Microbial metabolism and pathogenesis in inflammatory bowel diseases

How Enterobacteriaceae *take advantage of a compromised mucosal tolerance and how this can play a role in future drug discovery*

Supervisors: Dr. A.D. Kraneveld and Dr. R. Oozeer
Author: C. de Waard, Bsc.
Utrecht University
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Abstract
The prevalence of inflammatory bowel diseases (IBD) has been rising in developed countries. Although extensive research has shown that the onset of IBD depends on genetic susceptibility, environmental risk factors and the function of the microbiota, treatment of these diseases has shown limited results. In order to improve the treatment of IBD it is necessary to have a better understanding of the metabolism and survival strategies of bacteria like *Salmonella typhimurium* and adherent-invasive *Escherichia coli* (AIEC), two important bacterial strains that play a role in the onset and remission of inflammation in IBD. There are indications that both *S. typhimurium* and AIEC utilize an energy source to promote their own growth, that cannot be used by the commensal flora. This hypothesis gives rise to new treatment strategies for IBD. To compete with these pathogens, the administration of an extra fermentable carbon source, like oligosaccharides (prebiotics), in combination with the use of selected probiotic strains (together called synbiotics) should be investigated.

1. Introduction

The human body has to cope with the presence of bacteria every day. Mucosae, specially the gastrointestinal tract, are densely colonized with microorganisms. Bacteria can be beneficial or pathogenic and the immune system needs to elicit an adequate response to these inhabitants. Both pathogens and commensals challenge this delicate balance every day by interacting with host cells and influencing intracellular pathways. Studying how bacteria, and particularly pathogenic bacteria, interact in these pathways is a cornerstone in understanding the development and progression of both infectious diseases and autoimmune diseases.

In the last decade the prevalence of inflammatory bowel diseases (IBD) in developed countries has been rising. Extensive research has been done and it is clear that the onset of IBD depends on both genetic susceptibility and environmental risk factors (Xavier, 2007). The intestinal microbiota plays a key role in the progression to these diseases, thus the focus of research lies mainly on the microorganisms that are present in the intestines.

So far, clinical trials that aim to alter the microbiota to a composition that is found in healthy subjects, show conflicting results (Sartor, 2004; Marteau, 2003). This may be due to the ineffectiveness of current approaches to change the microbiota permanently. For instance, probiotics have a limited impact on the overall structure and activity of the microbiota. In addition, there is a possibility that the western-lifestyle diet further limits the alteration of the bacterial composition in the gut. Moreover, most intervention studies are performed with generic probiotic strains that are not selected for IBD. This is due to the lack of microbial biomarkers that facilitate the screening for relevant strains. Therefore it is necessary to have a better understanding of the metabolism and survival strategies of bacteria that play an important role in the onset of inflammation in IBD (Sartor, 2004; Marteau, 2003).

This mini review focuses on the survival mechanisms of *Salmonella typhimurium* and adherent-invasive *Escherichia coli* (AIEC). We have focussed on these two bacterial strains because of their similar way to influence host cell response in their benefit. As far as known, both *S. typhimurium* and AIEC play important roles in IBD (Chassaing, 2011). Hence, characterizing the role of these bacteria gives a better insight in the etiology of IBD and provides new potential targets for drug development in the future.

2. Inflammatory bowel disease

Inflammatory bowel diseases are characterized by a chronic inflammation of the intestinal tract. The two major forms of idiopathic IBD are Crohn's disease (CD) and ulcerative colitis (UC). Key features of CD are patchy and segmental lesions which can occur at any site of the gastrointestinal tract. The earliest lesions can be observed over Peyer's patches and mostly the proximal ileum is affected. Furthermore, granulomas consisting of aggregated macrophages can be found throughout the intestines. UC is characterized by a diffuse, superficial mucosal ulceration that begins in the rectum and extends proximally in a continuous manner. In histological stains, depletion of goblet cell mucin and infiltration of neutrophils in the lamina propria and crypts can be observed (Xavier, 2007).

Known risk factors for developing IBD are diet, hygiene, smoking, appendectomy, childhood infections and atypical mycobacterial infections (Loftus, 2004; Xavier, 2007). The highest prevalence and incidence can be found in Europe and North America. A rising incidence is

noticeable in Southern Europe, Asia and the developing countries (Molodecky, 2012). The western diet (a diet high in fats, sugar and protein but low in fibers) is an important contributor to the development of inflammatory diseases (Macia, 2012; Nicherson, 2012). Additionally, the high rate of antibiotic use, especially during childhood, adds to the high incidence of IBD in developed countries (Xavier, 2007; Macia, 2012).

Besides the environmental factors that play a role, also the genetic background is essential in the onset of IBD. Genome wide screening resulted in a list of genes that are involved in the development of IBD. These genes relate to pathways that affect intestinal homeostasis and epithelial barrier integrity, for instance NOD2-dependent innate immunity (mutations in the "sensor" genes CARD15 and NOD2); the inflammasome pathway and autophagy (Saleh, 2011).

3. The microbiome

Next to the characteristics that find their origin in the host, also the microbial environment is involved in the development of IBD. During the evolution of mankind, a symbiosis has developed between the host and the intestinal microbiota. Nutrition is the main factor shaping the composition of the microbiota, while genetic background, the immune system and environmental factors contribute to the stability. In turn, the microbiota is a major element contributing to the homeostasis of the host immunity and physiology on a local and systemic level (Simren, 2013; Nicholson, 2012).

The microbiota of a newborn is seeded at birth. A variety of factors influence the composition of the infants microbiota, like the way of delivery, mode of feeding or the use of antibiotics. For instance, the microbiota of children that are delivered vaginally is enriched with favourable *Lactobacillus* spp. and *Prevotella* spp., whereas children delivered via a caesarian section have a microbiota that contains *Staphylococcus* spp., which relates to the bacterial composition of the mother's skin (Nicholson, 2012; Chassard, 2014). The first colonizers are essentially aerobic bacteria. The composition is gradually changed to a more diverse, anaerobic-dominated microbial community. During adulthood the microbiota appears to be stable. In the late phase of life the composition changes towards a facultative anaerobe community, where the ratio Bacteroidetes to Firmicutes shifts and the amount of bifidobacteria decreases (Honda & Littman, 2012; Nicholson, 2012).

In a healthy adult, the microbiota is dominated by 4 phyla, see table 1. When developing IBD, a shift in the composition of the microbiota occurs. The diversity of the microbiota decreases and the amount of mucosa associated bacterial strains rise (Chassaing, 2011). Furthermore, a depletion of anaerobics has been found, while there is a rise in facultative anaerobic enterobacteriaceae (Winter, 2013).

Table 1. Major bacterial genera of the microbiota in a healthy adult (Honda & Littman, 2012; Simren, 2013)**.**

Phylum	**Genera**
Bacteroidetes	*Bacteroides* spp.
	Prevotella spp.
Firmicutes	*Clostridium* cluster IV (*C. leptum*, *Faecalibacterium prausnitzii*)
	Clostridium cluster XIVa (*C. coccoides*)
	Lactobacilli
	Bacillus spp.
Proteobacteria	*Escherichia* spp.
Actinobacteria	*Bifidobacterium* spp.

Next to the changes in the composition of the 4 dominating phyla, also pathogenic bacteria get the opportunity to colonize the gut (see table 2) (Honda & Littman, 2012). Opportunistic/pathogenic bacteria that are commonly found in subjects with IBD are enterotoxigenic strains of *Bacteroides fragilis*, *Klebsiella pneumoniae*, *Proteus mirabilis, Ruminococcus gnavus* and *Ruminococcus torques* (Honda & Littman, 2012; Winter, 2010). Additionally there is a rise noticeable in the amounts of the Proteobacteria *Salmonella typhimurium*, *Campylobacter* spp., adherent-invasive *Escherichia coli* (AIEC) and *Helicobacter hepaticus* (Honda & Littman, 2012; Winter, 2013; Chassaing, 2011).

Table 2. Bacterial shift in the microbiota of patients with IBD (Honda & Littman, 2012; Simren, 2013; Nicholson, 2012; Chassaing, 2011)

Decline in:	**Rise in:**
Bifidobacterium spp.	Actinobacteria (but no rise in *Bifidobacterium* spp.)
Bacteroides spp.	Proteobacteria (adherent-invasive *E. coli* (AIEC))
Firmicutes (*Lactobacilli* and *Faecalibacterium prausnitzii)*	Bacteroidetes (*Prevotellaceae* and *Porphyromonadaceae*)

Although *E. coli* can be present as a commensal without rarely causing any symptoms of disease, there are pathogenic strains that can initiate inflammation. Strains that are highly prevalent in subjects with IBD are mucosa-associated *E. coli* (MAEC). While MAEC are present in both UC and CD, the presence of AIEC, a subtype of mucosa-associated *E. coli*, is restricted to CD (Rolhion, 2007; Martinez-Medina, 2009). The criteria for inclusion in the group of AIEC are 1) the actin-dependent ability to adhere and invade intestinal epithelial cells, 2) the ability to invade and survive in macrophages without causing cell death, and 3) the ability to initiate the release of large amounts of TNF-α by macrophages (Glasser, 2001;

Martinez-Medina, 2009; Rolhion, 2007). These characteristics make AIEC a very interesting link between the innate immune system and the onset of inflammation in IBD.

Another strain of bacteria that can invade epithelia is *S. typhimurium*, a sulfate-reducing bacterium (SRB) that is highly present in patients with UC (Rowan, 2009). In the gut, hydrogen sulfide (H_2S) is produced endogenously and by strains of gram negative, anaerobic bacteria. These bacteria produce H2S by metabolizing short-chain fatty acids (SCFA) (Medani, 2011). When the gut is additionally colonized by SRB, a rise of H_2S can be seen as a byproduct of the metabolism of SRB. Recently there has been a lot of focus on the role of H_2S in the intestines. Small amounts seem to be necessary for optimal gut function, since hydrogen sulfide has shown to regulate gut motility, epithelial cell health and inflammation (Fiorucci, 2006; Shen, 2013). On the other side higher concentrations of H_2S in the gut can cause tissue damage and inflammation. It is known that H_2S can inhibit the oxidation of butyrate and other SCFAs, and impair phagocytosis and bacterial killing (Rowan, 2009; Medani, 2011; Chen, 2010). The inhibition of SCFA oxidation leads to an 'energy deficiency hypothesis' in UC, which makes H_2S concentrations increase, impairing the butyrate production and resulting in energy deficiency for colonocytes, since they rely for 70% on butyrate as an energy source (Rowan, 2009; Medani, 2011). Therefore, characterizing the survival strategies of *S. typhimurium* can provide new insights in the etiology and treatment of IBD.

4. The mucosal layer in the gut

Before pathogenic bacteria like AIEC and *S. typhimurium* have the opportunity to colonize the gut, they first have to disturb intestinal homeostasis. The human body maintains homeostasis by sustaining an intestinal barrier with the external environment and controlling the exposure of bacteria to host tissue. In an anatomical manner this is established by stratification of the intestinal mucosae, which minimizes direct contact of bacteria with the epithelial surface, and compartmentalization, which constrains invasive bacteria to intestinal sites and limits the exposure of these bacteria to the systemic immune system (Hooper, 2012). This compartmentalization is established by a bilayer of mucus that is present in the colon, the glycocalyx. The dense, inner layer of the polysaccharide bilayer is firmly attached to the epithelium and is devoid of bacteria (Saleh, 2011; Hooper, 2012). The luminal side consists of large amounts of secretory immunoglobulin A, glycoproteins, proteoglycans, peptides and enzymes. These components are packed in a network of glycoprotein Mucin, which is produced by goblet cells. Often the glycocalyx in patients with IBD is affected (Macia, 2012). For instance, less defensins are present in the mucus layer of CD patients compared to controls and these defensins are less capable of eliminating invading *E. coli* (Wang, 2007). Furthermore, there are indications that multidrug resistance 1 (MDR1) protein and transmembrane MUCIN-3 are associated with UC. These factors compromise the defence against pathogens and therefore play a role in the onset of IBD (Knight, 2008).

Underneath the glycocalyx lie the epithelial barrier and the lamina propria. The epithelial layer consists of absorptive enterocytes, microfold cells (M cells), multipotent crypt cells, antimicrobial and growth factor producing paneth cells and hormones producing enteroendocrine cells (Natividad, 2013). The paracellular pathway of the epithelial barrier is sealed by tight junction proteins, adherens junctions and desmosomes which regulate the integrity and structure of the monolayer. Intracellularly the junction proteins are connected with actin. Modulation of tight junction proteins or actin can result in an increased

permeability which can either be used by immune cells to invade the lumen and decrease the rate of infection, or by microorganisms to invade the host (Natividad, 2013; Turner, 2009; Berkes, 2003). Pathogens can disrupt the tight junctions by altering the cellular cytoskeleton and induce actin rearrangements, and by affecting directly the tight junction proteins (Berkes, 2003). For instance *E. coli* forms a pedestal lesion when invading an epithelial cell. This results in intracellular actin rearrangements that alters the structure of the cell and therefore also the structure of tight junctions, ultimately leading to increased permeability that facilitates bacterial passage through the epithelial barrier (Blikslager, 2007). Not only microorganisms can influence the permeability of the epithelial layer, also the genetic profile and the immune system play important roles in maintaining membrane integrity. Cytokines, especially TNF-α, IL-1 and IL-6 can impair barrier stability. The functional reason for this is enabling phagocytic cells to reach the side of infection. Due to the high release of TNF-α in IBD, the overall effect is that membrane integrity is impaired and pathogens are able to invade the host. This concept is further proven in the treatment of CD, where the membrane integrity can be improved by administering anti-TNF-α (Turner, 2009).

The second barrier a pathogen needs to overcome when invading the gut is the extensive presence of (innate) immune cells in the lamina propria that underlies the epithelial cell layer. Relevant players are the epithelial cell itself, macrophages, neutrophils and dendritic cells. The dendritic cells fall outside the scope of this article, an extensive review about these cells is written by Banchereau (Banchereau, 2000).

4.1 Epithelial cells

On top of forming a physical barrier with the external environment, intestinal epithelial cells (IECs) also produce a range of proteins that sustain homeostasis in the gut. Proteins that are secreted by IECs are defensins, lysozymes, C-type lectins and cathelicidins. Their role is mainly to act as a natural antibiotic. Next to this, these proteins can also function as immuno-modulators and they have the ability to shape the composition of the microbiota (Sartor, 2008; Natividad, 2013).

IECs are a source of inflammatory and anti-inflammatory mediators in the gut. For instance, the release of TGF-β and IL-8 contributes to the attraction of macrophages and neutrophils respectively. In order to suppress the initiated inflammatory response and prevent damage to the host, anti-inflammatory mediators are released. These mediators are mostly derived from fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) known as omega-3 (fish) oils (Serhan, 2005; Blikslager, 2007).

Besides their role in secreting immunomodulators, IECs are also important in sensing the environment via toll-like receptor signalling (TLR-signalling). The chronic activation of TLR by continuous bacterial passage through the epithelial layer should be prevented, as constant triggering of TLRs lead to the expression of the proinflammatory cytokines TNF-α and IL-1β, causing destruction of epithelium, loss of barrier integrity and the recruitment of macrophages and neutrophils (Honda & Littman, 2012; Fischer, 2006; Blikslager, 2007). Therefore, epithelial cells show a minimal expression of apically present pathogen recognition receptors, like TLR4 and lipopolysaccharide co-receptor CD14. IECs can signal to the systemic immune system by the activation of TLR3, TLR7, TLR8 and TLR9, which are intracellular pathogen recognition receptors (Artis, 2008). This way IECs are only able to signal when a pathogen is invading the mucosal layer and it could be one of the reasons why

IECs are hyporesponsive to commensal bacteria that do not invade the epithelial layer (Artis, 2008; Fischer, 2006).

4.2 Macrophages

Macrophages are attracted to the lamina propria by TGF-β that is released by epithelial cells. Monocytes from the blood migrate to the lamina propria and are able to differentiate to either residential, intestinal macrophages or macrophages that produce proinflammatory mediators according to the amount of TGF-β that is present. Intestine residential macrophages are able to survive for weeks. These macrophages are present in the extracellular matrix of the lamina propria and show a different phenotype than macrophages that can be found elsewhere in the human body. To control the presentation of antigens to the systemic immune system, residential macrophages show a limited expression of LPS co-receptor CD14 and receptors for immunoglobulin A and G (Smythies, 2005; Smith, 2005; Smith, 2011). Furthermore, residential macrophages have an impaired ability to produce proinflammatory cytokines and chemotaxis when they encounter a pathogen. The result is that residential macrophages cannot migrate to different sites of the intestines and therefore dendritic cells are the main presenters of antigens to T cells under healthy conditions (Smith, 2005). Although this type of macrophages show inflammation anergy, they do play a pivotal role in the response to infection since they are highly phagocytic and important scavengers of invaded *S. typhimurium* and *E. coli* (Smythies, 2005). This is done is a similar manner as IECs do, namely through the expression of intracellular TLR7-9 (Smith, 2011).

During an infection, more macrophages are recruited to the site of infection by cysteine-containing ligands (CC chemokines), non-cysteine-containing chemotactic ligands and pathogen derived peptides (Smith, 2005). The newly recruited macrophages show a distinct phenotype than their residential counterpart due to the increased release of TGB-β by IECs. Newly attracted macrophages can produce proinflammatory cytokines like TNF-α and IL-8. This phenomenon can particularly be seen in CD, where at least one third of the macrophages in the lamina propria show proinflammatory properties and CD14 expression, indicating that an elevated recruitment of monocytes is ongoing (Smith, 2005).

The release of proinflammatory cytokines by macrophages is not sufficient to induce the vast inflammatory response that is seen in IBD. Accordingly, other signals are necessary. As mentioned before, an important producer of inflammatory mediators is the epithelial cell. Upon TLR activation, IL-8 is released which attracts neutrophils to the site of infection (Berkers, 2003).

4.3 Neutrophils

Neutrophils are phagocytic cells that are essential for resistance against bacterial and fungal infections (Segal, 2005). They accumulate in the lamina propria and subepithelium, where they decrease the infection by uptaking pathogenic bacteria and causing a respiratory burst. Additionally, they are a source of the proinflammatory cytokines TNF-α, IFN-γ and IL-8 indicating that they can alter tight junctions and the progression of inflammation (Blikslager, 2007; Hanauer, 2006).

The respiratory burst is essential for the functioning of a neutrophil. For years it has been thought that the reactive oxygen species (ROS) and reactive nitrogen species (RNS) are responsible for the killing of bacteria. Recently it got to the attention that also the proteases

inside the neutrophilic vacuole play an important role in the clearance of microorganisms. The moment a neutrophil engulfs a bacterium, it interacts with cytosolic proteins that activate electron transport through the NADPH oxidase NOX2 (Segal, 2005; Bedard, 2007). NOX family NADPH oxidases use oxygen as an electron acceptor and the product is superoxide, a ROS (Bedard, 2007). After the activation of NOX2, degranulation occurs due to pH elevation in the vacuole and the flux of different ions across the vacuole membrane. This process activates the proteases and together with the ROS that are produced, the microorganisms get cleared (Segal, 2005).

While decreasing the infection, neutrophils can also damage the intestinal tissue. During their migration to the site of infection, or when saturated, they release vacuoles that contain ROS, RNS and proteases that can damage the surrounding tissue or even cause cellular necrosis. Moreover, the extensive accumulation of neutrophils in the subepithelial space can impair epithelial membrane integrity, giving microorganisms the opportunity to invade the host. Therefore, the function and recruitment of neutrophils should be tightly controlled. This is established in different manners by macrophages and neutrophils themselves. The neutrophil is capable of secreting a lipoxin that signals to macrophages to inhibit further recruitment. Additionally, this lipoxin promotes macrophages to take up the saturated neutrophil in order to prevent damage of the surrounding tissue (Blikslager, 2007; Serhan, 2005).

In IBD, inhibition of neutrophil recruitment and clearance of neutrophils is either impaired or malfunctioning. For instance, it has been proposed that the enhanced release of granulocyte colony-stimulating factor by epithelial cells could be a cause of the delayed clearance of neutrophils (Ina, 1999). Additionally, the adherence of neutrophils to endothelial cells is enhanced in patients with IBD, by the upregulated expression of neutrophil adherence effector proteins P-selectin and intercellular adhesion molecule 1 (ICAM 1) (Naito, 2007). The result of these processes is the formation of the characteristic granulomas we see in subjects with IBD (Hanauer, 2006).

5. Survival strategies of *S. typhimurium* and AIEC

As aforementioned, both AIEC and *S. typhimurium* have similar survival mechanisms to overcome colonization resistance. These mechanisms are shown in figures 1 and 2, which are presented below.

5.1 The sulfate-reducing bacterium S. typhimurium

S. typhimurium is a facultative anaerobic, sulfate-reducing, Gram-negative bacterium that can survive in the gastrointestinal tract under immunocompromised conditions. Infection can lead to gastroenteritis, an infection of the ileum and colon that manifests as fever and diarrhea (Santos, 2009). Enteric pathogens, like *S. typhimurium*, have the ability to actively induce inflammation, changing the composition of the microbiota and as a result, increase the amount of proteobacteria and give rise to a more favourable environment for survival.

The active inducement of inflammation by *S. typhimurium* is necessary to overcome colonization resistance (Stecher, 2007). To induce inflammation *Salmonella* makes use of two main characteristics, the invasion of the epithelial barrier via M cells and survival inside epithelial cells or macrophages. This is conducted via two Type III secretion systems (T3SS). Type 1 (T3SS-1) is involved in the invasion of the bacterium and actin arrangements

inside the host cell. Type 2 (T3SS-2) is concerned with survival in the host cell, trafficking of *Salmonella* containing vacuoles and anaerobic growth (Santos, 2009; Thiennimitr, 2011; Bruno 2009).

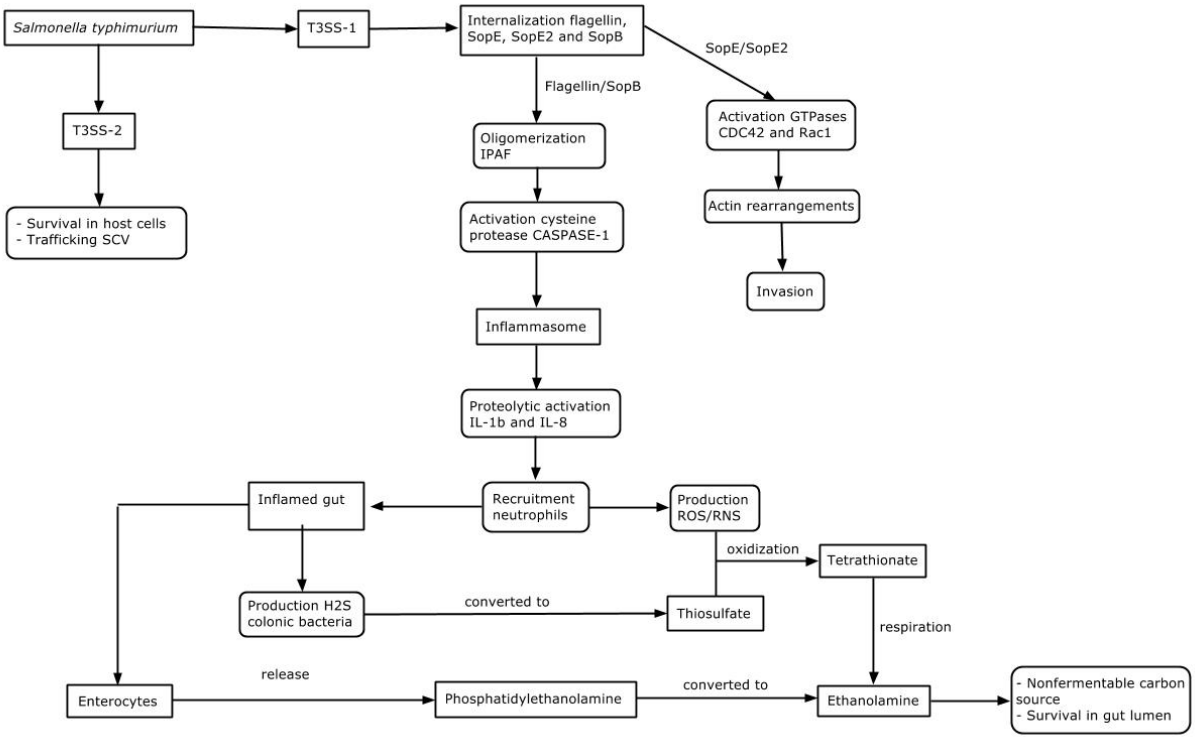

Figure 1. The survival strategies of *S. typhimurium*. Two type III secretion systems (T3SS) of S. typhimurium are involved in the survival in the gut lumen. T3SS-2 plays a role in the trafficking of Salmonella containing vacuoles (SCV) across host cell membranes, where T3SS-1 is important in eliciting an immune response. The immune response is initiated by a variety of effector proteins. SopE and SopE2 are involved in the invasion of macrophages by activating the GTPases CDC42 and Rac1. Flagellin and SopB are internalized in the macrophage where they interact with IPAF, a cytosolic pattern-recognition receptor. This results in the activation of inflammasome Caspase-1 and the recruitment of neutrophils to the side of infection. Neutrophils produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) as a response to Salmonella invasion. The vast inflammatory response causes an inflamed gut and elevated concentrations of hydrogen sulfide (H_2S). Hydrogen sulfide is converted to thiosulfate by rhodanese enzyme, after which thiosulfate is oxidized by the ROS/RNS from the neutrophils. This ultimately leads to the respiratory electron acceptor tetrathionate, used by Salmonella to respire ethanolamine as a nonfermentable carbon source and to outcompete the commensal bacteria.

To survive, *S. typhimurium* needs to elicit a highly controlled and specific immune response, wherein activation of TLR5 is avoided (Lebeer, 2010). Flagellin and T3 effector proteins SopE, SopE2 and SopB are internalized by T3SS-1. The effector proteins start a reaction cascade by activating the GTPases CDC42 and Rac1, which result in ruffling of the cell membrane provoked by actin rearrangements (Santos, 2009; Zhou, 2001). This process enhances the entrance of bacteria in those host cells. In addition, flagellin and SopB cause oligomerization of Ipaf, a cytosolic pattern-recognition receptor, that leads to activation of the Caspase-1 inflammasome (Gewirtz, 2001; Franchi, 2006). Caspase-1 in turn proteolytically activates IL-1β and IL-8, leading to a proinflammatory response, the recruitment of neutrophils and cell

death (Santos, 2009; Lyons, 2004; Andersen-Nissen, 2005). Although this will ultimately result in the death of *S. typhimurium* that reside inside the macrophage, the sacrifice is needed for the survival of the bacteria in the gut lumen. The inflammatory response leads to a more favourable environment for *S. typhimurium* due to an increased pH and a decrease in SCFAs, caused by an overproduction of inflammatory cytokines and H_2S (Honda & Littman, 2012; Winter, 2010; Thiennimitr, 2011; Abrahams, 2006). Under these circumstances *S. typhimurium* can outcompete the commensal microbiota, which further alters the composition of the microbiota and aggravates the symptoms associated with IBD (Honda & Littman, 2012).

The nutritional competition with the commensal flora is in favour of *S. typhimurium*, because the inflammatory response creates a new energy source that the commensal flora is not able to use. Important in this process is the presence of H_2S. The narrow concentration range from physiological feasible to highly toxic makes that the body need efficient mechanisms to clear H_2S. The clearance is mainly established by two enzymes, rhodanese and thiosulfate sulfurtransferase enzyme (Kolluru, 2013; Medani, 2011). Rhodanese activity is the rate limiting step in this process. In patients with UC, the activity of rhodanese is not increased nor is the expression of the enzyme, which can explain the high amounts of H_2S that are present in the gut (Medani, 2011). The end product of the enzymatic conversion of H_2S is thiosulfate ($S_2O_3^{2-}$). When thiosulfate is oxidized, tetrathionate ($S_4O_6^{2-}$) is formed (Winter, 2012; Stoffels, 2012; Thiennimitr, 2011). This oxidization is facilitated by ROS, that are products of the respiratory burst of neutrophils that was caused by the invaded *S. typhimurium*. Tetrathionate can be used by *S. typhimurium* as a respiratory electron acceptor to utilize the nonfermentable carbon source ethanolamine out of phosphatidylethanolamine (Winter, 2010; Thiennimitr, 2011). Phosphatidylethanolamine is released from the tips of the villi of enterocytes and it is one of the most abundant phospholipids in membranes. It can be converted to ethanolamine (C_2H_7NO) by phosphodiesterases, which are present in the majority of *Salmonella* and *E. coli* strains (Furne, 2001; Roof, 1988; Garsin, 2010).

So by invading the host, *S. typhimurium* induces an inflammatory response that leads to the production of a respiratory electron acceptor that can be used to bypass nutritional competition with the commensal microbiota in the gut lumen, thereby promoting it's own growth and survival inside the gut.

5.2 Adherent-invasive Escherichia coli

AIEC present in patients with CD are able to adhere and invade IECs, replicate within macrophages and form a biofilm (Glasser, 2001; Martinez-Medina, 2009; Rolhion, 2007; Chassaing, 2013). The invasion of AIEC is caused by the disorganization of actin and displacement of occludin and E-cadherin, which results in the loss of integrity of the monolayer (Sasaki, 2007). Invasion is further stimulated by the interaction with the host outer membrane vesicles protein OmpA and heat shock glycoprotein gp96, which are localized on the endoplasmatic reticulum (Rolhion, 2010).

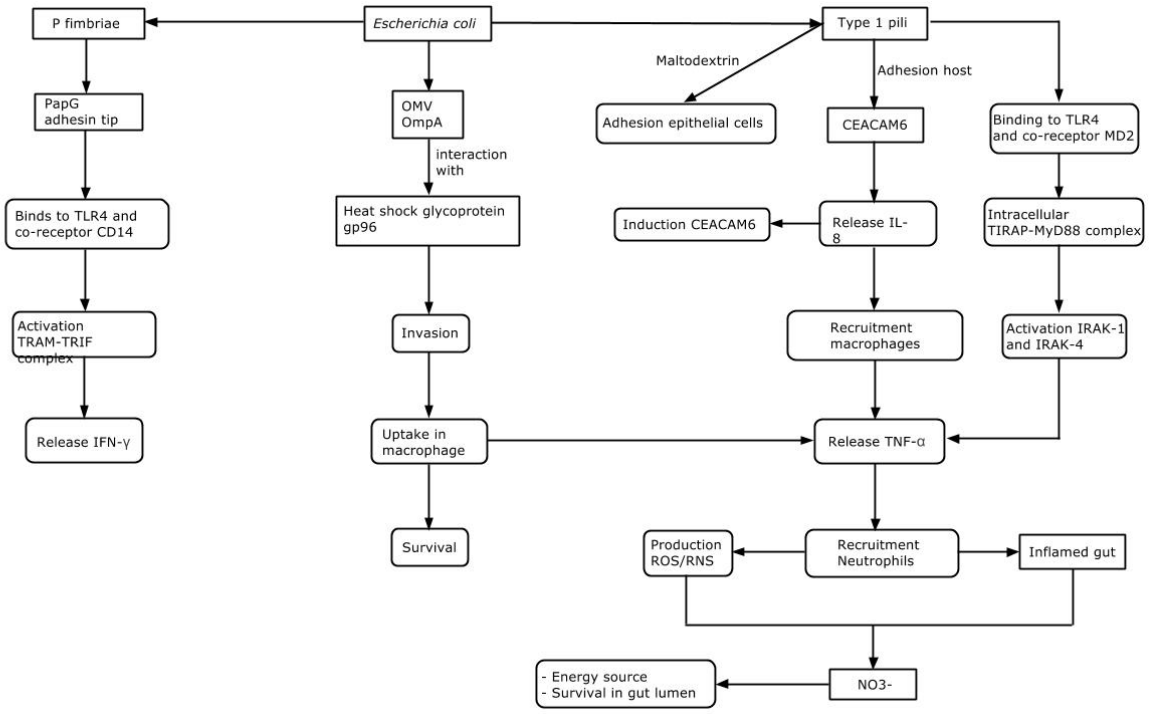

Figure 2. The survival strategies of adherent-invasive *Escherichia coli* (AIEC). For the survival in the gut *E. coli* relies mainly on two mechanisms. First AIEC invades macrophages by interacting with host outer membrane protein OmpA and heat shock protein gp96. The uptake in the macrophage results in an enhanced release of tumor necrosis factor alpha (TNF-α). This release is further enhanced by the adhesion of AIEC to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) and the interaction with toll-like receptor 4. Neutrophils are recruited to the side of infection and the inflamed gut and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by neutrophils result in a favourable environment for AIEC. Chemical reactions between ROS and RNS in the gut lead to higher levels of nitrate (NO_3^-), S-oxides and N-oxides, which AIEC can use as an energy source to potentially outgrow the commensal microbiota and survive in the gut lumen.

In addition to promoting invasion, AIEC also makes use of virulence factors like P fimbriae, type 1 pili and flagella to elicit invasion and survival. Type 1 pili can undergo an interaction with carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), which is present on the apical surface of ileal epithelial cells (Sartor, 2008). This interaction stimulates the release of IL-8, attracting neutrophils and macrophages that release TNF-α and IFN-γ (Bringer, 2012; Chassaing, 2013). Furthermore, it has been proven that the release of IL-8 induces the expression of CEACAM6, further enhancing the adhesion and invasion of AIEC (Chassaing, 2013; Rolhion, 2010; Barnich, 2007). Recently it has been found that the adhesion of AIEC can also be established independently from CEACAM6, by the dietary component maltodextrin (Nickerson, 2012).

Type 1 pili do not only cause adherence to - and invasion of IECs, but they can also elicit an inflammatory immune response by binding to TLR4. Under healthy conditions TLR4 is only expressed by IECs in very small amounts, but under chronic inflammatory conditions TLR4 expression is increased (Cario, 2010). Upon binding of AIEC with TLR4 and the co-receptor MD2, the MyD88 pathway is activated and via NF-kB TNF-α is released (see figure 2 for a more detailed description). Also P fimbriae have the ability to signal through TLR4. The PapG

adhesin tip of P fimbriae bind to the glycosphingolipid co-receptor and then signals in a MyD88 independent manner. The TRIF pathway is activated which results in the release of IFN-γ (Cario, 2010; Sartor, 2008). Although this gives the impression that the body is successfully fighting of the infection, AIEC actually uses this immune response in its benefit.

For the survival in the gut, AIEC relies on 2 mechanisms. After invasion of the intestinal barrier, *E. coli* needs to avoid clearance by the innate immune system. This is done by the entrance in macrophages and/or epithelial cells. Macrophages that are infected with AIEC secrete large amounts of TNF-α and IL-8 which enhance the inflammatory response in the intestines by attracting neutrophils. Furthermore, the release of TNF-α stimulates the intracellular replication of AIEC. Intracellular survival is achieved due to genetically impaired autophagy in subjects with CD, preventing or delaying cell death of the macrophage which results in the formation of granulomas (Glasser, 2001; Bringer, 2012).

The attraction of neutrophils is needed for the survival of AIEC in the gut lumen. The inflammatory response of neutrophils causes a rise in highly oxidized by products, like nitrate, ROS superoxide and RNS nitrogen oxide. In the colon these compounds react with each other to intermediate product peroxynitrite ($ONOO^-$), which further reacts to nitrate (NO_3^-) or it can oxidize organic sulfides and tertiary amines to S-oxides and N-oxides. Facultative anaerobic enterobacteriaceae, like AIEC, possess a variety of reductases and therefore they can use these by products for growth. This way they have an advantage over the commensal bacteria, increasing their survival in the gut and potentially outgrow the resident flora (Winter, 2013).

6. Possible targets and treatment

So far, there is no cure for IBD and current (long-term) treatment of the disease is based on the relief of symptoms and the prevention of remission. This is established in a stepwise manner, in which different drugs are prescribed. The first step of treatment consists of the administration of anti-inflammatory 5-aminosalicylates (5-ASA) and, when necessary, antibiotics. Antibiotics are only prescribed in cases of CD complications, like bacterial overgrowth. 5-ASA are highly bowel-specific and therefore they do not cause severe side effects. Next to this, being derived from salicylic acid, there are indications that 5-ASA compounds can also function as an antioxidant to scavenge the ROS that are released by neutrophils during inflammation. Parallel to these drugs, probiotics can be prescribed since combined administration of probiotics and 5-ASA can alleviate symptoms in patients suffering from UC (Medscape, 2013; Bernstein, 2009).

When the disease cannot be kept under control with the administration of 5-ASA, corticosteroids are prescribed. Steroids are known for their rapid response, the downside is the occurrence of side effects and their high risk for complications when used for a longer period of time. Therefore, therapy with corticosteroids is normally for a short period of time and in low doses. During exacerbations, higher doses of corticosteroids can be prescribed. If this step of the treatment fails or when it takes too long to control the inflammation, a last resort for the healthcare practitioner is the prescription of immune-modifying agents, like thiopurines and anti-TNF-α (Kozuch, 2008; MedScape, 2013).

Thus far, a few recommendations are made according to changes in diet and lifestyle, because the link between IBD and diet/lifestyle is poorly understood. Suggestions are to

lower the intake of oligosaccharides (fibers, prebiotics) during acute inflammation and reduce emotional stress where possible. Both recommendations might relieve symptoms, but are not known to prevent remission. Furthermore, there are indications that the administration of vitamin B12, vitamin D and certain strains of probiotics (*E. coli* Nissle 1917, *Bifidobacteria* spp. and *Lactobacilli*) can have a positive effect on symptom severity and the occurrence of remission (Sartor, 2004; Laparra, 2009; Bernstein, 2009; MedScape, 2013).

6.1 Competition for nutrients

Based on the survival strategies of both *S. typhimurium* and AIEC, it can be interesting to look at new treatment strategies for IBD. An important feature is the competition for nutrients. Focussing on a nutritional intervention for IBD can possibly reduce the prescription of corticosteroids and the occurrence of remission and is therefore more favourable and less invasive for the patient.

Since both *S. typhimurium* and AIEC survive by utilizing an energy source that cannot be used by the commensal microbiota, one of the treatment strategies could be to provide an extra carbon source during remission. According to the energy deficiency theory, high concentrations of H_2S inhibit the formation of butyrate and other SCFAs out of dietary polysaccharides. Butyrate is positively involved in colonic cell growth, cell differentiation and an adequate anti-inflammatory response (Laparra, 2009). Therefore, providing fermentable dietary polysaccharides, e.g. non-starch polysaccharides, resistant starch and oligosaccharides, can lower the inflammatory response and positively influence the regeneration of the intestinal mucosae. Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides derived from lactose, promote growth of bifidobacteria. Moreover, they may structurally mimic pathogen binding sites on IECs and thereby my inhibit the adhesion of pathogens like *S. typhimurium* and AIEC. In addition, inulin derived fructo-oligosaccharide (FOS) promote the growth of *Lactobacilli* and reduce the production of pro-inflammatory markers (Laparra, 2009).

The overall effect of prebiotics could be enhanced by administering specific strains of probiotics at the same time. Used strains should be selected for persistence in the gastrointestinal tract and their anti-inflammatory and antibacterial characteristics. For instance, *E. coli* Nissle 1917 exhibits an anti-inflammatory effect and the presence of large amounts of both *Bifidobacteria* spp. and *Lactobacilli* is associated with a healthy gut environment (Sartor, 2004; Bernstein, 2009). Dosing solely probiotics has a minimal effect on the overall composition of the microbiota and shows a limited effect on symptom improvement, therefore combination treatment with prebiotics should be considered (Marteau, 2003). The application of conventional treatment should not be forgotten. For instance, the simultaneous use of synbiotics (pre- and probiotics) with 5-ASA could be an interesting approach when patients do not suffer from active inflammation. With this approach synbiotics will support the overall intestinal health and promote homeostasis in the gut, where 5-ASA has an anti-inflammatory effect and can possibly scavenge the ROS that are released by neutrophils in the early phase of remission, thereby preventing disease flare. Thus, the application of synbiotics, consisting of selected probiotic strains and the fermentable carbohydrate substrates GOS and FOS, in combination with 5-ASA should be further explored.

7. Conclusion

The gut microbiota has an enormous impact on the health status of the host. Via metabolic functions and interactions with the immune system, the microbiota maintains gut homeostasis in cooperation with the hosts immune system. Subjects with IBD show a compromised mucosal tolerance in the gut and a decreased diversity of health-promoting bacterial strains. Pathogens like *S. typhimurium* and AIEC try to take advantage of a compromised mucosal tolerance and use the hosts immune response in their benefit to outgrow the commensal flora, leading to inflammation and disease flare.

There are indications that both *S. typhimurium* and AIEC utilize an energy source, that cannot be used by the commensal flora, to promote their own growth. This hypothesis gives rise to new treatment strategies for IBD. In order to compete with pathogenic bacteria, the administration of an extra fermentable carbon source, like GOS and FOS, in combination with the use of selected probiotic strains (together called synbiotics) should be investigated. Additionally, the application of synbiotics with 5-ASA could further reduce the occurrence of remission and should therefore be tested in well developed *in vivo* studies.

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