ROLE OF TOLL-LIKE RECEPTORS IN THE DEVELOPMENT OF IMMUNPATHOGENIC GASTROINTESTINAL DISEASES

Ph.D. Thesis

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1. INTRODUCTION

The intestinal mucosa must rapidly recognise detrimental pathogenic threats to the lumen to initiate controlled immune responses but maintain hyporesponsiveness to harmless luminal commensals and food antigens. Exaggerated inflammatory responses in the absence of pathogenic bacteria would be otherwise deleterious. If the balance shifts from tolerogenic to immunogenic responses, several inflammatory and autoimmune disorders may be developed, such as coeliac disease, inflammatory bowel disease and necrotizing enterocolitis.

Charles Janeway Jr first suggested that so-called pattern recognition receptors (PRRs) may play an essential role in allowing innate immune cells to discriminate between „self” and microbial „non-self” based on the recognition of broadly conserved molecular patterns (PAMPs). CD14, which is a membrane-associated glycosylphosphatidylinositol-linked protein, was the first described PRR. Toll-like receptors (TLRs) comprise a class of transmembrane PRRs, while nucleotide-binding oligomerization domains (NOD1 and NOD2) are a structurally distinct family of intracellular PRRs.

Intestinal epithelial tolerance versus intolerance towards luminal bacterial ligands are distinctly mediated through TLRs and NOD2. Recognition of PAMPs by these receptors initiates a signaling pathway, which leads to activation of several adaptor proteins and transcription factors and finally production of proinflammatory cytokines-, chemokines-, type I interferons- and antibacterial peptides, as well as induction of angiogenesis and apoptosis.

In health, TLR signalling protects the intestinal epithelial barrier and confers commensal tolerance whereas NOD2 signalling exerts antimicrobial activity and prevents pathogenic invasion. Aberrant TLR and/or NOD2 signalling induce tissue damage and barrier destruction through exaggerated cytokine and chemokine production and by loss of commensal mediated colonic epithelial progenitor responses, leading to acute and chronic intestinal inflammation with many different clinical phenotypes.
2. AIMS

We investigated the expression of Toll-like receptor (TLR) 2, TLR3 and TLR4 in the duodenal mucosa of children with coeliac disease, as well as in the colonic mucosa of children with inflammatory bowel disease. Moreover, we also investigated the association of genetic polymorphisms of CD14, TLR4 and caspase recruitment domain (CARD) 15 with necrotizing enterocolitis (NEC) in very low birth weight infants. Our most important questions were as follows:

1. How does the mRNA expression and protein levels of TLR2, TLR3 and TLR4 change in the duodenal mucosa of children with coeliac disease (CD) compared to controls?
2. Is there any difference in the mRNA expression and protein levels of TLR2, TLR3 and TLR4 between the duodenal mucosa of children with untreated CD and treated CD?
3. Is there any alteration in the mRNA expression and protein levels of TLR2, TLR3 and TLR4 in the colonic mucosa of children with inflammatory bowel disease (IBD) compared to controls?
4. Is there any traceable difference in the mRNA expression and protein levels of TLR2, TLR3 and TLR4 between the colonic mucosa of children with freshly diagnosed IBD (fdIBD) and active IBD after relapse on treatment (rIBD)?
5. Is there any detectable variation in the mRNA expression and protein levels of TLR2, TLR3 and TLR4 either in the inflamed or non-inflamed colonic mucosa of children with fdIBD and rIBD?
6. Is there any perceptible difference in the mRNA expression and protein levels of TLR2, TLR3 and TLR4 between the colonic mucosa of children with Crohn’s disease and ulcerative colitis?
7. Is there any association between genetic polymorphisms of CD14 (CD14 C\textsuperscript{260}T), TLR4 (TLR4 A\textsuperscript{896}G and C\textsuperscript{1196}T) and CARD15 (CARD15 G\textsuperscript{2722}C, C\textsuperscript{2104}T and 3020insC) with the risk and severity of NEC in very low birth weight infants?
3. METHODS

3.1. Patients

3.1.1. Investigation of TLR2, TLR3 and TLR4 expression in the duodenal mucosa of children with coeliac disease

16 children (boys: 6, girls: 10) with untreated CD (median age 9 years, range 4-15), 9 children (boys: 4, girls: 5) with treated CD (median age 6 years, range 3-14) underwent duodenal biopsy sampling. Biopsy samples of untreated CD patients were taken at the time of diagnosis before the introduction of gluten free diet. The diagnosis of CD was based on ESPGHAN criteria. All these patients had anti-endomysium IgA positivity and subtotal or total villous atrophy of the intestinal mucosa (Marsh III.b or c). In all patients on a gluten free diet full clinical remission was observed, no serum anti-endomysium antibodies were detected and at the duodenal biopsy a normal villous structure was observed. The control group consisted of 10 children (boys: 4, girls: 6) with median age 10 years (range 4-15), who were investigated for either growth retardation or chronic diarrhoea and an upper gastrointestinal endoscopy was part of their diagnostic procedure. The intestinal mucosa was normal in all of them and no significant age- or sex-related differences were observed among children with untreated CD, treated CD and controls.

3.1.2. Investigation of TLR2, TLR3 and TLR4 expression in the colonic mucosa of children with inflammatory bowel disease

12 children [boys: 8, girls: 4; median age 13 years, range 6-18] with freshly diagnosed IBD (fdIBD) and 23 children [boys: 14, girls: 9; median age 15 years, range 8-18] with active IBD after relapse on treatment (rIBD) underwent colonic biopsy sampling from macroscopically inflamed and non-inflamed mucosa. In the fdIBD group, 8 children had a diagnosis of Crohn’s disease (C) and 4 children had a diagnosis of ulcerative colitis (UC), before any medication. The rIBD group consisted of 16 children with C and 7 children with UC. In the rIBD group, 7 children with C and 3
children with UC were receiving prednisolone and 5-aminosalicylate (5-ASA), 5 children with C and 4 children with UC were receiving prednisolone, azathioprine and 5-ASA, 2 children with C were receiving azathioprine and 5-ASA and 2 children with C receiving 5-ASA. The control group consisted of 8 children [boys: 5, girls: 3, median age 14 years, range 6-16] with macroscopically and histologically normal intestine, who had been referred with rectal bleeding or obstipation. No significant age- or sex-related differences were observed among children with fIIBD, children with rIBD and controls.

3.1.3. Association of genetic polymorphisms of CD14, TLR4 and CARD15 and necrotizing enterocolitis in very low birth weight infants

We reviewed the records of 118 very low birth weight (VLBW) infants with birth weight 1,500 g or less born between 1995 and 1999 at the Second Department of Gynecology and Obstetrics, Semmelweis University, Budapest, Hungary. NEC (stage I = 14, stage II = 19, stage III = 8) was diagnosed in 41 infants using Bell's classification system. Seventy-seven VLBW infants had no symptoms of NEC. Clinical characteristics were similar in VLBW infants with or without NEC. Sepsis was diagnosed more frequently in NEC infants, whereas the prevalence of other perinatal complications was similar in VLBW infants irrespective of the presence of NEC. We compared genotype distributions of VLBW infants with those of 146 healthy term infants.

3.2. Semiquantitative and real-time reverse transcription- polymerase chain reaction (RT-PCR)

3.2.1. RNA isolation and reverse transcription

Total RNA was isolated from the colonic biopsy samples by RNeasy Total RNA Isolations Kit (Qiagen). The quality and quantity of the RNA were photometrically confirmed. One microgram of total RNA was reverse-transcripted to generate first-strand complementary DNA (cDNA).
3.2.2. *Semiquantitative PCR (human GAPDH, TLR2, TLR3, TLR4)*

PCRs were performed with specific primer pairs for TLR2, TLR3 and TLR4 in a total volume of 50 μl. To test the cDNAs (for representation and full length genes) RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed. PCR products were separated by electrophoresis on agarose gels and visualised by staining ethidium bromide under UV illumination. The mRNA expression of each gene was determined by computerized densitometry using Gel-Pro Analyzer version 3.1. The integrated optical density of each PCR product was normalized to that of GAPDH for the same biopsy sample.

3.2.3. *SYBR Green real-time PCR (human TLR2, TLR3, TLR4)*

The TLR PCRs were performed in glass capillaries, in a final volume of 20 μl containing 1μl cDNA on a Light Cycler system. Specific primer pairs for TLR2, TLR3 and TLR4 were equivalent to those, which were used for the semiquantitative PCR. After each Light Cycler run, PCR products were separated by electrophoresis on agarose gels and visualized by staining with ethidium bromide Results were analyzed by using Light-Cycler software version 3.5.3.

3.2.4. *Real-time PCR-fluorescence resonance energy transfer (FRET) (human GAPDH)*

The GAPDH PCR was performed in glass capillaries, in a final volume of 20 μl containing 1μl cDNA on a Light Cycler system. Probes were labelled with the Light Cycler Red 640 fluorophore and fluorescein. After each Light Cycler run, PCR products were separated by electrophoresis on agarose gels and visualized by staining with ethidium bromide. Results were analyzed by using Light-Cycler software version 3.5.3.

3.3. **Western blot**

Colonic and duodenal biopsy specimens were homogenized in chilled extraction buffer and then the homogenate was centrifuged at 10000 g for 10 min at 4ºC to pellet
cell nuclei and large cellular fragments. Protein concentration of the supernatants was
determined by Bradford assay. Samples were solubilized in a sample buffer and then
were electrophoretically resolved on a 10% SDS-polyacrylamide gel and transferred to
nitrocellulose membranes, which were blocked in 5% non-fat dry milk. Equal protein
loading to the gel was confirmed by staining with a goat polyclonal antibody raised
against actin. The membranes were incubated with goat polyclonal TLR2, TLR3 or
TLR4 antibodies. Blots were then washed and incubated with peroxidase conjugated
anti-goat secondary antibody. Immunoreactive bands were visualized using the
ECLplus Western blotting detection system of Amersham Pharmacia. Computerized
densitometry of the specific bands were analysed with Gel-Pro Analyser 3.1 software.
The values were normalized to an internal standard and expressed as the relative optical
density.

3.4. PCR-restriction fragment length polymorphism (PCR-RFLP)

3.4.1. DNS isolation

Blood spots were taken on the fifth postnatal day for metabolic screening or
after the introduction of oral feeding and then were stored at the Metabolic Screening
Program. We used remnant dried blood samples for genotyping. DNA was extracted
from filter papers with an extracting agent (Chelex-100) according to the manufacturer's
instructions.

3.4.2. Detection of CD14 C-260T, TLR4 A+896G and C+1196T, as well as CARD15
G+2722C, C+2104T and CARD15 3020insC SNPs with PCR-RFLP

PCR amplifications were performed in a total volume of 50 µl containing 2 µl
DNA. PCR products were restricted by 10 units of the following restriction enzymes:
HaeIII (CD14 C-260T), NcoI (TLR4 A+896G), HinfI (TLR4 C+1196T), HhaI (CARD15
G+2722C), MspI (CARD15 C+2104T) and Apal (CARD15 3020insC). The restricted PCR
products were separated on agarose gels and visualized under UV illumination, stained
with ethidium bromide.
3.5. Statistical analysis

After testing the normality with Shapiro-Wilk’s test, non parametric Mann-Whitney $U$-test was used to determine the levels of difference among all groups for TLR2, TLR3 and TLR4 mRNA expression and protein levels in the duodenal and colonic biopsy samples.

Hardy-Weinberg equilibrium of genotypes and linkage disequilibrium of the tested SNPs were tested using Arlequin software. Allelic and genotype frequencies in VLBW (with and without NEC) and healthy term infants were compared by chi-square test; continuous variables were compared by Mann-Whitney $U$ test. Logistic regression analysis was used for the evaluation of independent effects of clinical characteristics and investigated SNPs on risk and severity of NEC. P values less than or equal to 0.05 were considered statistically significant.
4. RESULTS

4.1. Investigation of TLR2, TLR3 and TLR4 expression in the duodenal mucosa of children with coeliac disease

4.1.1. TLR2, TLR3 and TLR4 mRNA expression

TLR2 and TLR4 mRNA expression were significantly increased in the duodenal mucosa of children with untreated CD and treated CD compared to controls. We found even higher TLR2 and TLR4 mRNA levels in the duodenal mucosa of children with treated CD than in untreated CD. TLR3 mRNA expression was significantly increased in the duodenal mucosa of children with treated CD, but remained unchanged in the duodenal mucosa of children with untreated CD.

4.1.2. TLR2, TLR3 and TLR4 protein levels

TLR2 protein levels were 2 times higher in the duodenal mucosa of children with untreated CD than in controls. We found even 4 times higher TLR2 protein levels in the duodenal mucosa of children with treated CD than in untreated CD. We were able to detect TLR3 protein only in the biopsy specimens of treated CD patients. We found 3-fold elevation of the TLR4 protein levels in the duodenal mucosa of children with untreated CD in comparison to controls. TLR4 protein levels were 3 times higher in the duodenal mucosa of children with treated CD than in untreated CD.
4.2. Investigation of TLR2, TLR3 and TLR4 expression in the colonic mucosa of children with inflammatory bowel disease

4.2.1. TLR2, TLR3 and TLR4 mRNA expression

As we found no difference in the mRNA expression of TLR2, TLR3 and TLR4 in the colonic mucosa of children with Crohn’s disease or ulcerative colitis, reported findings were not specified for these subgroups. TLR2 and TLR4 mRNA expression were significantly increased in the inflamed colonic mucosa of children with fdIBD and rIBD compared to controls. In the non-inflamed mucosa of children with fdIBD and rIBD, TLR2 and TLR4 mRNA expression were similar to controls. TLR2 and TLR4 mRNA expression also did not differ between children with fdIBD or rIBD in either inflamed or non-inflamed colonic mucosa. TLR3 mRNA expression remained unchanged in all groups studied.

4.2.2. TLR2, TLR3 and TLR4 protein levels

We observed also no difference in the protein levels of TLR2, TLR3 and TLR4 in the colonic mucosa of children with Crohn’s disease or ulcerative colitis, therefore reported findings were not specified for these subgroups. TLR2 protein levels in the inflamed colonic mucosa of children with fdIBD and rIBD were about 9 times higher than in controls. We found about 5-fold elevation of the TLR4 protein levels in the inflamed colonic mucosa of children with fdIBD and rIBD compared to controls. In the non-inflamed mucosa of children with fdIBD and rIBD, TLR2 and TLR4 protein levels were similar to controls. TLR2 and TLR4 protein levels in either inflamed and non-inflamed mucosa were similar in children with fdIBD and rIBD. TLR3 protein levels did not changed significantly in the investigated groups.
4.3. Association of genetic polymorphisms of CD14, TLR4 and CARD15 and necrotizing enterocolitis in very low birth weight infants

The prevalence of CD14 C^{260}T, TLR4 A^{896}G and C^{1196}T, as well as CARD15 G^{2722}C, C^{2104}T and 3020insC SNPs was similar in VLBW infants and in healthy newborns. Furthermore, genotypes were not associated with gestational age of VLBW infants. In VLBW population, we found no association between genotype and risk of NEC. The severity of NEC was not influenced by carrier state of any of the tested SNPs. Logistic regression analysis revealed independent association between sepsis and NEC. No association was found between the investigated genotypes and patent ductus arteriosus, bronchopulmonary dysplasia or intracranial hemorrhage
5. SUMMARY, CONCLUSIONS

5.1. Investigation of TLR2, TLR3 and TLR4 expression in the duodenal mucosa of children with coeliac disease

1. We firstly demonstrated increased TLR2 and TLR4 mRNA expression and elevated TLR2 and TLR4 protein levels in the duodenal mucosa of children with untreated coeliac disease (CD) and treated CD compared to controls. Increased TLR2 and TLR4 expression may prevent gliadin induced Th2-type inflammation by upregulation of Th1 responses and may sensitise epithelial or subepithelial cells to gliadin inducing mucosal hypersecretion of interleukine (IL)-15 and persistent activation of intraepithelial lymphocytes in coeliac patients.

2. Interestingly, TLR2 and TLR4 mRNA and protein levels were even higher in the duodenal mucosa of children with treated CD compared to untreated CD. Our results of increased expression of TLR2 and TLR4 even in treated CD may indicate the primary role of these PRRs in the pathogenesis of CD.

3. Since TLR3 mRNA expression was unaltered in the duodenal mucosa of children with untreated CD compared to controls and we were able to detect TLR3 protein only in the biopsy specimens of treated CD patients, TLR3 mediated virus infection maybe not a major element in the pathogenesis of CD.

4. TLR2 and TLR4 proteins were barely detectable in the duodenal mucosa of all controls, while we demonstrated elevated TLR2 and TLR4 protein levels in children with untreated CD and treated CD. Our findings suggest that expression of TLR2 and TLR4 in the duodenum is similar to those in distal part of the intestine either under normal condition or during inflammation.
5.2. Investigation of TLR2, TLR3 and TLR4 expression in the colonic mucosa of children with inflammatory bowel disease

5. Increased TLR2 and TLR4 mRNA expression and higher protein levels were demonstrated in the inflamed colonic mucosa of children with freshly diagnosed (fd) IBD and active IBD after relapse on treatment (rIBD) compared to controls. In accordance with previous data, our results support the potential implication of the elevated TLR2 and TLR4 expression in the pathomechanism of IBD. Increased TLR2 and TLR4 expression may promote the recognition of endogenous luminal bacteria and enhance the immune response against them.

6. We observed similar TLR2 and TLR4 mRNA expression and protein levels in the non-inflamed colonic mucosa of children with fdIBD and rIBD to controls. Moreover, TLR2 and TLR4 mRNA expression and protein levels did not differ between children with fdIBD or rIBD in either inflamed or non-inflamed colonic mucosa. The non elevated expression of these PRRs in the non-inflamed colonic mucosa may imply that the increased TLR2 and TLR4 expression are not the cause, but rather the consequence of IBD.

7. We demonstrated that the TLR3 mRNA expression and protein levels remained unchanged in the colonic mucosa of children with fdIBD and rIBD in comparison to controls. Our findings suggest that TLR3 mediated virus infection is not a major element in the pathogenesis of IBD.

8. No difference in the mRNA expression and protein levels of TLR2, TLR3 and TLR4 in the colonic mucosa of children with Crohn’s disease (C) or ulcerative colitis (UC) were observed. These data suggest that in the background of Th1 shift in C and Th2 shift in UC is not the discrepant TLR2, TLR3 or TLR4 mucosal expression.
5.3. Association of genetic polymorphisms of CD14, TLR4 and CARD15 and necrotizing enterocolitis in very low birth weight infants

9. We firstly investigated the association of CD14 C$^{-260}$T-, as well as TLR4 A$^{+896}$G and C$^{+1196}$T SNPs with the risk and severity of NEC in very low birth weight (VLBW) infants. Only one study tested the significance of CARD15 G$^{+2722}$C, C$^{+2104}$T, as well as 3020insC SNPs in NEC and our findings confirm their results.

10. We observed that the prevalence of CD14 C$^{-260}$T, TLR4 A$^{+896}$G and C$^{+1196}$T, as well as CARD15 G$^{+2722}$C, C$^{+2104}$T and 3020insC SNPs did not differ between VLBW infants with or without NEC. Furthermore, these SNPs were not associated with the stage of NEC. We could not demonstrate any association between other perinatal complications or risk factors (patent ductus arteriosus, bronchopulmonary dysplasia or intracranial hemorrhage) and the investigated SNPs.

11. Our results suggest that the carrier state of the tested CD14, TLR4 and CARD15 SNPs is not associated with NEC risk in VLBW infants. Further investigation with larger cohorts of VLBW subjects with NEC would be helpful to better define the role of these SNPs in perinatal pathology.
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7. PUBLICATIONS

7.1. Articles


7.2. Citated abstracts


