

***Escherichia coli* infection, intimin, and Toll-like receptors**

V. Bueno^{1,*} and O. Augusto Sant'Anna²

¹ UNIFESP Federal University of São Paulo, Immunology Division

² INSTITUTO BUTANTA, Immunochemistry Laboratory

*Author correspondence: Valquiria Bueno. Rua Botucatu 862 4º andar São Paulo SP BRAZIL – ZIP 04023-900; Phone: 55 11 5549 6073 email: valquiria@nefro.epm.br

In order to control bacterial grown the small intestine possess: motility for prevention of stasis of nutrients and bacteria, a barrier created by mucus, and antibacterial molecules secreted by epithelial cells. Macrophages and dendritic cells reside in lamina propria and are responsible for the innate immune response in the mucosa.

Bacterial infection leads host cells to express Toll-like receptors (TLRs) on the outer membrane surfaces. TLRs interact with the conserved regions of pathogenic microorganisms (pathogen-associated molecular patterns, PAMPs) including lipopolysaccharides, peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, and double-stranded RNA.

The recognition of PAMPs by TLRs initiates the induction of inflammatory responses in the host cells through activation of signal transduction pathways mediated by transcription factor (nuclear factor- κ B, NF- κ B). Considering TLRs expressed in human cells TLR1, TLR2, TLR4, TLR5, and TLR6 are positioned near trans-membrane domains and are vital to the defense response of cells against external pathogens.

Enteropathogenic *Escherichia coli* (EPEC) cause diarrheal disease by altering enterocyte physiology and producing mucosal inflammation. *E.coli* establishes type III secretions system (TTSS) through which bacterial factors are translocated into cell and modifies several host functions. One of the proteins injected is Tir and this factor is directed into the host cell membrane binding intimin. Tir-intimin mediates adherence and triggers the reorganization of the cytoskeleton. Activation of TLRs by intimin could lead to cytokine and other factors secretion by host cells which potentiate the immune response.

Inflammation induced by bacteria results from the balance of positive and negative factors. In this chapter we discuss *E.coli* infection and intimin as one of the main factors of TLRs activation and immune response induction.

Keywords *Escherichia coli*, intimin, toll-like receptors, immune system

1. Introduction

In small intestine, it is observed a crescent load of bacteria from proximal to distal regions. Intestine motility, mucus, and anti-bacterial molecules are used to maintain the bacterial count low. Epithelial cells facing the luminal surface of the intestine form the first barrier against pathogens. Enterocytes (absorption), goblet cells (mucus), enteroendocrine cells (hormone), and Paneth cells (anti-microbial peptides) form the epithelium (intestinal mucosa). Lamina propria localized underneath the epithelium contains macrophages and dendritic cells. Peyer's patches (mucosa-associated lymphoid tissue, MALT), T and B cells constitute the adaptive immune system. Toll-like receptors (TLRs) are proteins that recognize evolutionary conserved patterns in microorganisms. TLRs are expressed in intestinal epithelial cells (surface or endosomes) and in immune cells present at the lamina propria. TLR stimulus causes nuclear factor kappa B (NF- κ B) activation, cytokine production, and chemokine secretion with the attraction of inflammatory cells to intestinal site [Reviewed in 1].

Flagellin, intimins, and possibly other components of enteropathogenic *Escherichia coli* (EPEC) are capable of trigger the activation of intestinal epithelial cells and immune system. These bacterial structures contain pathogen-associated molecular patterns (PAMPs) recognized by host pathogen-recognition receptors (PRRs) such as toll-like receptors (TLRs) amongst others. As an example Andersen-Nissen et al. [2] showed that TLR5 is stimulated mainly by EPEC flagellin N-terminal D1 domain, centered on amino acids 89-96 and mutations in this PAMP could provide bacteria evasion from TLR5 recognition.

In mice ileal biopsies it was observed the staining for TLR5 both in the apical and basolateral side of the epithelium. The application *ex vivo* of flagellin (full-length) extracts in the apical side induced basolateral secretion of a cytokine (KC) with human IL-8 similar properties. Flagellin mutant (*fliC*) was not capable to induce KC secretion confirming that flagellin was the bacterial product (PAMP) responsible for the pro-inflammatory response [3].

Human biopsy showed that intestinal epithelial cells from normal mucosa constitutively expressed TLR5 suggesting that animal findings are reproducible in humans [4].

After TLRs stimulation downstream effectors such as NF- κ B are activated. NF- κ B associated with I κ B (inhibitory protein) is present in cytoplasm and after stimulus I κ B suffers phosphorylation, ubiquitination, and degradation providing translocation of the free NF- κ B to cell nucleus. Nuclear NF- κ B regulates the expression of several genes.

Cytokines and chemokines production is stimulated and their secretion cause inflammatory cells infiltration (i.e. neutrophils) aiming bacterial destruction but also leading to tissue damage.

2. Intestine – attaching and effacing process caused by pathogens

Enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *Escherichia coli* (EPEC), and *Citrobacter rodentium* are a family of attaching and effacing (A/E) pathogens [5, 6].

Enteropathogenic *Escherichia coli* are a major cause of human infant diarrhea in developing countries besides of its pathogenic effect in other animals [7].

Diarrheal disease accounts for 1.3 million infant deaths each year worldwide [8] and the pathology includes altered transport of water and electrolytes, disruption of the intestinal barrier and inflammation [9, 10].

EPEC initial cell adhesion to intestinal cells occurs through adhesins and bacterial appendages, including the flagellum [11].

EPEC cause attaching and effacing (A/E) lesions on enterocytes which are mediated by tight bacterial adherence to epithelial cells, destruction of the absorptive microvilli, and development of actin pedestal structures at the site of bacterial adherence. A/E formation requires the locus for enterocyte effacement pathogenicity island (LEE) which contains the *eae* gene coding for the bacterial outer membrane protein intimin responsible for bacterial adherence. LEE also encodes for the type III secretion system (TTSS), translocated intimin receptor (Tir) and secreted effector proteins (Esp) such as EspA, EspB, and EspD required for signal transduction in host cells and for A/E lesions [5].

Intimin carboxyl-terminal amino acids are variable and thus different types of intimin are defined considering EPEC and Enterohemorrhagic *Escherichia coli* (EHEC) from animal and human sources [12]. Antibodies against LEE-encoded proteins in endemic areas are present in patients infected with EPEC or EHEC but also in healthy individuals suggesting population exposure to proteins expressed by *E.coli* strains. Guirro et al. [13] observed in children with no signs of infection that IgG antibodies against the intimin conserved region were present in 65% whereas IgG antibodies for the variable region of intimin γ (EHEC) were detectable in 18.5% suggesting the potential immunogenicity of these antigens and their possible use for human vaccination targets. For that purpose is essential the better understanding of *E.coli* virulence factors, immune system activation, and pathophysiology of the resulting diarrhea due to EPEC infection.

3. Enteropathogenic *Escherichia coli* virulence factors

EPEC lack genes to produce shiga toxin, instead they present the virulence factors LEE-encoded and non-LEE encoded effector proteins which subvert and modulate cellular and barrier properties of the host. The locus of enterocyte effacement (LEE) encode for a large genomic pathogenicity island. Genes present in LEE encode for the outer membrane adhesion (intimin), type III secretion system (TTSS) with Esc and Sep proteins, chaperones (Ces proteins), translocators (Esp A, EspB, EspD) and effector proteins (EspF, EspG, EspH, Map, EspZ), the translocated intimin receptor (Tir), the regulatory proteins Ler (LEE-encoded regulator), GrIR (global regular of LEE proteins, repressor) and GrlA (global regulator of LEE proteins, activator). EspA acts as a needle from the bacterial surface to the host cell plasma membrane whereas EspB and EspD are pore-forming structures [14].

In addition, the presence of the plasmid *E.coli* adherence factor (EAF) is unique of typical EPEC with the operon *bfp* encoding for the type IV-bundle forming pillus (BFP) and the operon *per* responsible for the transcriptional activator plasmid encoded regulator (Per) whereas atypical EPEC does not present these operons. Most of the typical EPEC belong to classic O:H serotypes and the presence of BFP leads to the localized adherence (LA) phenotype. Atypical EPEC display adherence patterns such as localized-like (LAL) associated with *E.coli* common pilus and adhesins [15, 16].

Effector encoded outside the LEE region, the non-LEE-encode (Nle) effector genes, localized in six pathogenicity islands scattered throughout the genome are NleA-H, EspG2/Orf3, Cif, EsJ and EspL. NleA inhibit protein secretion, EspJ inhibits phagocytosis, NleE and NIH activate innate immune responses [14, 17].

4. EPEC virulence factors and host immune system activation

Most of the studies in EPEC have used intestinal epithelial cell lines such Caco-2, T84, and HT-29 and identify flagellin present in bacteria flagella as the major factor activating the intestinal inflammatory response [18, 19, 20]. Hayashi et al. [21] showed that the virulence factor flagellin is recognized by TLR5 and this activation mobilized the nuclear factor NF-kappa B and stimulated TNF- α (tumoral necrosis factor) production. IL-8 expression was also induced by flagellin in polarized intestinal epithelia [22]. Considering the inflammatory and chemoattractant effects of these secreted factors it has been attributed to flagellin the role of activating innate immune response after EPEC infection.

Schuller et al. [23] showed that human intestinal biopsy apically infected *ex vivo* with EPEC presented bacteria A/E lesions and expressed a higher level of IL-8 mRNA when compared to non-infected cells. Disruption in flagellin gene

(*fliC*) produced *fliC*-negative EPEC which caused the same attachment and effacement lesions in *ex vivo* biopsy but decreased levels of IL-8 mRNA. TLR5 localization (apical and basolateral) was maintained after EPEC and adhering bacteria was observed in proximity to TLR5 suggesting a direct interaction. These results confirm the partial effect of flagellin-induced inflammation after EPEC infection and suggest that other bacterial factors could contribute to initiate the immune response.

5. Flagellin, Toll-like receptor 5 (TLR5), and inflammatory response to EPEC

In the flagellar structure flagellin is the primary protein component extending from the outer membrane of Gram-negative bacteria. In addition to the bacteria movement flagellin contributes to their virulence through mechanisms such as attaching to host cells and assisting in bacteria invasion.

Flagellin gene sequences from different Gram-negative bacteria have been compared to show that they share highly conserved regions amino-terminal and carboxyl-terminal. A central hypervariable (D3) region is present and associated with antigenic variation and evasion of the host immune response. Three domains have been identified: D1 and D2 are discontinuous and formed when residues in to the amino-terminal and carboxyl-terminal are juxtaposed (hairpin structure). D3 acts as a “blade” of the flagellar paddle. The linear arrangement is amino domain (ND)1, ND2, ND3, carboxyl domain (CD)2, and CD1 [24, 25].

Flagellin is the structural protein forming flagellar filaments and is produced by commensal/pathogenic bacteria. Polarized flagellin present in flagella become monomers and through amino- and carboxyl-conserved regions binds to TLR5 in the host epithelial intestinal cells initiating the process of immune system activation [reviewed in 26].

Using Caco-2 human colonic carcinoma cells cultured with EPEC, Steiner et al. [27] observed the secretion of IL-8. After isolating bacteria flagella it was confirmed that depolymerized flagella was the responsible for the chemokine IL-8 release by Caco-2 cells. Authors showed that the EPEC flagella presented similar predicted sequences from the *S.dysenteriae* flagellin *fliC*. In addition, it was shown in Caco-2BBE cells that flagellin causing inflammatory response required the highly conserved N and C D1 and D2 regions.

T84 cell line in the presence of purified flagellin secreted IL-8 only when the basolateral surface was exposed whereas the apical cell surface in contact with flagellin caused no IL-8 release. It was also shown that blocking NF- κ B activation decreased significantly IL-8 secretion. From all TLRs (1-10) cDNA tested only TLR5 was associated with proinflammatory gene expression (NF- κ B) in response to flagellin. Furthermore, basolateral surface cells staining for TLR5 was observed which matches the polarity of the response to flagellin supporting the hypothesis that flagellin activation of intestinal epithelia is mediated by TLR5 [28].

There is still a controversy whether the localization of TLR5 in the cell surface (basolateral/apical) is a limitation for flagellin binding. Aiming to clarify this controversy Eaves-Pyles et al. [29] showed that flagellin deposition apically in Caco-2BBE cells did not prevent engulfment. There was no change in the integrity of cell barrier suggesting a trans-cellular route for flagellin. This internalization was possible only in the presence of TLR5 as blocking or silencing (siTLR5) this receptor maintained flagellin on cell apical surface. Flagellin was co-localized with endosomal and lysosomal compartments and was not translocated to the basolateral surface suggesting degradation of the protein. Flagellin addition either to apical or basolateral surface of epithelial cells induced IL-8 release from both surfaces.

6. Intimin

Intimin, a 94-kDa outer membrane protein, is an essential virulence factor in the pathogenesis of enteric bacterial infections and is coded by *eae* gene. Despite of the establishment of 27 *eae* variants encoding distinct intimin types and subtypes it is not completely clear whether alleles are related to specific clinic characteristics. Intimin alleles are used for diagnosis, pathogenesis, epidemiology, and studies in cloning and immunology. Intimin molecule has 939 amino acids and the conserved N-terminal region (aa 1-558) works as a membrane anchor whereas the C-terminal (Int-280, aa 659-939) is associated to different intimin subtypes. C-terminal region has proximal immunoglobulin-like domains (immuno-dominant) and also a distal C-terminal lectin-like region responsible for binding to Tir (translocated receptor). Intimins variants are: α 1, α 2, β 1, β 2 (ξ R/ β 2B), β 3, γ 1, γ 2, δ (δ / β 2O), ϵ 1, ϵ 2 (vR/ ϵ 2), ϵ 3, ϵ 4, ϵ 5 (ξ B), ζ , η 1, η 2, θ , ι 1, ι 2 (μ R/ ι 2), κ , λ , μ B, vB, o, π , ρ and σ [12, 30-37].

Considering that intimin could be used as a target for vaccination, several authors have studied the effect of intimin deletion or truncated structure.

Ramirez et al. [38] showed that enterocytes from rabbits infected with wild EPEC presented increased mRNA expression of IL-1 β , IL-6, IL-8, and TNF- α with a discrete increase of IL-10. Intimin mutant bacteria caused in rabbits enterocytes increased mRNA expression of IL-10 whereas the expression of IL-1 β , IL-6, IL-8, and TNF- α were significantly decreased besides of lower intestinal infiltrates than in wild EPEC. In addition, intimin mutant-infected rabbits did not present mucoid bloody watery diarrhea and weight loss which were the clinical signs observed in rabbits infected with wild EPEC. Authors concluded that the cytokine profile involved in diarrhea caused by EPEC is intimin-

dependent. Also, in atypical EPEC (1551-2) expressing intimin subtype omicron (o) it was observed invasiveness of HeLa cells whereas EPEC mutant for intimin was not capable of invasion [39].

Using intestinal epithelial cell line (HT-29) in contact with intimin mutant EPEC Salazar-Gonzalez et al. [40] observed changes in factors downstream TLR5 activation (ERK1/2 and NF- κ B). Besides of flagellin, intimin was required to maintain NF- κ B activated during EPEC infection. Secretion of IL-1 and IL-8 was significantly decreased in mutants and TNF- α sustained production was observed only in wild EPEC. Authors concluded that intimin modulates TLR5 activation and intimate adherence alters the immune response.

These findings suggest that not only flagellin but also intimin are responsible for the inflammatory response mediated by EPEC infection. Moreover, changes in flagellin and intimin domains could favor bacterial escape from the host immune system and survival. On the other hand, PRRs (i.e. toll-like receptors) expressed in host cells constitute a selective force driving the evolution in bacteria genes associated with diversity.

7. Concluding remarks

The immune response is a complex network of cells, molecules, and factors interacting strategically to eliminate pathogens. Molecules that “perceive” such as toll-like-receptors (TLRs), T cell receptor (TCR), B cell receptor (BCR) play an essential role in pathogen recognition. TLRs are pathogen-recognition receptors (PRRs) for conserved structures (pathogen-associated molecular patterns, PAMPs) expressed by pathogens. TLRs expression in intestinal epithelial cells, dendritic cells, and macrophages is essential for the innate immune response initial activation.

Infectious processes are probably the main source of selective pressure on the immune system evolution and vice versa the immune network exerts selective pressure on functional features of microorganisms’ virulence, infectivity and toxicity.

Pathogens fate relies on their capacity to “escape” from molecules that “perceive” in immune system. As an example, bacteria flagellin recognized by TLR5 expressed in human epithelial cells causing in turn immune system activation can be mutated and favor bacteria survival. As shown by Andersen-Nissen et al. [2] *C. jejuni*, *H. pylori*, and *Bartonella bacilliformis* make flagellin that are not recognized by TLR5 and in consequence lose motility. In addition, these bacteria possess compensatory amino acid changes in other regions of flagellin for motility preservation. For *E.coli* the down-regulation of flagellin expression in biofilms eliminates motility based in flagella but also provides evasion of TLR5 recognition [41].

These findings suggest that the evasion of TLR5 contribute to persistence of bacteria at mucosal surfaces. Considering the complex pleiotropic actions of the immune system one can imagine that other processes of bacterial elimination occur.

In conclusion, immune response is the co-evolutionary result from both vertebrates and microorganisms and comprises the establishment of constant commensal and symbiotic relationships. Homeostasis in bacterial-epithelial interactions contributes for this evolutionary process and thus inflammatory positive factors such as increase in TLR5 expression, NF- κ B translocation to nucleus, cytokines/chemokines secretion, and infiltration of neutrophils can be counterbalanced for instance by the expression of TLR inhibitory molecules and the downstream TLR5 activation MyD88 factor lower expression in intestinal macrophages.

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