, other forms of cell death such as apoptosis could occur, LDH being trapped within granules during apoptosis is not increased in cell supernatants. Therefore, we cannot exclude the occurrence of apoptosis in our experimental conditions. To eliminate the possibility that NS model induce apoptosis, further experiments should analyse the mRNA levels of caspases and apoptosis assays such as Tunel or Annexin V assay.

As a protective mechanism upon autophagy induction, intestinal cells might reduce their activity to be able to keep essential mechanisms for cell maintenance. Thus, we confirmed here that although autophagy induction is not responsible for trypsin activity increase, it does modulate cysteine proteases balance. Indeed, cysteine protease activity has also been found upregulated in IBD patients (Menzel et al. 2006; Flood et al. 2015). In further experiments, we could use Caco-2 cells bearing the ATG16L1 mutation, and investigate proteolytic activity (both trypsin-like and cysteine activities) released by those cells, compared to wild-type controls. These cells carry the
same ATG16L1 polymorphism associated with CD, and constitute therefore a very useful tool to study the cellular mechanisms associating potentially epithelial cell proteases and CD pathogenesis.

Although **ER-stress** is active at basal levels, prolonged stress might contribute to IBD pathogenesis by compromising protein secretion, mediating intestinal epithelial cell apoptosis, mucosal barrier dysfunction and induction of inflammatory response in the gut (Cao 2016, 2015). Polarized Caco-2 cell line stimulated with Tunicamycin or Thapsigargin, two well-known ER-stress inducers, increased the release of trypsin-like activity at the apical side. Similar results are observed in HT-29mtx mucus-secreting cell line. ER-stress induction was confirmed by mRNA expression of XBP1s, CHOP, ATF6 and -4. These results demonstrate that aberrant ER-stress modulates trypsin proteolytic activity. Moreover, our results showed upregulation of PRSS-1, -2 and elastase 2 after 6 hours of ER-stress stimulation. mRNA levels of PRSS-3 and matriptase-1 remained unchanged in Caco2 cells at this time-point, while elafin expression was downregulated. However, mRNA levels of PRSS-3 increased 24 hours after induction in Caco2 cells, and was significantly up-regulated as soon as 6h after ER stress induction in HT29MTX cells. Together these results showed that ER stress profoundly altered proteolytic homeostasis in intestinal epithelial cells, not only at the granule secretion level, but also at the transcriptional level.

**The impact of ER-stress on intestinal barrier homeostasis**

In the present study, we better analyzed the impact of **ER-stress on epithelial biology**. IBD are characterized by impaired intestinal barrier including altered intestinal permeability, activated inflammatory response and disruption of the gut shield along with decreased AMPs and thinner mucus layer. In both cell lines and animal models, deletion of UPR factors lead to spontaneous inflammation and exacerbated colitis. Knockdown of XBP1 in ileal epithelium causes hyperactivity of IRE1a increasing inflammatory response in the gut and activating JNK and NF-κB (A. Kaser et al. 2008). Monolayers of polarized Caco-2 cells treated with Thapsigargin increased permeability compared to control. It has been demonstrated that proteases can increase intestinal permeability either directly by degradation of tight junctions or by receptor-mediated signaling pathway. In colonic biopsies of IBS patients, permeability was found increased pointing out to cysteine proteases from microbiota as the main responsible for TJ degradation (Piche et al. 2009; Z. Zhang et al. 2000; Wan et al. 1999). Other studies have shown the ability of PAR2 activation to increase intestinal permeability via MLC phosphorylation (Cenac et al. 2004). Thus, both hypothesis highlight the lumen as possible main location for proteases responsible for the increased intestinal permeability either by TJ degradation (Annaházi et al. 2013) or by PAR2 via MLCK involvement. Our data showed that stressed Caco-2 cell monolayers pre-treated with ML-7, a MLCK inhibitor, normalized permeability compared to control conditions. These results suggest that trypsin activity alters permeability via MLCK rather than degrading TJ, and are in favor of a receptor-mediated mechanism to explain the permeability effects of ER stress-associated trypsin activity. Indeed, our other results confirmed the implication of both PAR2 and PAR4 in ER stress-associated increased permeability, while previous studies have clearly demonstrated that PAR2 activation is linked to MLC phosphorylation (Cenac et al. 2004)

Importantly, our work reports for the first time that ER-stress is associated to increased permeability, and provides mechanisms associating the release of trypsin-like activity and the activation of PAR2 and PAR4.
Intestinal epithelia produce and release specific weapon peptides against pathogens, in the mucus of the gut. Although, Paneth cells are specialized for the storage and secretion of AMPs and goblet cells are responsible for mucus and AMPs secretion, *enterocytes* are also able to produce and secrete a variety of them. As previously mentioned, several publications have shown that aberrant UPR in IEC causes accumulation of MUC2 precursor on goblet cells (Tsuru et al. 2013). Epithelial cells constitutively express HBD1, while HBD2 is induced under inflammation (O’Neil et al. 1999). Rat model of DSS-induced colitis displayed increased expression of TFF3, a bioactive peptide responsible for maintaining intestinal epithelial homeostasis, in inflamed colonic mucosa (Renes et al. 2002). Moreover *in vitro* experiments demonstrated that TFF3 regulates permeability by upregulating claudin-1 and ZO-1, two crucial TJ for intestinal integrity and downregulating claudin-2, known to increase TEER (Meyer zum Büschenfelde, Tauber, and Huber 2006; Xu et al. 2012). Therefore, we investigated whether ER-stress induction modified AMPs or mucus gene expression. Stressed caco-2 cells displayed upregulated mRNA levels of HBD2, MUC2 and TFF3 while HBD1 remained unchanged. These results demonstrated that ER-stress is involved in the alteration of intestinal function.

We further analyzed whether ER-stress had an impact on the inflammatory response. CXCL8, a neutrophil chemoattractant factor, is involved in the initialization of inflammation, released from different cell subtypes in response to inflammatory stimuli. Besides pro-inflammatory effects, it has been suggested that CXCL8 is involved in immature intestinal development including intestinal maturation, differentiation, migration and prevention from injury and cell death (Nguyen et al. 2014; Maheshwari et al. 2004, 2002). High quantities of CXCL8, also named as IL-8, are found in inflammatory mucosa of IBD patients. Our *in vitro* results showed increased CXCL8 in the apical and basal supernatant of ER-stress cells. Both pro-inflammatory factors are induced by the transcription factor NF-κB, which activation induces further ER-stress and PAR downstream pathways (Mahdi, Rizvi, and Parveen 2016). We confirmed that ER-stress induction promotes inflammatory signs. We, thus, suggest that increased release of CXCL8 at the basal side of polarized IECs could attract inflammatory cells to fight possible bacteria infiltration, while apical CXCL8 release might be involved in restoration of intestinal homeostasis and repair.

**Trypsin-3 a protease increased in UC and stressed caco-2 cells**

A major aim of our studies was to determine the type of serine protease increased in supernatants of stressed epithelial cells of IBD patients.

As mentioned before, colonic biopsies of UC and CD patients displayed increased trypsin activity in IECs. By immunohistochemistry, our results showed trypsin-3 detected in epithelial cells of UC but not in CD biopsies. Moreover, relative mRNA expression of PRSS-3 is downregulated in patients with UC compared to control or CD (data from Dr. A. Denadi Souza, post-doctoral fellow in the laboratory, data not shown). These results suggested that abnormal ER-stress is associated with increased trypsin-3 protein in epithelial cells and outside epithelial cells, and that it could be used as a marker to discriminate between UC and CD. Although little is known about trypsin-3, it has been found involved in several pathologies such as IBS or breast, lung, prostate, and pancreatic cancer. mRNA isoform of trypsin-3, PRSS-3, plays an important role in progression, transendothelial migration, metastasis and prognosis of human cancer besides neuronal sensing, visceral hypersensitivity and intestinal permeability (Hockla et al. 2012; Rolland-Fourcade et al. 2017; Cottrell et al. 2004; Takeuchi, Shuman, and Craik 1999; Diederichs et al. 2004; Jiang et al. 2010). Our data reported increased ER-stress signalling pathway, confirmed by XBP1s expression,
in UC but not CD. These data suggest that the correlation between trypsin-3 release and abnormal ER-stress response is a feature of UC but not CD.

Moreover, our in vitro model also exhibited trypsin-3 as a trypsin type increased in stressed cells supernatant. By activity-based prove approach, supernatant from stressed cells showed a unique and specific band at molecular weight of 33 kDa compared to control or stressed samples treated with AEBSF, a serine protease inhibitor used as negative control to verify the specificity of active trypsin proteases. 33 kDa corresponds to the known molecular weight of trypsin-3. In addition, trypsin activity was lost in stressed supernatants treated with ABP, confirming that ABP binds effectively to the active protease site detected in trypsin activity assays. ABP result was confirmed by western blot, detecting trypsin-3 levels increased compared to non-stressed supernatants. In addition, just like ER stress-associated trypsin activity, the release of trypsin-3 was polarized on the apical compartment. Intracellular levels of trypsin-3 remained unchanged in stressed and non-stressed conditions, indicating that the first level of regulation is the release of trypsin-3 intracellular stocks rather than the level of intracellular protein expression. Although trypsin-3 appears as a serious candidate to explain the increased proteolytic activity associated with ER stress, one cannot rule out the possible involvement of other proteases. In particular, mRNA levels of PRSS1 and -2 were upregulated upon ER stress induction. We could not assess specifically the expression of these proteases as no specific antibodies are available to discriminate between trypsin-1, trypsin-2 and the trypsin common precursors. It is therefore not known whether PRSS1 and PRSS2 are translated into proteins.

Our results point to a polarized release of trypsin-3 at the apical compartment of enterocyte monolayers under ER stress conditions. The next step would be to identify whether specific inhibition of trypsin-3 in intestinal epithelial cells submitted to ER stress would completely inhibit the released trypsin-like activity and the ER-associated effects. However, no specific inhibitors have been reported so far for trypsin-3 and the antibody we used was not able to block trypsin-3 activity (in enzyme assays performed with pure trypsin-3). We have tried to use a shRNA approach in Caco2 cells, in order to down-regulate the expression of Trypsin-3, but the expression of PRSS1 and PRSS2 were also down-regulated by this approach. Selective inhibition of trypsin-3 activity could also be useful in in situ zymography of UC patients, in order to determine whether or not trypsin-3 is the protease responsible for increased activity in intestinal epithelial cells from those patients. Therefore, although strong presumptions point out trypsin-3 as the molecular target to inhibit ER stress disruptive effects associated with UC, we still need better tools to fully confirm this hypothesis.

The association between ER stress-induced increased trypsin proteolytic activity and intestinal biology

We aimed at determining whether dys-regulation of intestinal barrier was due to ER-stress itself or due to the large amount of trypsin released at the apical side of polarized enterocytes. Stressed caco-2 cells cultured in a transwell co-treated with AEBSF or Leupeptin, two serine proteases inhibitors, had a restored paracellular permeability, compared to un-stressed cells. These results confirm that disruption of intestinal barrier is due to increased serine proteases. However, surprisingly, inhibition of serine proteolytic activity aggravates the secretion of CXCL8, both in the apical and basal compartments of stressed supernatants. Previous studies have shown that specific components, such as LPS or transforming growth factor-β act as anti-inflammatory mediator attenuating the production of IL-1β and CXCL8 in response to inflammation (Rautava et
al. 2012, 2011; Fujihara et al. 2003). By proteolytic processing, some proteases are required to mature some antimicrobial peptides such as the human defensin-5 or to increase the chemotactic activity of CXCL8 (proteinase-3) or CXCL5 (cathepsin G) (Manna and Ramesh 2005; Nufer, Corbett, and Walz 1999). In contrast, proteases like cathepsin G, proteinase-3 or elastase 2 can also degrade cytokines including IL-6 or TNFα as well as some proteins involved in cellular contacts (Bank et al. 1999; Chin et al. 2008). We thus, could hypothesise that active proteases degrade CXCL8, mediating anti-inflammatory effects. The results generated with the PAR2 and PAR4 antagonists contradicted this hypothesis since PAR blockade also increased the release of CXCL8 in cell culture media after ER stress, suggesting that a PAR-mediated effect is responsible for the enhanced CXCL8 release.

**Activation of PARs by trypsin might be the molecular mechanism to disrupt intestinal homeostasis**

Protease mechanism of action is transmitted through the cleavage of protein substrates or receptors which modulates irreversibly their function. Beside molecular maturation or degradation as mentioned above, protease-mediated signalling can be established through the proteolytic cleavage of the Protease-activated receptor (PAR). PARs are widely expressed along the GI on several cell types including enterocytes, endothelial cells, neurons and inflammatory cells among others. The activation of the receptor is involved in GI physiological processes as visceral sensitivity, secretion, motility, intestinal permeability and gut immune response (Coelho et al. 2002; Nathalie Vergnolle and Chignard 2006). Our cell culture studies evidenced that induction of ER-stress increased the gene transcription of PAR2 and -4 and moreover, inhibition of serine proteases normalized their mRNA expression at 24 and 6 hours, respectively. Likewise observed in previous data, our results have shown a reduced permeability when stressed cells were pre-treated with antagonists of PAR2 and -4. Surprisingly, pre-treatment with antagonist PAR1 aggravated paracellular permeability compared to stressed-cells. A recent study from the lab demonstrated that trypsin-3 cleaves and activates PAR2, leading to visceral hypersensitivity (Rolland-Fourcade et al. 2017). We confirm that in our model, trypsin-3 also regulates permeability through PAR2 and -4.

As mentioned above, stressed Caco-2 cells treated with ML-7 normalized permeability, suggesting that PAR-2 and -4 activated by the trypsin activity, can activate MLCK signaling pathway disrupting TJ (Cenac et al. 2004). Although PAR-1-mediated mechanism is poorly understood, PAR-1 activation induces apoptosis leading to intestinal barrier dysfunction (Chin et al. 2003). Thus, it is surprising that blockade of PAR1 in caco-2 cells monolayers increased intestinal permeability since our data suggest a protective role. However, we hypothesized that inhibition of PAR1 let the other two receptors PAR2 and -4 more available for trypsin, thus, due to over-activation of PAR2 and -4, permeability could be increased. Nonetheless, the use of PAR1 antagonist in this study had some limitations, since we notably suspected a detrimental effect of this PAR1 antagonist. My team have used the same antagonist to treat organoids for 72 hours, detecting cell death caused by this PAR1 antagonist. Although in this project the time course used was 6 hours, cells could start undergoing apoptosis, therefore altering the results. Thus, permeability is increased and CXCL8 decreased when cells are pre-treated with PAR1 antagonist. CHO cells carrying the firefly luciferase PAR2 evidenced that ER-stress supernatant was able to cleave the receptor compared to control supernatants. Firefly luciferase PAR4 were not available in the lab, thus we cannot conclude on whether ER-stress supernatant can cleaves it. Taken
together, these analyses demonstrated that PAR2 and PAR4 might be activated by trypsin-3, released due to ER-stress induction, which in turn, enhanced permeability by a MLCK-dependent mechanism.

**Interaction between ER-stress and trypsin proteases.**

Finally, we investigated whether excessive release of active trypsin was able to modulate the induction of ER-stress. First, we have shown that serine protease inhibitor do not modulate ER-stress markers in stressed cells. Second, caco-2 cells treated with trypsin-3 failed to induce any signs of ER-stress. Moreover, levels of CXCL8 remained stable in cells treated with trypsin-3. Thus, we demonstrated that ER-stress is able to trigger the release of Trypsin-3 while trypsin-3 is unable to induce ER-stress and that trypsin-3 alone, without an inflammatory context, is not able to induce CXCL8.

**Trypsin-3 inhibitor as a possible treatment for UC patients**

Patients with abnormal activity of ER-stress display increased trypsin-3 in intestinal epithelial cells. Increased trypsin proteolytic activity is responsible for intestinal barrier alteration while ER-stress induces inflammation. Our results suggest that Trypsin-3 inhibition could be beneficial in the course of IBD, and specifically for UC patients. However, one has to wonder which patients might benefit the most from such treatment, and when would be the best time and in which combination with potential other treatments this approach could be used? Pentasa (Mesalamine) is used to induce remission in mild to moderate active UC. Ingested orally, pentasa is slowly released into the body, acting locally in the colon instead of systemically. The mechanisms of action of Mesalamine include the inhibition of lipoxygenase and cyclooxygenase pathways, cytokine inhibition (interleukin-1, interleukin-2, TNF). It also acts as a potent antioxidant and a free-radical scavenger, as well as a Peroxisome proliferator-activated receptor (PPAR)-gamma. Through all these mechanisms, Mesalamine treatment usually achieve a good inhibition of cell recruitment and a fair repair of damaged tissues. However, the effects that Mesalamine treatments have on epithelial homeostasis are most likely indirect. No direct effect for this treatment on epithelial repair or on reinforcement of epithelial barrier have ever been demonstrated. Therefore, there might be some space for a combinatory treatment in mild and moderate UC patients that would target a fast repair of epithelial functions and integrity. Anti-trypsin-3 treatment might well fit within that space. While classical anti-inflammatory drugs such as Mesalamine would suppress the inflammation and inflammatory cell recruitment, trypsin-3 inhibition would repair and reconstruct rapidly the damaged mucosa.

ER-stress inhibition though would not be a good strategy since ER stress is an essential cellular mechanism, especially for secreting cells like Paneth and Goblet cells. Anti-inflammatory drugs have to be taken during active phases, until remission is achieved. But one can imagine that low doses of trypsin-3 inhibitors could be taken even in remission phases. Since ER-stress is constitutively active in these patients, trypsin-3 inhibition would lower the pro-inflammatory effects of constitutive up-regulation of ER stress associated with UC and might maintain remissions for a longer period.
Conclusion

Among the three main cellular pathways associated with IBD (NOD2, Autophagy and ER stress), we demonstrated that only ER stress is associated with increased trypsin-like activity. Most importantly, we have identified here the downstream mechanisms of ER stress in intestinal epithelial cells. Our results point to trypsin activity, and in particular trypsin-3 release, as possible new therapeutic targets in the context of intestinal inflammation that involves ER stress.
Figure 15. The three main IBD pathways and trypsin activity. Under physiological conditions, basal levels of trypsin-like are released. Nod2 activation does not modify trypsin activity secretion. Autophagy reduces trypsin proteolytic activity released by IECs and their genetic expression. ER-stress induces the secretion of trypsin activity in the apical compartment of polarized IECs together with mRNA upregulation of PRSS1,-2, elastase 2A, downregulation of elafin and no changes of PRSS3.
Figure 16. The impact of ER-stress on intestinal biology. ER-stress increased paracellular permeability which was normalized blocking MLCK with ML-7. ER-stress also enhanced the secretion of CXCL8 apically and basally and upregulated TFF3, HBD2 and MUC2.
Figure 17. **Impact of proteases on intestinal biology.** Trypsin-3 increases permeability by activating PAR-2 and -4, which in turn, phosphorylates MLCK. By inhibiting serine proteases (AEBSF), PAR-2 (GB83) or PAR-4 (ML354) permeability is normalized to basal levels. Inhibition of proteases restore mRNA of TFF3 but not HB2 or MUC2. Inhibition of serine proteases or PAR-2 and PAR-4 increases CxLC-8 secretion at the apical and basal side of IEC.
**Figure 18. Impact of ER-stress on trypsin activity secretion.** ER-stress induces trypsin-3 release but trypsin-3 is not able to induce ER-stress.
Annex
Portfolio

Article Publications
Alexandre Denadai-Souza, Chrystelle Bonnart, Núria Solà Tapias et al. • Functional Proteomic Profiling of Secreted Serine Proteases in Health and Inflammatory Bowel Disease. Scientific Report

Núria Solà Tapias et al. • Ulcerative colitis-associated endoplasmic reticulum stress induces trypsin activity affecting intestinal epithelial response. • In preparation

Oral presentations
Presented by N. Solà Tapias

Presented by N. Vergnolle

April 2017 Experimental Biology • Chicago, USA • Solà Tapias N, et al. Autophagy controls epithelial proteolytic homeostasis of intestinal mucosa.

Poster presentations
Presented by N. Solà Tapias

Presented by N. Vergnolle
Oct 2016 United European Gastroenterology (UEG) • Vienna, Austria • Solà Tapias N, et al. Endoplasmic reticulum stress enhances the release of serine proteases by epithelial cells altering the intestinal barrier function.

May 2016 Digestive Disease Week (DDW) • San Diego, USA • Solà Tapias N, et al. Endoplasmic reticulum stress alters the gut barrier function by modulating the proteolytic activity of intestinal.

May 2016 Digestive Disease Week (DDW) • San Diego, USA • Solà Tapias N, et al. Increase of cysteine protease activity induced by autophagy alters the intestinal barrier homeostasis.
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Functional Proteomic Profiling of Secreted Serine Proteases in Health and Inflammatory Bowel Disease

Alexandre Denadai-Souza*¹; Chrystelle Bonnart*¹; Núria Solà Tapia¹; Marlène Marcellin²; Brendan Gilmore³; Laurent Alric⁴; Delphine Bonnet¹; Odile Burlet-Schiltz²; Morley D. Hollenberg⁵, Nathalie Vergnolle#$¹,⁵; Céline Deraison#¹

¹IRSD, U1220, Université de Toulouse, INSERM, INRA, ENVT, UPS, Toulouse, France.

²Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, France.

³School of Pharmacy, Queen’s University, Belfast, Ireland.

⁴Pôle Digestif, CHU Purpan, Toulouse, France.

⁵Department of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada.

* Authors contributed equally

# Joint senior authors

$ Correspondance: Dr Nathalie Vergnolle, Inserm UMR-1220, Institut de Recherche en Santé Digestive, CS60039 CHU Purpan Toulouse, Cedex-3 31024, France.

Nathalie.vergnolle@inserm.fr

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Abstract

While proteases are essential in gastrointestinal physiology, accumulating evidence indicates that dysregulated proteolysis plays a pivotal role in the pathophysiology of inflammatory bowel disease (IBD). Nonetheless, the identity of overactive proteases released by human colonic mucosa remains largely unknown. Studies of protease abundance have primarily investigated expression profiles, not taking into account their enzymatic activity. Herein we have used serine protease-targeted activity-based probes (ABPs) coupled with mass spectral analysis to identify active forms of proteases secreted by the colonic mucosa of healthy controls and IBD patients. Profiling of (Pro-Lys)-APB bound proteases revealed that most of hyperactive proteases from IBD secretome are clustered at 28-kDa. We identified seven active serine proteases: cathepsin G, plasma kallikrein, plasmin, tryptase, chymotrypsin-like elastase 3A, aminopeptidase B, and thrombin. Only cathepsin G and thrombin were overactive in supernatants from IBD patient tissues compared to healthy controls. Gene expression analysis highlighted the transcription of genes encoding these proteases into intestinal mucosae. The functional ABP-targeted proteomic approach that we have used to identify active proteases in human colonic samples bears directly on the understanding of the role these enzymes may play in the pathophysiology of IBD.
Introduction

The degradome represents almost 2% of protein coding genes in the human genome, with at least 588 genes coding for proteases. Among them, one of the largest classes is represented by 184 genes encoding serine proteases, which are characterized by the presence of a nucleophilic serine in their reactive site. Since the hydrolysis of peptide bonds is an irreversible process, the expression and activity of proteases are tightly regulated. For instance, these enzymes often exist as inactive zymogens (pro-forms), which must be activated by proteolytic cleavage. A large array of endogenous protease inhibitors also exists that can control cell and tissue proteolysis.

Proteases are essential mediators in gastrointestinal physiology, being produced and released by the pancreas, in order to be activated in the intestinal lumen for digestive purposes. Proteolytic activity is also detected within mucosal tissues in healthy conditions and is thought to play a role in mucus consistency and mucosal antigen processing. Otherwise, in intestinal pathophysiological contexts such as inflammatory bowel disease (IBD), proteolytic homeostasis can be disrupted in tissues. Increased serine protease activity has been demonstrated in colonic tissues from Crohn’s disease (CD) or Ulcerative Colitis (UC) patients. Some of these studies also demonstrated that the reestablishment of the proteolytic homeostasis by the local delivery of recombinant protease inhibitors reduces the severity of experimentally-induced colitis, thus highlighting the importance of these enzymes both as central mediators of IBD pathophysiology, and as potential therapeutic targets.

The identity of overactive serine proteases in intestinal tissues remains elusive. In situ zymography assays demonstrated that the increased IBD-associated elastolytic activity was mostly present within the epithelium. This is an interesting finding, given that most studies aimed at identifying upregulated proteases in inflammatory diseases have focused on...
enzymes highly expressed by infiltrating immune cells. Thus, gene and protein expressions of several proteases released primarily by leukocytes (including neutrophil elastase, proteinase-3, cathepsin G, tryptase, chymase or granzymes) have been found to be upregulated in IBD\textsuperscript{2}. Additionally, genetic studies have supported an association of protease genes with IBD risk\textsuperscript{7,8}. Nevertheless, the major limitations of such studies based on expression analysis are due to the fact that mRNA or protein levels for proteases do not necessarily reflect their activity status. Indeed, variations of zymogen activation or local availability of endogenous inhibitors can drastically modify biological activity.

Therefore, the identity and implication of proteases in health and diseases, including IBD, have to come from studies investigating the \textit{in situ} net activity of these enzymes\textsuperscript{9}. The development of functional proteomic assays based on Activity-Based Probes (ABPs) now allows such approaches, monitoring the availability of enzyme active sites in biological samples\textsuperscript{10-13}.

The ABP structure possesses a reactive group that mimics enzymatic substrate and covalently binds to active proteases. Additionally, the ABP reactive group is associated to a biotin motif via a spacer, in such a way that bound active enzymes thus become biotinylated and can be visualized and/or immobilized by avidin-based affinity chromatography. Further mass spectral analysis could then determine the enzyme sequence. Obviously, detection of active proteases is dependent to their affinity towards the ABP that is used. We have previously used this approach successfully to identify active serine proteases upregulated in the setting of a murine model of infectious colitis\textsuperscript{14} and to determine the sequences of serine proteases present in complex allergenic cockroach extracts\textsuperscript{15}. Here, we performed a study to profile and identify active serine proteases secreted by the colonic mucosa of control and IBD patients by using ABPs.
Results

Validation of the sensitivity for detecting Trypsin-like activity using a Biotin-PK-DPP activity-based serine protease probe: signal intensity correlates with trypsin activity level

The ABP biotin-PK-DPP synthesized for the present study$^{16}$ was of sufficient reactivity to detect a level of 2.5 mU of trypsin from bovine pancreatic trypsin. The ABP signal intensity was proportional to increasing concentrations of trypsin, and was eliminated by the serine protease irreversible inhibitor AEBSF (Figure 1A, 1B and supplementary figure 1).

Secreted serine protease activity is upregulated in IBD colonic mucosa

Colonic tissue supernatants from control patients exhibited a baseline proteolytic activity, which was increased in samples from CD and UC patients (Figure 2). To characterize the serine proteases underlying this increase of proteolytic activity, we initially performed ABP proteomic profiling assays with these samples. Since ABPs react only with active enzymes, the bands corresponding to proteases were discriminated from non-specific labelling by pre-incubating the samples in parallel with the serine protease inhibitor, AEBSF. Therefore, the signal intensity of protease bands from AEBSF-treated samples was reduced or absent in comparison to the sample not treated with this irreversible serine protease inhibitor. As a whole, bands representing putative serine proteases ranged from 12 to 250 kDa (Figure 3A). A distribution analysis of putative proteases according to their molecular weight regrouped them in 10 main clusters, with mean molecular weights of 15, 24, 28, 32, 36, 68, 100, 126, 140 and 250 kDa. The majority of serine proteases were grouped into the 28, 32 and 36 kDa clusters (Figure 3B). Once these clusters were analysed in individual groups of patients, some differences became evident. Likewise, the cluster 1 (15 kDa) was only detected in IBD samples. The cluster 6 (68 kDa) was more prominent in UC samples, while cluster 9 (140 kDa) was only detected in CD samples.
Next, we focused on the number of AEBSF-sensitive bands, which were diminished in the presence of the protease inhibitor, as opposed to the labelled bands which were not affected by AEBSF incubation. In all samples there were biotin-labelled constituents in the 50 and 65 kD range which appeared to yield a comparable streptavadin-biotin reactivity, for which the signal was not diminished by AEBSF treatment (Figure 3A). Relative to those AEBSF-resistant signals present in all samples, quite distinct AEBSF-sensitive ABP labelling profiles were observed for samples obtained from the CD and UC individuals either compared between diseases or compared to controls (Figure 3A). In particular, the CD-derived samples contained a unique ABP-labelled constituent in the 15 kDa range comparable to a labelled component found in the trypsin preparation, that might represent a cleaved, catalytically active fragment of trypsin (supplementary figure 1). Other higher molecular mass AEBSF-sensitive ABP-labelled bands also distinguished the CD samples from the UC and control samples. This distinction was quantified further by analysing the AEBSF-sensitivity of labelled bands for constituents clustered in the mass regions of 28 (cluster 3), 32 (cluster 4), 68 (cluster 6) and 140 (cluster 9) kDa. According to this analysis, differences in the percentage of AEBSF-sensitive bands present in control versus CD samples were observed for clusters 3 (28 kDa: 63% reduction in Controls vs. 100% inhibition by AEBSF, in CD) and 9 (140 kDa: 8% reduction in Controls vs. 25%, in CD). Changes in the percentage of AEBSF-sensitive bands between controls and UC were observed for cluster 6 (68 kDa; 13% reduction caused by AEBSF in Controls vs. 42% reduction in UC) (Figure 3C).

We then defined the activity index of each cluster bands by considering the intensity in the absence versus in the presence of AEBSF (Figure 3C). In control samples, the most active cluster was at 32 kDa (Figure 3A and C, cluster 4). The comparison of the activity index between control and IBD samples revealed an increased proteolytic activity index associated with some clusters. For instance, the activity index for clusters 3 and 9 (28 and 140 kDa, respectively) increased in CD samples. That said, although for bands in the 32 and 68 kDa (clusters 4 and 6) a number
of AEBSF-sensitive labelled bands appeared to differ between the CD and UC-derived samples, the difference in the activity index did not quite reach statistical significance (Figure 3C).

**ABP-reactive enzymes identified by LC-MS/MS analysis**

Unbiased mass spectrometric analysis identified 6 proteases from S1 family in samples from colonic biopsy supernatants. These proteases were considered active according to the ability of AEBSF to block labelling, with an activity index >2 and P<0.05 (Table 1). Here, the activity index was defined by the ratio -/+ AEBSF of the quantity of positive peptides identified by LC-MS-MS analysis. This group of identified active proteases includes thrombin, cathepsin G, kallikrein-1 (also named plasma kallikrein), plasmin, chymotrypsin-like elastase family member 3A and tryptase. Additionally, aminopeptidase B (also called arginyl aminopeptidase), a lysine-cleaving protease from the M01 family was also identified as active. Overall, thrombin was the most active protease identified, and its activity was particularly prominent in CD. Similarly, aminopeptidase B was highly active specifically in association with UC (Table 1).

**Active secreted proteases identified by ABP labelling are expressed by the intestinal mucosae**

Gene expression experiments were carried out to investigate whether or not the proteases identified as active were expressed in the human colonic mucosa. RT-PCR products were detected for the 7 proteases, wherein amplicons with expected base pair numbers were amplified from colonic mucosa.
Discussion

Mass spectrometry proteomic approaches have been applied to IBD tissues, identifying global changes in proteome for these pathologies\textsuperscript{17-20}. Using such approaches, only few proteases were identified. Their relative abundance seems to be secondary to immune cell infiltration as they are major components of innate immune cells. As a matter of course, the major drawback of classical proteomic approaches remains the lack of information about protease activity. As a consequence, the implication of these enzymes in the pathophysiology of human diseases has been only marginally characterized to date. Herein, we used a biotinylated ABP capable of interacting with lysine-cleaving proteases (biotin-PK-DPP), a catalytic feature of most serine proteases and some proteases from other classes\textsuperscript{21}. Furthermore, experiments performed with increasing amounts of active trypsin clearly demonstrated that the signal intensity generated by this ABP augmented accordingly, thus highlighting that this probe can unveil varying activity levels of lysine-cleaving proteases. The ABP proteomic gel profiles revealed the presence of bands with a broad molecular weight distribution, either sensitive or not to AEBSF inhibition. Because streptavidin which is used to reveal biotinylated bands, can bind non-specifically to proteins\textsuperscript{22,23}, and because we cannot fully exclude that ABP might bind non-specifically to some proteins in a complex mixture, the use of inhibitory AEBSF pre-treatment in counterpart samples was instrumental at discriminating active protease bands.

In previous work using activity-based probes to identify active serine proteases associated with intestinal inflammation in an infectious model of rodent colitis, we established a role for host serine proteases and their signalling target, protease-activated receptor-2 (PAR2), in driving the inflammatory response\textsuperscript{14}. The results we report here establish proof of principle, that a comparable approach can be used to evaluate patient-derived tissue samples. Our work considerably extends our previous observations which showed that explants from individuals with IBD secrete increased lysine-targeted protease activity. Our main finding is that compared with non-diseased tissues, serine protease-targeted activity-based probes reveal a distinct set of
active serine proteases secreted by colonic tissues derived from individuals with either Crohn’s disease or ulcerative colitis. These data amplify in molecular terms, the initial finding that the secretome of tissues derived from IBD patients contained increased trypsin-like activity, relative to controls. Our data also complement observations by others reporting the increased presence of cathepsin-G in faeces of patients with ulcerative colitis. Several studies have documented high levels of tryptase in IBD mucosae. However, increased level of tryptase in IBD mucosae was reported based on immunoassays. Active tryptase was not detected in the secretome from UC biopsies in this study. Our results suggest that concomitantly with exocytosis of tryptase, endogenous inhibitors could also be present in the granules or in the vicinity of activated mast cells, leading to a quick neutralization of enzymatic activity. While from protein or mRNA expression studies, tryptase could appear as a potential molecular target for IBD, our results suggest on the contrary that active tryptase is not present in patient’s tissues.

The ABP-tagged enzymes that were distinct in the secretome from the Crohn’s disease and ulcerative colitis-derived tissues, compared with disease-free tissues, fell into four clusters. One cluster represented by a protease in the 10 kDa range was found only in tissues from CD individuals (Figure 3A) and three others, two of which (clusters 3 and 9, in the range of 28 and 140 kDa) were associated with Crohn’s disease and a fourth (cluster 6, in the range of 68 kDa), which was associated with samples from ulcerative colitis individuals. In addition, within these clusters, the scatter-plots, with groups of points well above baseline, suggest that a subset of individuals may be present within each cluster. It will be of importance to follow the clinical outcomes of the individuals with high activation profiles within the clusters, compared with the others in each patient group.

The expression of all serine proteases identified by ABP labelling, namely thrombin, cathepsin G, plasma kallikrein, plasmin, tryptase and chymotrypsin-like elastase family member
3A, were also found as mRNA transcripts in extracts of colon biopsy tissue (Table 1 and Figure 4). Thus, all of the ABP-labelled enzymes can be both produced and secreted in situ by mucosal tissue. Most of these identified serine proteases are well-established activators of Protease-Activated Receptors (PARs), which have been implicated in IBD pathophysiology\textsuperscript{16,17}. Further, the enzymes like cathepsin G, in addition to regulating PAR activity, can play an inflammatory role via either the processing/activation of cytokines, chemokines and growth factors (e.g. the convertase action of cathepsin-G for generating alarmin-IL-33) or by the cleavage/inactivation of such mediators\textsuperscript{27}. It will be important to validate whether or not the active proteases we have identified in the tissue secretome would also be found as active enzymes in fecal samples, so as to provide for a ‘biomarker’ to follow disease progression.

A high level of active thrombin was particularly detected in the secretome from CD mucosal biopsies and to a lesser extent from the ulcerative colitis-derived tissues. Biopsies from IBD patients were collected in macroscopic inflamed areas, where ulceration or erythema is observed, which can be associated to blood vessel leakage. At the site of colitis, circulating pro-thrombin could be activated by tissue factor Xa expressed on cells like monocytes, dendritic cells, platelets, endothelial cells and vascular smooth muscle cells. Of particular note, thrombin was also identified at colonic tissue mRNA transcript level. This extrahepatic source of thrombin could therefore also participate to modulation of innate immunity, notably via PARs 1 and 4 receptor cleavage. Activation of these receptors could result in intestinal epithelial cell apoptosis and barrier disruption\textsuperscript{18} and might cause either an inflammatory or anti-inflammatory response in the tissues\textsuperscript{19}. The exact role that thrombin might play in Crohn’s disease and ulcerative colitis, diseases that have been associated with increased thrombosis\textsuperscript{20,21}, remains to be clarified.

Surprisingly, one protease from a class other than serine protease was identified as active in samples from UC patients: the metalloopeptidase aminopeptidase B. This enzyme is known to display endopeptidase activity after an Arg or a Lys residue\textsuperscript{28} and this could explain
the fact that it binds to the ABP we have used here. To the best of our knowledge, the role of aminopeptidase B in IBD pathophysiology has not been evaluated yet.

In summary, the functional proteomic approach employed herein allowed for the identification of a consistent proteomic profile of active serine proteases secreted by the colonic mucosa of healthy and IBD patients. Additionally, the use of ABP labelling in conjunction with mass spectral proteomic analysis resulted in the identification of unique active proteases selectively secreted from the colitis-derived samples, compared with samples from disease-free individuals. This approach identified not only proteases previously established as putative candidates in IBD pathophysiology, but also enzymes not yet appreciated in this context. In this way, the results presented herein pave the way for future studies aimed at understanding the roles of these proteases in IBD pathophysiology. This study also revealed strong differences between CD patients and UC in terms of profiles of active proteases that could dictate distinct and specific therapies for these 2 sub-categories of IBD patients in the forthcoming targeted therapy strategies. The approach proposed here can be applied to other diseases and other tissues, in order to identify active secreted proteases that may play roles in other inflammatory diseases, so as to serve as potential new therapeutic targets.
Methods

Patients and colonic samples
This work and tissue collection received ethical approval. All methods were performed in accordance with guidelines and regulations from the French Ethics Committee (Comité d’Ethique sur les Recherches Non Interventionnelles) (Identifier: NCT01990716). Colonic tissue samples were obtained from well-characterized CD and UC patients undergoing colonoscopy or colonic resection procedures at the Toulouse Hospital Centre (France). Colonic tissue samples from individuals undergoing colon cancer screening who were otherwise healthy were used as controls. Written and verbal informed consent was obtained before enrolment in the study. Fresh colonic tissue samples were rinsed in isotonic sterile Hanks’ balanced salt solution pH 7.4 (HBSS) and were then immediately incubated in 2 ml of HBSS (containing Ca$^{2+}$ and Mg$^{2+}$) at 37°C for 60 min. Freshly isolated colonic tissue specimens were quick-frozen in RP1 buffer (Macherey-Nagel, GmbH) and stored at −80°C until use for RNA extraction.

Measurement of protein concentration
The concentration of protein in colonic tissue supernatants was determined by using the Pierce Protein BCA Assay Kit, according to instructions (Thermo Scientific).

Measurement of proteolytic activity
The proteolytic activity was measured in colonic tissue supernatant samples with 0.1 mM N-p-Tosyl-GPR-amino-4-methylcoumarin hydrochloride as substrate in 50 mM Tris, 10 mM CaCl$_2$, pH=8 (Sigma-Aldrich$^{29}$). Substrate cleavage was calculated by the change in fluorescence (excitation: 355 nm, emission: 460 nm), measured over 30 min at 37°C on a Varioskan Flash microplate reader (Thermo Fisher Scientific). Sample values were interpolated into a linear regression generated with a standard curve of TPCK-treated trypsin from bovine pancreas (8-500 mU/mL; Sigma-Aldrich). Data were expressed as mU of trypsin-like activity per mg of protein.
**Activity-Based Probe reaction**

The Biotin-PK-DPP serine protease activity-based probe was obtained from the laboratory of Dr. Nigel W. Bunnett (Columbia University, USA), with the participation of Dr. Laura Edgington-Mitchell (Monash University, Australia) and synthetized as previously described \(^{30}\). This probe presents a relative selectivity towards Enzyme Class 3.4.21.4 and EC 3.4.21.5 \(^{16,30}\).

Colonic supernatants (40 µg of protein) were diluted in 100 mM Tris-HCl, 1 mM CaCl\(_2\), pH=8 to a final volume of 900 µL and then split into duplicates. Each duplicate (450 µL) was then pretreated or not with a final concentration of 4 mM AEBSF (SIGMA) during 15 min at 37ºC under stirring (1000 rpm). The pre-incubation with this irreversible broad-spectrum serine protease inhibitor allows the identification of active proteases, since enzyme inhibition abrogates their interaction with the ABP, impacting the intensity signal of bands in proteomic profiles and of peptides retrieved by mass spectrometry. Then, the ABP biotin-PK-DPP was added to each reaction to a final concentration of 1 µM, and each replicate sample, containing 20 ug of protein, was incubated for 60 min at 37ºC under stirring (1000 rpm).

**Functional proteomic profiling**

The reaction product was then precipitated in 15% trichloroacetic acid at 4ºC during 90 min. The pellet was washed twice in cold acetone (-20ºC) and solubilized in 20 µL of protein solving buffer with tris-(2-carboxyethyl)-phosphine hydrochloride (PSB-TCEP; Macherey-Nagel, GmbH). Samples were then heated at 95ºC for 5 min, clarified by centrifugation at 12000 x g for 5 min and the solubilized sample was loaded into 4-20% Mini-Protean TGX precast gels (Bio-Rad, GmbH). After electrophoresis, the proteins were blotted onto nitrocellulose membranes by using the Trans-Blot Transfer Turbo System (Bio-Rad). Membranes were incubated with streptavidin-HRP (Life Technologies), and bands were visualized with ECL Prime Western Blot Detection Reagent (GE Healthcare Life Sciences) and quantified by chemiluminescence yield (Chemidoc XRS; Bio-Rad). The molecular weight and intensity of each band was determined with the Image Lab Software v5 (Bio-Rad). The bands
corresponding to active proteases were identified by their sensitivity to AEBSF. Additionally, the activity index of each protease-corresponding band was estimated by the calculation of a ratio between the volumetric densitometry of the fluorescent signal generated by untreated vs AEBSF-pretreated duplicates (-/+AEBSF). An activity index of 0 was given to cluster bands that were not detected in specific samples.

**Mass spectrometry analysis**

For mass spectrometry analysis, colonic supernatants from 3 representative patients per group were pooled and submitted to an ABP reaction in a final volume of 4.0 mL, as described above. In the following, 3.8 mL of the reaction product were incubated with 50 μL of pre-washed Dynabeads MyOne Streptavidin C1 (Invitrogen, USA) for 60 min at room temperature under stirring (1000 rpm). The beads were washed 5 times with 1 mL of phosphate buffered saline pH=7.2. As a control procedure for the ABP reaction and following steps, 200 μL of ABP-labelled secretome fluids (before incubation with beads), bead supernatant and buffer from the first wash were recovered, precipitated and analysed by proteomic profiling. The pellets containing the ABP-protease complexes adsorbed to the magnetic beads were washed twice with 50 mM ammonium bicarbonate buffer (Sigma-Aldrich, USA), and then suspended in 6 M urea and 25 mM DTT (Sigma-Aldrich). After 60 min under stirring (850 rpm) at room temperature, the samples were alkylated by the incubation in 90 mM iodoacetamide (Sigma-Aldrich) during 30 min in the dark. Bead-bound samples were then washed twice as described above and submitted to overnight proteolysis at 37°C in ammonium bicarbonate buffer (50 mM, pH=8.5) containing 1 μg of trypsin (Promega, USA) per sample. The supernatants were collected, dried under vacuum and solubilized in 2% acetonitrile and 0.05% trifluoroacetic acid (Sigma-Aldrich), for further analysis.

The resulting peptides were analysed with a NanoLC (Ultimate 3000 RSLCnano system Thermo Scientific) coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher...
Scientific, Bremen, Germany). Peptides extracts (5 μL) were loaded on a C18 precolumn (300 μm inner diameter x 5 mm; Thermo Scientific) in a solvent made of 2% acetonitrile and 0.05% trifluoroacetic acid, at a flow rate of 20 μl/min. After 5 min of desalting, the precolumn was switched online with the analytical C-18 column (75 μm inner diameter x 50 cm; Reprosil) equilibrated in 95% of solvent A (0.2% formic acid) and 5% of solvent B (80% acetonitrile and 0.2% formic acid). The peptides were eluted using a 5-50% gradient of solvent B over 105 min at a flow rate of 300 nL/min. The LTQ Orbitrap Velos was operated in a data-dependent acquisition mode with Xcalibur software. MS survey scans were acquired in the Orbitrap on the 350–1800 m/z range, with the resolution set to 60,000. The 20 most intense ions per survey scan were selected for fragmentation by collision-induced fragmentation and MS/MS spectra were acquired in the linear ion trap. A 60s dynamic exclusion was used to prevent repetitive selection of the same peptide. Triplicate LC-MS measurements were performed for each sample.

**Protein identification and quantification**

Raw MS files were processed with MaxQuant software (version 1.5.2.8) for database search with the Andromeda search engine and for quantitative analysis. Data were searched against human entries in the Swissprot protein database (release UniProtKB/Swiss-Prot 2015-12; 20200 entries). Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine, protein N-terminal acetylation were set as variable modifications. Specificity of trypsin digestion was set for cleavage after K or R, and two missed trypsin cleavage sites were allowed. The precursor mass tolerance was set to 20 ppm for the first search, 5 ppm for the main Andromeda database search and minimum peptide length was set to 7 amino acids. Andromeda results were validated by the target-decoy approach using a reverse database at both a peptide and a protein false-discovery rates of 1%. For label-free relative quantification of the samples, the match between runs option of MaxQuant was enabled with a time window of 0.7 min, to allow cross-assignment of MS features detected in the different runs.
To perform relative quantification between proteins identified, we used the “Intensity” metric from the MaxQuant “protein group.txt” output (sum of peptide intensity values for each protein). Quantitative data were first normalized and missing protein intensity values were replaced by a constant noise value that was determined independently for each sample as the lowest value of the total protein population. Enrichment ratios between AEBSF not treated and AEBSF treated samples were calculated from the mean protein intensities derived from three technical replicate experiments. A potential active protease was selected based on an enrichment ratio $> 2$ (Intensity AEBSF not treated vs. treated) and a Student’s t-test P-value $< 0.05$ over the triplicates.

**RT-PCR**

Total RNA was extracted from human colonic tissue samples with the Nucleospin RNA/Protein Kit (Macherey-Nagel, GmbH). DNase-treated RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Resulting cDNA samples were amplified by conventional PCR with Taq DNA polymerase (Invitrogen, USA) and sequence-specific primer pairs (Supplementary Table 1). As a control procedure, RT reactions were performed without the addition of enzyme to the mix.

**Statistical analysis**

For the enzyme kinetics and proteomic profiles assays, each dot represents the data from an individual patient. All data were used to calculate the values expressed as mean±SEM. Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) or Kruskal-Wallis, followed by multi comparison tests, as indicated in figure legends. Outliers were identified by the method of ROUT with Q settled at 1%. GraphPad Prism v.6 software was used for analysis. Statistical significance was accepted at p<0.05.
Author’s contributions

AD-S, CB, MH, NV and CD participated to study design, data acquisition, analysis, interpretation and drafted the manuscript; NS-T; MM, LA; DB and OB-S participated on data acquisition and analysis; BG and MDH participated to ABP design. All authors read and approved the final manuscript.

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Additional Information

All authors declare they have no competing financial interests related to the present study.
References


Figure legends

Figure 1. Validation of biotin-PK-DPP sensitivity of detection of trypsin-like enzymes

A. 1µM PK-ABP was incubated with an increasing concentration of trypsin and the biotinylated trypsin product was visualized by electrophoresis followed by detection using streptavidin-linked horseradish peroxidase and ECL. B. Trypsin was treated first with the broad-spectrum serine protease inhibitor AEBSF (4mM) prior to its reaction with ABP and ECL detection.

Figure 2. Measurement of trypsin-like activity released by human colonic mucosa.

Trypsin-like activity detected in supernatants from colonic tissue samples of control or IBD patients (n=11-16). Data were analysed by ANOVA followed by the multi comparison test of Holm-Sidak. *P<0.05 vs. control.

Figure 3. Proteomic profiling of serine proteases released by the human colonic mucosa.

A. Representative ABP proteomic profile, showing the differential repertoire of ABP-labelled serine proteases secreted from control or IBD colonic tissue samples along with the positive trypsin control (20 mU of trypsin). The red arrowheads point to bands corresponding to active proteases, as verified by the inhibitory effects of pre-treatment of the samples with AEBSF (4mM). B. Clustering of ABP-labelled serine proteases according to size (kDa). C. Graphic representation of protease size clusters along with their activity index determined by the impact of enzyme inhibition (-/+AEBSF). The percentage of AEBSF-inhibited bands per patient is represented by the pie graphs. The empty circles represent patients wherein bands within the cluster were not detected (negative), a 0 value was given to these samples as per
their activity index. Activity index data were analysed by Kruskal-Wallis followed by the multi comparison test of Dunn. *P<0.05, **P<0.01, vs. control; #P<0.05 vs. CD.

Table 1. Active ABP-labelled serine proteases secreted from the colonic mucosa of control and IBD patients. The list shows the active ABP-labelled proteases identified by LC-MS/MS analysis of pooled supernatant samples from control and IBD patients, showing the respective protease family, gene symbol, protein name, predicted molecular weight and the activity index reflecting the sensitivity of ABP labelling to protease inhibition (-/+AEBSF ratio).

Figure 4. Colonic RNA expression for proteases identified as active. Analytical agarose-gel electrophoresis of RT-PCR products amplified from cDNAs prepared from human colonic mucosa tissue samples are shown with arrows denoting the predicted size (base pairs: bp) of the PCR product. Negative controls (noted -) consisted of RT reactions performed in the absence of enzyme. Positive expression was confirmed using cDNAs prepared from human tissue sources known to express the target proteases.
Figure 1

A

0.5  1  2.5  5  10  20 mU

25 kDa

Trypsin concentration

B

-  +  AEBSF

25 kDa

Trypsin

50 mU

Figure 2

Trypsin-like activity (U/mg of protein)

Ctrl  CD  UC

*
## Table 1

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Figure 4
**Supplemental Figure 1.** Full-length gels of Figure 1. Pictures correspond to merged pictures between the luminescent signal and colorimetric signal, in order to have the ECL signals and the prestained molecular weight ladder present on the same picture. In the cropped figure 1, only ECL signal picture is depicted, this might explain difference in background, notably the presence of smudge in panel A which is not present in the cropped corresponding panel A in Figure 1.
**Supplementary Table 1.**

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**Supplementary Table 1. Sequences of oligonucleotides used for RT-PCR experiments.** The human gene symbols, oligonucleotide sequences, amplicon size in base pairs (bp), individual or multiple splice variants targeted by each primer pair and respective NCBI accession numbers are indicated.
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Crohn’s disease (CD) and Ulcerative colitis (UC) are two forms of Inflammatory Bowel Disease (IBD), a chronic inflammatory pathology affecting the digestive tract. Patients suffer from relapsing flares, diarrhea, abdominal pain and bleeding. Although the molecular mechanisms of IBD are poorly understood, recent data suggest that IBD occurs in genetically predisposed individuals developing an abnormal immune response to intestinal microbes after, being exposed to specific environmental triggers. Genetic studies have reported more than 170 polymorphisms susceptible to be involved in IBD pathogenesis. The strongest associations have highlighted three main pathways altered in IBD including bacterial sensing (NOD2, CD), autophagy (ATG16L1 and IRGM, CD) and endoplasmic reticulum stress (ER-Stress) (XBP1, UC). The role of intestinal barrier function is also strongly implicated in IBD pathogenesis, and is modulated by factors present in the lumen derived from microbiota, food or at a molecular level, by factors such as proteases. In IBD pathophysiology, the inflammatory process is characterized by impaired intestinal biology including disruption of tight junctions and leaky gut, decreased amount of Paneth and Goblet cells, and translocation of luminal antigens triggering inflammation. Previous studies have demonstrated an increased level of active serine proteases in the stools and tissues of IBD patients, supposing that proteases originate from infiltrated immune cells, pancreatic secretion or microbiota. However, our team has reported that intestinal epithelial cells are a major source of serine proteases, in particular trypsin-like enzymes, are released by a stressed epithelium in pathogenic context such as irritable bowel syndrome.

In this project, we aimed at better understanding whether the three main pathways involved in IBD (Nod2, autophagy, ER-stress) could be linked to an epithelial release of trypsin and reciprocally, if epithelial trypsin is able to induce or modulate these three IBD pathways.

We confirmed that trypsin-like activity was significantly higher in biopsies from UC and CD patients compared to healthy controls. In Caco-2 monolayers cultured in transwells, secreted trypsin-like proteolytic activity remained stable upon NOD2 stimulation but decreased under autophagy induction. Thapsigargin (Tg) stimulation a well-known ER-stress inducer, enhanced the apical release of trypsin-like activity in Caco2 cells. Activity-based probe assay identified a unique band at 33 KDa in ER-Stress-induced Caco-2 supernatants. This band showed specificity for Trypsin-3 in western blot. In UC patients, immunohistochemistry of colonic biopsies showed that Trypsin-3 was detectable mainly in epithelial cells, and up-regulated compared to biopsies from healthy controls and CD. Similarly, only UC patients displayed altered ER-stress with increased XBP1s mRNA levels. In Caco-2 cells, ER-Stress induction provoked increased paracellular permeability, CXCL8 release, antimicrobial peptides (AMP) (TFF-3 and HBD2), and mucins (MUC2) dysregulation. Serine protease inhibitor AEBSF inhibited Tg-induced increased permeability and AMP dysregulation, while Caco2 cells were increased. In Caco-2, Tg-induced ER-Stress increased PAR-2 and -4 mRNA expression, PAR4 control levels were restored in the presence of AEBSF. ER-Stress-associated increased paracellular permeability was suppressed by PAR2 and/or -4 antagonist treatment, while CXCL8 was aggravated. Trypsin-3 did not induce ER stress in Caco2.

Our data showed that in intestinal epithelial cells, ER-Stress increased trypsin-3 expression and trypsin proteolytic activity, which is responsible for altered barrier function and dysregulated AMP and mucin expressions. We identified PAR-2 and -4 activation as possible mechanisms by which ER-Stress contributed to epithelial pathophysiology. Trypsin-3 appears as a candidate protease overexpressed upon ER-Stress and in UC patients epithelium.


Nos résultats ont confirmé un excès de protéases à sérine dans les cellules épithéliales de patients atteint de MC ou de RGH. In vitro, surdes monocouches de cellules Caco-2, l’induction de l’autophagie diminuait la libération apicale de protéase de type trypsine, alors que le sensor bactériens NOD2 n’avait aucun effet. À l’inverse, une stimulation du Stress du réticulum endoplasmique (SRE) par la Thapsigargin, induisait une libération accrue de protéases actives de type trypsine au pôle apical des cellules. L’utilisation d’ABP (Activity-based probe), emprisonnant les protéases actives de type trypsine dans des surnageants apicaux de Caco-2 stimulées par la Thapsigargin, a montré une importante sécrétion d’une protéase unique au poids moléculaire de 33-KDa. Dans le blé, la présence augmentée de Trypsin-3 était identifiée dans ces surnageants, de même que dans les monocouches de patients atteints de RCH comparé à des échantillons contrôles ou CD. Seul les monocouches de patients RCH présentaient également une induction du SRE. Sur les monocouches de Caco-2, l’induction du SRE augmentait la perméabilité paracellulaire, la sécrétion de CXCL8 et l’expression de PAC, de mucine et des récepteurs PAR2 et -4. Les inhibiteurs de protéases de type trypsine supprimaient l’augmentation de la perméabilité et l’expression des PAR, de la mucine 2 et des récepteurs PAR2 et -4 induite par le SRE, et augmentaient la sécrétion de CXCL8. Les antagonistes sélectifs des récepteurs PAR2 et/ou PAR4 inhibaient l’augmentation de la perméabilité et l’expression des PAR, de la mucine 2 et des récepteurs PAR2 et -4 induite par le SRE, mais augmentaient la sécrétion de CXCL8. Enfin, la Trypsin-3 ne modifiait pas les marqueurs de SRE.

En conclusion, l’induction d’un SRE dans les cellules épithéliales déclenche une libération apicale de Trypsine-3 et d’activité trypsine, responsable de l’altération de la fonction de barrière de la monocouche cellulaire. Nous avons identifié l’implication des récepteurs PAR2 et -4 (deux deux activables par la Trypsine-3) dans la rupture de l’homéostasie de l’épithélium intestinal. La Trypsine-3 semble être spécifiquement exprimée dans les colonocytes de patients RGH, cette surexpression pourrait être liée à une induction anormale du SRE.