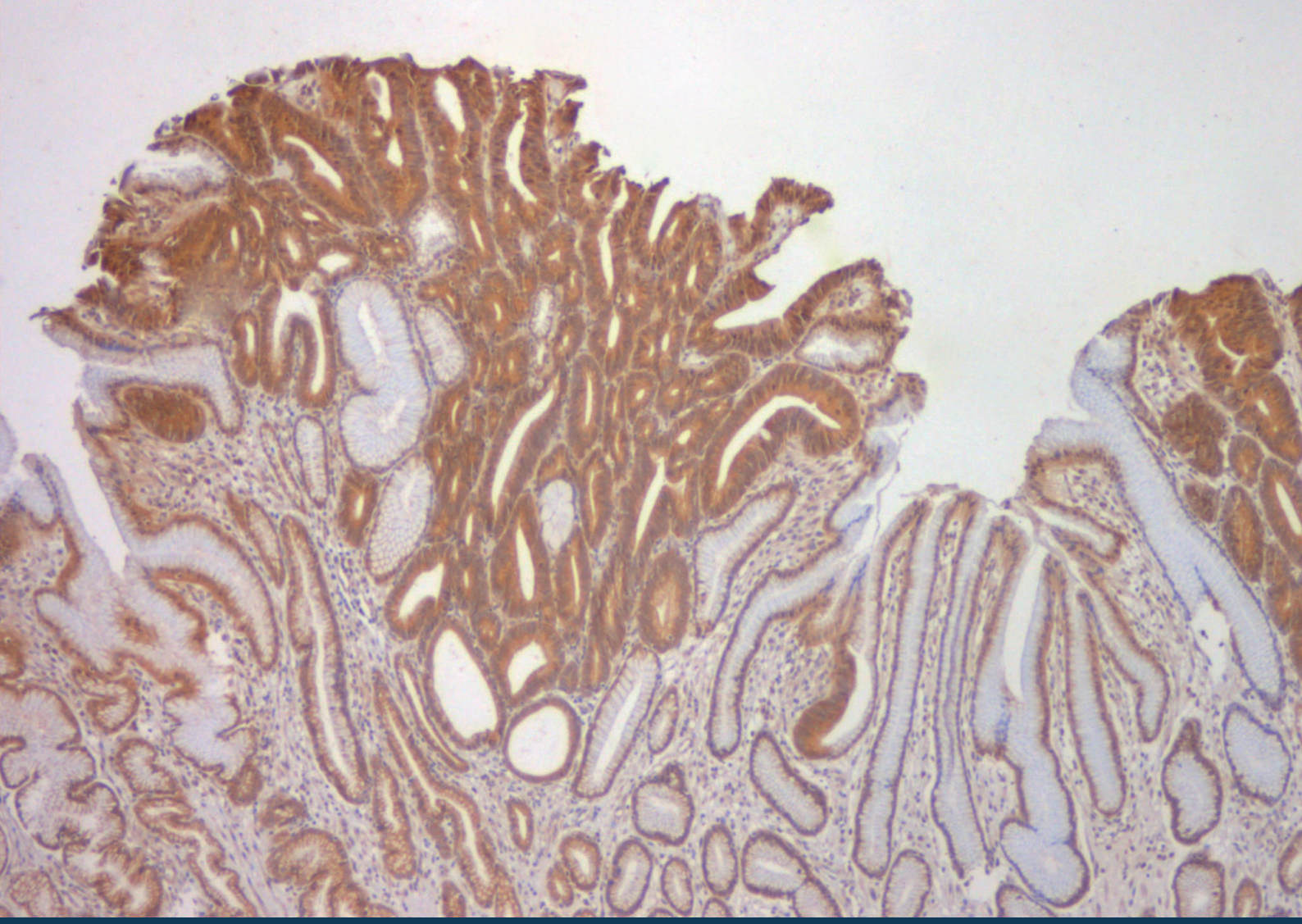

ROLE OF INNATE IMMUNITY RECEPTORS TOLL-LIKE 2 AND 4 IN GASTROINTESTINAL DISEASES

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ROLE OF INNATE IMMUNITY RECEPTORS TOLL-LIKE 2 AND 4 IN GASTROINTESTINAL DISEASES

RELEVÂNCIA DOS RECEPTORES DA IMUNIDADE INATA TOLL-LIKE 2 E 4 EM PATOLOGIA GASTROINTESTINAL

Pedro Pimentel Nunes

Faculdade de Medicina da Universidade do Porto

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On the front cover: Transition of normal gastric mucosa (right and below) to intestinal metaplasia (left and below) and then to dysplasia (up) with a growing intensity of TLRs expression being apparent. *Adapted with permission from Helicobacter (Pimentel-Nunes P et al, 2012)*

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Title: Role of Innate Immunity Receptors Toll-like 2 and 4 in Gastrointestinal Diseases.

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To my mother

To my brothers and my family

To my beautiful wife, Filipa

For you, father, my source of inspiration

Without your support, I would not be able to do it; you make my work worthwhile

Thank you!

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“Functional Polymorphisms of Toll-Like Receptors 2 and 4 Alter the Risk for Colorectal Carcinoma in Europeans.”

OUTLINE OF THE THESIS AND LIST OF PUBLICATIONS

This thesis is structured in the following way:

In the **Summary** a brief description of the thesis will be presented.

Chapter I, Introduction, constitutes a general introduction for the thesis. First, the relevance of several gastrointestinal diseases on the society will be highlighted, with special emphasis on oncologic diseases - subchapter A. Then, a description of Toll-Like receptors (TLR) and of their signalling pathways will be made – subchapter B.

In **Chapter II, Toll Like Receptors as Therapeutics Targets in Gastrointestinal Diseases,** the review article that constitutes the genesis of all the original research that brought light to this thesis is presented. The rationale of this thesis will in this way be described in this chapter.

From **Chapter III to V**, the role of TLRs in several gastrointestinal diseases will be presented, according to the different organs (**III- Liver diseases; IV- Gastric Diseases; V- Colon Diseases**).

Chapter III, Toll-Like Receptors and Liver Disease, includes one systematic review (subchapter A), two original articles (subchapter B and D) and one letter to the editor (subchapter C). It shows that although progressive TLR activation may be essential for the development of chronic liver disease and hepatocellular carcinoma, attenuation of these receptors in immunological

cells may on the other hand contribute to the increase risk of infection in cirrhotic patients.

Chapter IV, Toll-Like Receptors and Gastric Disease, includes two original articles. The important role of TLR activation throughout the entire sequence of gastric carcinogenesis will become clear in this chapter both at the protein (subchapter A) and at the molecular level (subchapter B).

Chapter V, Toll-Like Receptors and Colon Disease, includes two original articles. In the first one it will be shown that colon carcinogenesis is associated with increasing levels of TLRs as well as decreased levels of TLRs inhibitors (subchapter A). The second one, confirming the important role of TLRs in colon carcinogenesis, demonstrates that TLR polymorphisms significantly influence the risk of colorectal cancer development (subchapter B).

In **Chapter VI, Conclusion**, an integrated discussion of all the articles will be provided and a general conclusion will be presented.

And, finally, in **Chapter VII, Future Research**, because all research gives answers but creates even more questions, potential lines of research will be discussed.

This dissertation includes material published in several peer-reviewed journals. The total list of publications is presented below. Furthermore, a book chapter and two original articles, in whom the author was actively involved during the period of this thesis and that have an indirect relation with it, are also presented followed by a brief explanation of the association between them and this dissertation.

The full list of publications is hereby presented:

1. **Chapter II**

Pimentel-Nunes P, Soares JB, Roncon-Albuquerque R Jr, Dinis-Ribeiro M, Leite-Moreira AF.

TOLL-LIKE RECEPTORS AS THERAPEUTIC TARGETS IN GASTROINTESTINAL DISEASES. *Expert Opin Ther Targets*. 2010 Apr;14(4):347-68. Review. PubMed PMID: 20146632.

Journal Impact Factor 2011: 3.7

Number of citations (July 2012): 8

2. **Chapter III, A**

Soares JB, Pimentel-Nunes P, Roncon-Albuquerque R, Leite-Moreira A.

THE ROLE OF LIPOPOLYSACCHARIDE/TOLL-LIKE RECEPTOR 4 SIGNALING IN CHRONIC LIVER DISEASES. *Hepatol Int*. 2010 Oct 21;4(4):659-72. Review. PubMed PMID: 21286336; PubMed Central; PMCID: PMC2994611.

Journal Impact Factor 2011: 2.6

Number of citations (July 2012): 5

3. **Chapter III, B**

Pimentel-Nunes P, Roncon-Albuquerque R Jr, Gonçalves N, Fernandes-Cerqueira C, Cardoso H, Bastos RP, Marques M, Marques C, Alexandre Sarmento J, Costa-Santos C, Macedo G, Pestana M, Dinis-Ribeiro M, Leite-Moreira AF. ATTENUATION OF TOLL-LIKE RECEPTOR 2-MEDIATED INNATE IMMUNE RESPONSE IN PATIENTS WITH ALCOHOLIC CHRONIC LIVER DISEASE. *Liver Int*. 2010 Aug;30(7):1003-11. PubMed PMID: 20492495.

Oral communication in the plenary session at the XXVIII Congresso Nacional de Gastrenterologia e Endoscopia Digestiva (Vilamoura, Portugal, 2008).

Poster presentation at Digestive Disease Week/110th Annual Meeting of the American-Gastroenterological-Association (Chicago, USA, 2009).

Poster presentation at Young Clinicians Program 2009 and at the 17th United European Gastroenterology week (London, UK, 2009).

Presented as abstract at Gastroenterology 136(5): A418-A418, 2009 and at Gut 2009; 58: P1917 – Suppl 2.

Prizes:

- *Best Basic Science Communication Award at the XXVIII Congresso Nacional de Gastrenterologia e Endoscopia Digestiva (Vilamoura, Portugal, 2008).*
- *Best Poster Presentation Award at Young Clinicians Program 2009 (London, UK, 2009).*

Journal Impact Factor 2011: 3.8

Number of citations (July 2012): 4

4. **Chapter III, C**

Pimentel-Nunes P, Roncon-Albuquerque R Jr, Dinis-Ribeiro M, Leite-Moreira AF.

ROLE OF TOLL-LIKE RECEPTOR IMPAIRMENT IN CIRRHOSIS INFECTION RISK: ARE WE MAKING PROGRESS? *Liver Int*. 2011 Jan;31(1):140-1. Review/Letter. PubMed PMID: 20825560.

5. **Chapter III, D**

Soares JB, Pimentel-Nunes P, Afonso L, Rolanda C, Lopes P, Roncon-Albuquerque R Jr, Gonçalves N, Boal-Carvalho I, Pardal F, Lopes S, Macedo G, Lara-Santos L, Henrique R, Moreira-Dias L, Gonçalves R, Dinis-Ribeiro M, Leite-Moreira AF.

INCREASED HEPATIC EXPRESSION OF TLR2 AND TLR4 IN THE HEPATIC INFLAMMATION-FIBROSIS-CARCINOMA SEQUENCE. *Innate Immun*. 2012;18(5):700-8. Epub 2012 Feb 13.PubMed PMID: 22330637.

Oral communication at the 13th Reunião Anual da Associação Portuguesa para o Estudo do Fígado (Porto, Portugal, 2010).

Oral communication at the XXX Congresso Nacional de Gastrenterologia e Endoscopia Digestiva (Vilamoura, Portugal, 2010).

Poster presentation at the 17th United European Gastroenterology week (London, UK, 2009).

Presented as abstract at Gut 2010; 59 (Suppl III) A203

Prizes:

- *Best oral communication at the 13th Reunião Anual da Associação Portuguesa para o Estudo do Fígado (Porto, Portugal, 2010)*

Journal Impact Factor 2011: 4.0

Number of citations (July 2012): 1

6. **Chapter IV, A**

Pimentel-Nunes P, Afonso L, Lopes P, Roncon-Albuquerque R Jr, Gonçalves N,Henrique R, Moreira-Dias L, Leite-Moreira AF, Dinis-Ribeiro M. INCREASED EXPRESSION OF TOLL-LIKE RECEPTORS (TLR) 2, 4 AND 5 IN GASTRIC DYSPLASIA. *Pathol Oncol Res*. 2011 Sep;17(3):677-83. PubMed PMID: 21455638.

Oral communication at the XXX Congresso Nacional de Gastrenterologia e Endoscopia Digestiva (Vilamoura, Portugal, 2010).

Poster presentation at the 18th United European Gastroenterology week (Barcelona, Spain, 2010).

Presented as abstract at Gut 2010; 59 (Suppl III) A283

Journal Impact Factor 2011: 1.4

Number of citations (July 2012): 1

7. **Chapter IV, B**

Pimentel-Nunes P, Gonçalves N, Boal-Carvalho I, Afonso L, Lopes P, Roncon-Albuquerque R Jr, Henrique R, Moreira-Dias L, Dinis-Ribeiro M, Leite-Moreira AF.

HELICOBACTER PYLORI INDUCES INCREASED EXPRESSION OF TOLL-LIKE RECEPTORS AND DECREASED TOLL-INTERACTING PROTEIN IN GASTRIC MUCOSA THAT PERSISTS THROUGHOUT GASTRIC CARCINOGENESIS. *Helicobacter*. 2012 Sep 3. [Epub ahead of print]; PMID: 23061653

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Journal Impact Factor 2011: 3.2

8. **Chapter V, A**

Pimentel-Nunes P, Gonçalves N, Boal-Carvalho I, Afonso L, Lopes P, Roncon-Albuquerque R Jr, Soares JB, Cardoso E, Henrique R, Moreira-Dias L, Dinis-Ribeiro M, Leite-Moreira AF.

DECREASED TOLL-INTERACTING PROTEIN AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA ARE ASSOCIATED WITH INCREASED EXPRESSION OF TOLL-LIKE RECEPTORS IN COLON CARCINOGENESIS. *J Clin Pathol*. 2012 Apr;65(4):302-8. PubMed PMID: 22228906.

Oral communication in the plenary session at the XXXI Congresso Nacional de Gastrenterologia e Endoscopia Digestiva (Estoril, Portugal, 2011).

Journal Impact Factor 2011: 2.3

9. **Chapter V, B**

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FUNCTIONAL POLYMORPHISMS OF TOLL-LIKE RECEPTORS 2 AND 4 ALTER THE RISK FOR COLORECTAL CARCINOMA IN EUROPEANS. *Dig Liver Dis*. 2012 Sep 18. [Epub ahead of print] PMID: 22999059

Oral communication in the plenary session at the XXX Congresso Nacional de Gastrenterologia e Endoscopia Digestiva (Vilamoura, Portugal, 2010).

Oral communication at the 18th United European Gastroenterology week (Barcelona, Spain, 2010).

Presented as abstract at Gut 2010; 59 (Suppl III) A101

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- *Travel Grant for Young Investigators at the 18th United European Gastroenterology week (Barcelona, Spain, 2010).*

The works related to this dissertation in which the author was involved are hereby presented:

1.Soares JB and Pimentel-Nunes P.

INNATE IMMUNITY IN ALCOHOLIC LIVER DISEASE. Chapter 3, pg 1-24 from the Book *Trends in Alcoholic Liver Disease Research - Clinical and Scientific Aspects*, ISBN 978-953-307-985-1, edited by Ichiro Shimizu, InTech, 2012.

This book chapter includes much information from the chapter III of this thesis. It is a complement to that chapter since it goes beyond the role of TLRs to include several other innate immunity molecules and receptors. On the other hand it is specifically related to alcoholic liver disease. The invitation to write this chapter of an international book happened as a consequence of all the research included in the chapter III of this dissertation.

2. Pimentel-Nunes P, Dinis-Ribeiro M, Soares JB, Marcos-Pinto R, Santos C, Rolanda C, Bastos RP, Areia M, Afonso L, Bergman J, Sharma P, Gotoda T, Henrique R, Moreira-Dias L.

A MULTICENTER VALIDATION OF AN ENDOSCOPIC CLASSIFICATION WITH NARROW BAND IMAGING FOR GASTRIC PRECANCEROUS AND CANCEROUS LESIONS. *Endoscopy*. 2012 Mar;44(3):236-46. Epub 2012 Jan 31. PubMed PMID: 22294194.

This article was essential for the construction of the article included in chapter IV, B. Indeed, it was the endoscopic classification developed in this paper that allowed the guided sampling of the different gastric lesions

and consequently the certainty that the samples being analyzed corresponded to the histology. Only after the validation of this endoscopic classification it was possible to initiate the last article to be written of this dissertation.

3. Dinis-Ribeiro M, Areia M, de Vries AC, Marcos-Pinto R, Monteiro-Soares M, O'Connor A, Pereira C, Pimentel-Nunes P, Correia R, Ensari A, Dumonceau JM, Machado JC, Macedo G, Malfertheiner P, Matysiak-Budnik T, Megraud F, Miki K, O'Morain C, Peek RM, Ponchon T, Ristimaki A, Rembacken B, Carneiro F, Kuipers EJ.
MANAGEMENT OF PRECANCEROUS CONDITIONS AND LESIONS IN THE STOMACH (MAPS): GUIDELINE FROM THE EUROPEAN SOCIETY OF GASTROINTESTINAL ENDOSCOPY (ESGE), EUROPEAN HELICOBACTER STUDY GROUP (EHSG), EUROPEAN SOCIETY OF PATHOLOGY (ESP), AND THE SOCIEDADE PORTUGUESA DE ENDOSCOPIA DIGESTIVA (SPED). *Endoscopy*. 2012 Jan;44(1):74-94. Epub 2011 Dec 23. PubMed PMID: 22198778; PubMed Central PMCID: PMC3367502.

During the elaboration of this dissertation, the author actively participated in these international guidelines for the management of precancerous gastric lesions. One of the conclusions was that further research concerning molecular markers for the development of gastric preneoplastic and neoplastic conditions was needed. The results of the chapter IV of this thesis suggest that in the future TLRs may be used as potential biomarkers for the development of gastric cancer.

Summary

INTRODUCTION: Gastrointestinal diseases are a major burden in modern society. Specifically, premalignant and malignant gastrointestinal pathologies are increasing and nowadays represent an important cause of disability in the population of most countries. Indeed, gastric, liver and colorectal cancers represent the 2nd, 3rd and 4th causes of death from cancer worldwide, respectively. For all the three tumours, precursors lesions are known and a sequence of progression from normal cells to cancer is described. Common to all three is a pro-inflammatory environment that facilitates the activation of oncogenic pathways and consequently cancer development and progression. Nevertheless, the molecular pathways that converge inflammation and cancer in these tumours are still not fully clarified. Toll-like receptors (TLRs) are the first line of interaction of the human cells with the different external and environmental agents. When activated by pathogen-associated molecular patterns of different microorganisms these receptors initiate pro-inflammatory and survival signalling pathways that at long term may create an oncogenic microenvironment for the cell. It is believed that gastrointestinal system express low levels of TLRs as well as high levels of these receptors inhibitors in order to prevent inadequate inflammatory reactions to commensal and diet bacteria. Nevertheless, how the expression of these receptors or their antagonists varies with the progression from normal epithelia to cancer in gastrointestinal organs is still not known.

AIM: To clarify the role of TLR2 and TLR4 in several gastrointestinal diseases, particularly pre-malignant and malignant disease of liver, stomach, and colon.

METHODS: 1. Liver - In order to determine the role of TLR2 and TLR4 in cirrhosis infection risk a population of 26 stable and 5 unstable alcoholic cirrhotic patients as well as 17 controls were selected. For the study of TLRs expression in the hepatic inflammation-fibrosis-carcinoma sequence 15 patients with unexplained transaminases elevation (control group), 22 with viral chronic hepatitis B or C, 14 with virus-induced severe fibrosis/cirrhosis and 10 with hepatocellular carcinoma were selected. Serum quantifications were made for several liver and inflammatory markers and for endotoxaemia. Stimulation of primary cultures of peripheral blood monocytes was made with TLR2 and TLR4 ligands and TNF-α production quantified afterwards. TLR protein expression was determined by flow cytometry (monocytes) or by immunohistochemistry (tissue samples). TLRs and related inflammatory molecules gene expression was evaluated by real time RT-PCR. **2. Stomach** - Histological database analysis (n=117) and biopsy samples obtained by endoscopy (n=80, 44 patients) from normal mucosa, *Helicobacter pylori* (HP) induced gastritis, metaplasia, dysplasia and adenocarcinoma were included for evaluation of TLRs in gastric carcinogenesis. **3. Colon** - Colon biopsy samples (n=90) from normal mucosa, normal mucosa adjacent to lesion, adenoma or carcinoma were obtained from 35 patients performing colonoscopy for evaluation of TLRs in colon carcinogenesis. Both in stomach and in colon gene quantification of TLR2, TLR4, TLR5, TOLLIP, PPAR-γ, NF-κB, TNF-α, COX-1, COX-2 and CDX2 was done by real-time RT-PCR while TLR2, TLR4 and TLR5 protein expression was quantified by immunohistochemistry. In order to determine the role of TLR2 and TLR4 polymorphisms in colorectal cancer development a Hospital

based multicentre case control study involving 193 colorectal cancer patients and 278 healthy individuals was performed. DNA samples were extracted from blood cells and genotyping of TLR2+597T>C, TLR2-4760T>C, TLR4-3745A>G, TLR2Arg753Gln and TLR4Asp299Gly was done. Functionality of risk polymorphisms was evaluated through production of TNF-α in cell culture.

RESULTS: **1. Liver** - Stable cirrhotic patients presented increased endotoxaemia with no differences in serum TNF-α or IL-10 when compared to controls. Both TLR2/TLR1 and TLR2/TLR6 activation induced TNF-α production by monocytes was blunted in stable cirrhosis (-40.1±13.5% and -66±20.4% respectively, $p<0.05$), but not TLR4 activation. Basal TNF-α mRNA expression was decreased in monocytes from cirrhotic patients (-50.1±21.0%, $p<0.05$), with no significant differences in the other studied genes. Results were similar in Child-Pugh A and B/C patients. Unstable patients presented increased serum levels of TNF-α (+141±48.2%, $p<0.05$) and both TLR2 and TLR4 diminished activation (-74.1±28% and -67±28%, respectively, $p<0.05$). As compared with control (expression = 1.0 arbitrary unit (AU)), we found an increased TLR2 and TLR4 mRNA expression in hepatitis (TLR2: 2.66±0.69 AU, $p=0.04$; TLR4: 3.11±0.79 AU, $p=0.03$) and cirrhosis (TLR2: 2.14±0.5 AU, $p=0.04$; TLR4: 1.74±0.27 AU, $p=0.008$). This was associated with an increased TNF-α and COX-2 mRNA expression in hepatitis (TNF-α: 3.24±0.79 AU, $p=0.02$; COX-2: 2.47±0.36 AU, $p=0.003$) and cirrhosis (TNF-α: 1.73±0.28 AU, $p=0.009$; COX-2: 1.8±0.35 AU, $p=0.04$). Immunohistochemistry confirmed increased protein expression of TLR2 and TLR4 in hepatitis and cirrhosis and a maintained expression of these

receptors in hepatocellular carcinoma. **2. Stomach** - When compared to normal mucosa, HP gastritis presented higher expression of TLR2 (2.23±0.36 AU), TLR4 (1.92±0.40 AU) and TNF-α (2.14±0.50 AU) and lower TOLLIP and PPARγ expression (0.72±0.12 AU) ($p<0.05$). Metaplasia and dysplasia/carcinoma presented higher expression of TLR2 (1.66±0.46 and 1.48±0.20 AU, $p<0.05$), lower expression of TOLLIP (0.66±0.09 and 0.52±0.04 AU, $p<0.05$) and PPARγ (0.73±0.12 and 0.63±0.10 AU, $p<0.05$). The significant trend for decrease of TOLLIP and PPARγ was associated with increasing levels of CDX2 from normal mucosa to carcinoma ($p<0.05$), translating that in diffuse and higher TLR2, TLR4 and TLR5 protein expression ($p<0.05$). An immunohistochemistry score of all TLRs' expression of 8 leads to a low (4%) false positive rate for the diagnosis of dysplasia in patients with precancerous conditions. **3. Colon** - When compared to colon normal mucosa, adjacent to lesion normal mucosa presented higher expression of COX-2 (1.86±0.3 AU) and TNF-α (1.44±0.18 AU) and lower TOLLIP expression (0.75±0.05 AU) ($p<0.05$). Adenoma and carcinoma presented higher expression of COX-2 (1.63±0.27 and 1.38±0.14 AU, $p<0.05$) and lower expression of TOLLIP (0.44±0.04 AU, $p<0.001$), translating that in diffuse and higher TLRs protein expression ($p<0.001$). Carcinoma additionally expressed higher TLR2 (2.31±0.32 AU, $p=0.006$) and lower PPARγ (0.56±0.12 AU, $p=0.003$). There was a statistical significant trend for decrease of TOLLIP ($p<0.001$) and PPARγ ($p=0.05$) from normal mucosa to adenoma/carcinoma. When concerning TLR2 and TLR4 risk polymorphisms analysis we found that TLR2+597CC homozygous had a 5-fold decreased risk (odds ratio (OR)=0.21, 95%CI: 0.09-0.50, $p<0.001$) and TLR4 299Gly

homozygous a 3-fold increased risk of colorectal cancer (OR=3.30, 95%CI: 1.18-9.28, p=0.015). In stratified analysis, TLR2+597CC genotype protective effect was even higher in overweight individuals (OR=0.17, 95%CI: 0.06-0.53, p<0.001) and in never smokers (OR=0.11, 95%CI: 0.02-0.51, p=0.001). Also, the increased risk effect for TLR4 299Gly homozygous genotype was higher in overweight individuals (OR=8.67, 95%CI: 1.11-87.85, p=0.011). TLR2+597T>C polymorphism conferred 41% less (p=0.03) and TLR4Asp299Gly 65% more TNF-α production (p=0.02). **CONCLUSION**: In cirrhosis, activation of immune cells TLR2 at an early stage and TLR4 in advanced stages of liver disease is compromised. This may constitute an important mechanism of acquired immunodeficiency in chronic liver disease patients. Although immune cells lower TLR activation may contribute for the risk of infection in cirrhosis, progression of liver disease in the inflammation-fibrosis-carcinoma sequence is associated with progressive parenchyma TLR expression. Furthermore, both in the stomach and in the colon the sequence of gastrointestinal carcinogenesis was associated with increased expression of TLRs and/or decreased expression of their antagonist molecules. Moreover, single nucleotide polymorphisms of these receptors may impact significantly the individual risk to develop gastrointestinal cancer. This previously not described data suggests that TLRs play an essential role in gastrointestinal carcinogenesis. In the future TLRs modulation may be an interesting therapeutic option not only to prevent infectious complications but more important to prevent gastrointestinal cancer development.

CHAPTER I - INTRODUCTION

"The important thing is not to stop questioning"

Albert Einstein (1879-1955)

A) GASTROINTESTINAL PATHOLOGIES AND THEIR IMPORTANCE ON SOCIETY – FOCUS ON ONCOLOGY

Gastroenterological diseases represent a major burden in the society. Although the majority are benign, recent trends suggest that malignant gastrointestinal pathologies are increasing and nowadays represent an important cause of death in the population (1, 2). The reasons for this is not only because the population is living longer but also because the human conduct is changing with increasing adoption of cancer-associated lifestyle behaviours like hyper-caloric and fat diets, sedentary lifestyle, alcohol, smoking and other environmental factors (3). Indeed, the gastrointestinal system is the first one to interact with several external agents, particularly from diet but also microbial agents, and so, it is not strange that higher consumption of potential noxious agents will in the first place damage gastrointestinal cells. Furthermore, some gastrointestinal organs like the liver have a central role in human body metabolism and are in that way exposed not only to external harmful agents but also to endogenous ones. When exposed to aggressive agents, these organs start pathways of defence that although potentially beneficial at short term, when chronically exposed to noxious agents they may initiate potential oncogenic intracellular pathways that eventually lead to cancer. In fact, when we look to the three organs that are studied in this dissertation, we realize that all of them share common pathways to cancer with a sequence of well-defined events and lesions that precede the phenotypical change to cancer. These aspects will be further described individually below.

Liver disease

The liver is a unique organ with an extraordinary capacity to answer to the aggression. Indeed, liver cells possess a regenerative capacity that no other organ can match (4). However, when chronically exposed to noxious agents, a sequence of inflammation and fibrosis begins that will eventually lead to cirrhosis, the final process of liver injury, independently of what was the aggressive agent. Indeed, the four most common agents of liver lesion (alcohol, steatosis/metabolic, viral hepatitis and auto-immunity) share this common pathway of inflammation-fibrosis-cirrhosis (5-7). The crucial step in this sequence appears to be activation of Kuppfer and stellate cells with production of several pro-inflammatory and fibrogenic factors (5-7).

Chronic liver disease represents a major burden in the society with incidences as high as 15 cases per 100 000 person year, with the incidence of cirrhosis in developed countries tending to increase (8, 9). The mortality by this disease remains high with cirrhosis being the 12th leading cause of death in the United States, with a mortality rate of 10 per 100,000 persons (10). The scenario in other developed countries, including Portugal, is similar or worse (11, 12). Indeed, Portugal ranked 5th in cirrhosis mortality rates with values as high as 30 per 100 000 person year in men and 10 per 100 000 person year in women (11, 12). Although the improved management of some complications of cirrhosis (like gastrointestinal bleeding) has decreased the mortality, fatality rates by cirrhosis remain high with 5-year mortality rates superior to 40% in most series mainly because other complications like infection and hepatocellular carcinoma have emerged (13-16).

Cirrhosis is frequently complicated by infections, which result in increased morbidity and mortality and place an economic burden on health care systems (7, 17-20). Actually, this disease is considered one of the most common forms of acquired immunodeficiency (7, 17-19). Several organic factors like ascites, hypoalbuminemia, intestinal bacterial overgrowth, increased intestinal permeability, bacterial translocation and increased endotoxaemia contribute for this susceptibility (17-19, 21, 22). However, many immunological factors like defects in polymorphonuclear leukocytes recruitment and activation, deficiencies in the complement system as well as defects in macrophage activation and adherence have been described (23-26). So, the infection risk in cirrhotic patients appears to be multifactorial and several factors may remain to discover.

In the liver, from the sequence of aggression-chronic inflammation-fibrosis-cancer, hepatocellular carcinoma has emerged as a major complication of cirrhosis, with an incidence rate of 3-5% per year (27). Indeed, the majority of these tumours occur in patients with chronic hepatitis and cirrhosis, highlighting the importance of a pro-inflammatory and a pro-fibrogenic milieu for the development of carcinoma (27-29). Although with a wide geographic variation, hepatocellular carcinoma is the sixth most prevalent cancer and the third most frequent cause of cancer-related death in the world (1, 27). In Portugal, hepatocellular carcinoma has an incidence of 2.2 per 100 000 person year and an almost equal incidence of mortality of 2.1 per 100 000 person year (30). Indeed, despite the great improvement in the diagnosis and therapy of this disease, the number of deaths per year by this cancer is virtually identical to its

incidence in almost every country, underscoring the high case-fatality rate of this tumour (1). Together with a rapid diagnosis and treatment, prevention of this tumour appears to be the challenge for the future. Only by knowing the molecular pathways that lead to cancer we will be able to efficaciously prevent this disease.

Gastric disease

Gastric pathologies have some unique characteristics mainly because many gastric diseases have a strong association with a bacteria infection – *Helicobacter pylori* (HP). Indeed, this bacteria discovered in the 1980s was rapidly associated to several gastric pathologies (31). In 1994 it was clearly recognized that HP was a major cause of gastroduodenal peptic ulcers and later that year the International Agency for Research on Cancer declared HP to be a group I human carcinogen for gastric adenocarcinoma (32). HP is considered one of the oldest bacteria to infect humans with genetic studies identifying this bacterium in the first human populations, more than 58 000 years ago (31). Even today with wide use of antibiotics, HP is estimated to infect more than 50% of the World's population, with prevalence's as high as 90% in some developing countries (31).

HP is a Gram-negative bacterium that adheres to the surface of gastric mucosa, without invasion of gastric epithelial cells, and that upon interaction with several innate immunity receptors such as Toll-like receptors (TLRs), causes inflammation of the mucosa that perpetuates as a chronic gastric inflammatory state (33, 34). In some patients this inflammation progresses leading to gastric atrophy and intestinal metaplasia, clearly established gastric premalignant gastric conditions (35-38). Indeed, Correa was the first one to describe a multistep pathway for the intestinal-type gastric adenocarcinoma, where HP is considered the initiator of the so-called Correa cascade of gastric carcinogenesis that involves chronic gastritis, atrophic gastritis, intestinal metaplasia, gastric dysplasia and, finally, intestinal-type gastric adenocarcinoma (39-42).

Gastric cancer is considered the number one infection related cancer in the world and even with conservative analyzes it is believed that 75% of all gastric cancers are attributable to HP infection, accounting for almost 660 000 gastric cancers annually worldwide (43). Although in developed countries gastric cancer rates have decreased substantially, mainly because HP infection is being actively controlled, gastric adenocarcinoma is still one of the most common cancers in the world being the fourth most common in men and the fifth in women (1, 2, 44, 45). Moreover, gastric adenocarcinoma ranks second in mortality representing 10% of all deaths for cancer (1, 2). In Portugal, gastric cancer is the fifth most common and lethal cancer with an incidence of 14 per 100 000 person year and a mortality of 10 per 100 000 person year, the highest among European countries (30). It was estimated that Portuguese inhabitants show a life-time risk for gastric cancer of approximately 2% (46). Taking altogether, gastric cancer acquires a relevant socioeconomic role in the world but particularly in Portugal among the developed countries.

Considering that HP infection is treatable with antibiotics, we might speculate that eradication at an early age might prevent most of gastric cancers in the future. Nevertheless, there is marked individual variability in the outcomes of this infection, with complications emerging only in 10-15% of infected persons, with fewer than 5% of infected persons developing cancer (31). Probably, the different outcomes possible following HP infection happen because HP infection involves complex interaction between bacterial, genetic and environmental factors (31). It is important to better define these factors and their interaction in order to better

prevent gastric cancer, since, considering all the side effects of antibiotics and also the possibility of generation of resistant microorganisms, it does not look ethical to treat all the infected persons when only few patients will benefit from that.

Colon disease

Colorectal cancer (CRC) is one of the most common cancers in the world, being the third most common in males and the second one in females. Its incidence rates are rapidly increasing in several areas in the world, particularly at developed countries, probably related to a combination of factors like diet, obesity and smoking ("the western lifestyle") (1, 2, 47). Although the mortality rates have been decreasing in several developed countries, mainly because increased awareness, implementation of screening programmes with early detection and improved treatment, CRC still ranks fourth in world cancer mortality rates, representing 8% of cancer deaths (1, 2, 30, 47-49). In Portugal CRC is the third most common cancer (the second one both in men and women, losing only to prostate and breast cancer, respectively) with incidence rates of 31 per 100 000 person year (30). The mortality rates in Portugal are of 15 per 100 000 person year (survival superior to 50%), similar results to other developed countries like the United States of America (30). It is estimated that in developed countries, including Portugal, the lifetime risk of CRC is about 5-8% (1, 2, 47). The risk factors for CRC are environmental and inherited with three patterns for presentation:

- Sporadic disease, accounting for 70% of the cases, in which there is no family history and where it is believed that environmental and dietary factors play a major role.

- Hereditary syndromes, like familiar adenomatous polyposis and Lynch syndrome, representing fewer than 10% of the cases, in which a causative genetic mutation is generally identified.

- Familial CRC, which accounts for 20-25% of the cases, where there is a clear familiar history with no germline mutation identified, with the risk of cancer being superior to general population but inferior to hereditary syndromes, probably reflecting environmental and genetic factors (50).

Probably because there are well-known hereditary syndromes leading to CRC, the understanding of the molecular events leading to CRC is one of the greatest among all the other tumours. Three distinct molecular mutagenic pathways are involved and described in colon carcinogenesis, sporadic or hereditary: Chromosomal instability (inactivating mutations of APC gene and sequential activation of oncogenes and inactivation of tumour suppressor genes); Microsatellite instability (mutations in DNA mismatch repair genes predisposing to mutations in genes with repetitive sequences); Epigenetic pathway (hypermethylation and gene silencing) (51). All these pathways appear to initiate an adenoma-carcinoma sequence where there is a progression from normal colonic mucosa to hyperproliferative epithelium, then to adenoma with increasing dysplastic changes, and finally carcinoma (50-53). Although these pathways are now extensively described, it is still not clear which factors initiate and promote tumour progression.

Indeed, a number of environmental risk factors have been described, yet, it is unknown how environment and diet influence genetic pathways and predispose to cancer. For example, age, male gender, race, obesity, diabetes and diet, among many others, are considered risk factors for CRC. Even so, the molecular mechanisms through which these factors increase the risk of cancer and influence colon carcinogenesis are still not known (50, 54-56). In contrast, inflammatory bowel disease is a risk factor for CRC

by promoting chronic inflammation and consequently predisposing to mutations (57, 58). So, inflammation (even subclinical inflammation) may be an important factor in the initiation and progression of the mutagenic pathways described, with significant influence in the adenoma-carcinoma sequence (59-61). Emerging data imply that colonic microbiota may be the link between risk factors, subclinical inflammation and CRC (62, 63). Indeed, older studies suggested the important role of bacteria for the development of CRC not only because cancer and adenomas present higher bacteria levels than normal mucosa but also because germ-free rats given carcinogens are protected from CRC (64, 65). Taking altogether we might speculate that risk factors for CRC, like diet, may change the colonic microbiota to a more aggressive one that through interaction with colonic mucosa promote activation of pro-inflammatory pathways which, at long term, will facilitate the progression of the adenoma-carcinoma sequence.

So, even though we have extensive knowledge of the molecular pathways leading to CRC and, even more important, we possess weapons capable of significantly reduce the incidence and mortality of CRC, like colonoscopy with polipectomy (66, 67), we still have many lines of research in this battle against CRC, one of the most common cancers in the world. Probably, we will have to better understand how the western lifestyle contributes to the risk of CRC. For that we will need to have a clear understanding of how microbiome changes with these factors and how the different bacteria interact with the gastrointestinal cells.

Epilogue

Malignant pathology of the liver, stomach and colon represent a major burden in the society and an important cause of death in the population. Even though we have an extensive knowledge of this tumours and we possess important therapeutic alternatives to fight them, the incidence and rates of mortality of this tumours are still very high and, in some cases, with a tendency to grow even more (Table I.1).

Indeed, in order to reduce its incidence and mortality, prevention appears to be the better way to fight cancer. In order to accomplish this we need to better understand the molecular pathways that lead to cancer.

For all the three tumours, precursor lesions are known and a sequence of progression from normal cells to cancer is well described and validated by scientific studies. Common to all three appears to be a pro-inflammatory stimulus that may facilitate the activation of oncogenic pathways and consequently cancer development and progression. If we were able to better understand how environmental factors interact with the normal epithelium to generate these precursor lesions and how these precursor lesions evolve and eventually lead to cancer, we will probably be able to better prevent cancer development.

Since TLRs are the first line of interaction of the human cells with the different external and environmental agents and that, when activated, initiate pro-inflammatory signalling pathways, we believe that TLRs may have an important role in the genesis and progression of gastrointestinal cancers. In the following chapters we will provide scientific evidence to prove that hypothesis.

Table I.1 - Epidemiology of the gastrointestinal cancers studied in this thesis (both sexes considered).

	INCIDENCE	MORTALITY	2030 NEW CASES*
GASTRIC CANCER			
World	14.0	10.3	1.75
Europe	7.9	5.6	1.36
Portugal	13.7	10.4	1.32
LIVER CANCER			
World	10.8	9.9	1.67
Europe	4.7	4.3	1.36
Portugal	2.2	2.1	1.34
COLON CANCER			
World	17.2	8.2	1.77
Europe	31.7	12.6	1.36
Portugal	31.4	14.6	1.34

Incidence presented as cases per 100 000 person year; *2030 new cases calculated as the proportion of: (estimated new cases in 2030)/(total cases in 2008). Data extracted from references (1) and (30).

B) TOLL LIKE RECEPTORS AND THEIR SIGNALING PATHWAYS

Innate Immunity

The human body is in permanent contact with million different microorganisms, all potential pathogenic under propitious circumstances. Without fast and effective mechanisms to prevent and respond to eventual microbial threats it would not be possible for humans to survive as a species. These mechanisms, that human share with several other living species are known as innate immunity mechanisms (68, 69).

In this way, innate immunity constitutes the several immunological responses that are present from birth and that are not a result of exposure to microorganisms, so they are not learned nor are a result from adaptation. Moreover, innate immunity is in clear contrast with the immunological response of T and B-lymphocytes, a process known as adaptive immunity. Although essential, this specific adaptive immune response takes days to weeks to develop whereas most bacteria rapidly multiply in a matter of minutes, emphasizing the important role of innate immunity. Indeed, innate immunity is an essential and crucial first line of defence against infection, quickly responding to potential attacks by several and different microorganisms (68, 69).

Innate immunity consists of a diversity of components like anatomic and physical barriers (e.g. tight junctions in the skin, epidermis, dermis, and mucous membranes, mucus itself), physiologic barriers (e.g., temperature, low pH, oxygen), humoral factors (e.g., pepsin, lysozyme, other anti-microbial substances, interferons, complement), phagocytic cells (e.g., neutrophils and macrophages), and some lymphocyte cells (e.g., natural killer [NK] and NKT cells). Although many of these factors can

prevent or destroy the invading pathogens non-specifically, we now know that the microbiological recognition by innate immunity is also a specific and highly coordinated process involving pattern recognition receptors (PRRs) that identify preserved structures of different pathogens, the so-called pathogen-associated molecular patterns (PAMPs) (70, 71). Moreover, this amazing specificity conferred by the recognition of PAMPs by PRRs is essential, not only for a more adequate initial control of a potential infection (innate immunity), but also for triggering the late antigen-specific acquired immunity (adaptive immunity), for controlling inflammation processes and for maintenance of a immunological homeostasis within the host (72, 73).

Many different PAMPs and PRRs have been identified. PAMPs include bacterial carbohydrates (e.g., lipopolysaccharide or LPS, mannose), bacterial peptides (flagellin), peptidoglycans and lipoteichoic acids (from Gram-positive bacteria), N-formylmethionine, lipoproteins and fungal glucans, and nucleic acids (e.g., bacterial or viral DNA or RNA) (69-71). On the other way, the PRRs can also be divided into 3 categories: secreted PRRs, membrane-bound PRRs, and phagocytic PRRs. Secreted PRRs are a group of proteins that kill pathogens through complement activation and opsonization of microbial cells for phagocytosis. Secreted PRRs include complements, pentraxins, and peptidoglycan-recognition proteins, which are mainly produced by hepatocytes and secreted into the blood stream. Membrane-bound or intracellular PRRs include TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-induced gene I-like helicases. Phagocytic (or endocytic) PRRs, which are expressed

on the surface of macrophages, neutrophils, and dendritic cells, can bind directly to pathogens, and this is followed by phagocytosis into lysosomal compartments and elimination. These phagocytic PRRs include scavenger receptors, macrophage mannose receptors, and ß-glucan receptors (69-71). Of all these PRRs, TLRs are the most studied and appear to be one of the most important families of PRRs.

The family of TLRs

The TLRs are surface molecules on eukaryotic cells, present in invertebrates and conserved in vertebrates, which were originally identified as homologs of *Drosophila* Toll molecule, an important component of antifungal defence mechanism (71, 74, 75). TLRs are considered the most important family of PRRs, with ten different TLRs being ubiquitously expressed in humans (71, 74-77). The existence of several TLRs enables the innate immunity system to recognize different groups of pathogens while initiating appropriate and distinct immunological responses, according to the PAMP recognized (70, 76, 78). In normal physiological conditions TLRs do not recognize self-ligands. However, after tissue lesion they may recognize endogenous antigens, the so-called damage-associated molecular patterns (DAMPs), and contribute to promote sterile inflammation (79, 80). Although initially described in several immunological cells, various studies have shown that different human tissues express these receptors, with the degree of expression varying from tissue to tissue (71, 74-77).

The structure of all TLRs is identical. TLRs are membrane-surface receptors consisting of a distinct leucine-rich repeat (LRR) extracellular domain that confers specificity to the receptor, a single transmembrane domain and a conserved toll/interleukin 1 (IL1) receptor (TIR) intracellular domain, homologous to the IL1 receptor (76, 77). In general, TLR2 recognizes PAMPs mainly from Gram positive bacteria, TLR4 is the receptor for Gram negative bacteria lipopolysacharide (LPS), TLR5 recognizes bacteria flagellin, TLR3, TLR7 and TLR8 recognize viral components namely double (TLR3) and single-stranded RNA (TLR7/8), TLR9 recognizes

unmethylated CpG DNA from bacteria and virus, and finally, TLR1 and TLR6 form heterodimers with TLR2 in order to sense tri-acyl (mycobacterium) and di-acyl lipopeptides (mycoplasma), respectively. In addition, TLR4 and TLR2 can detect a wide range of antigens not only from bacteria but also from fungus, parasites, virus (particularly TLR2) and DAMPs (particularly TLR4) (71, 81). TLR4, the receptor of LPS, is probably the most studied TLR. In order to recognize LPS, a complex interaction between TLR4 and LPS binding protein (LBP) with CD14 and MD2 co-receptors appears essential for innate immune activation in response to the LPS of Gram-negative bacteria (82-85). In Table I.2 we can see a brief description of the different TLRs, their location, ligands, signalling molecules and final products.

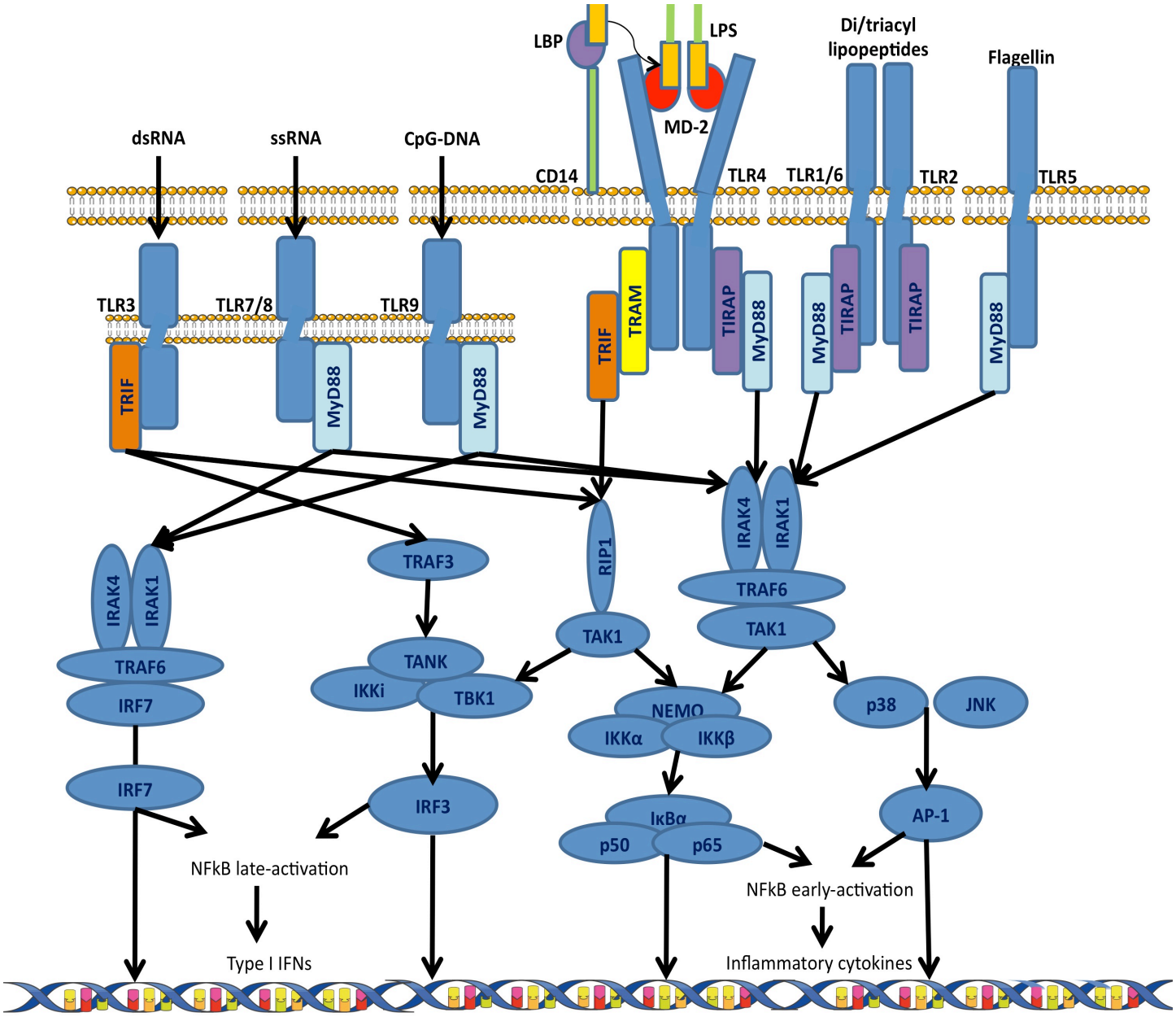
TABLE I.2 – Description and essential features of Toll-like receptors

RECEPTOR	LOCATION	LIGANDS	SIGNALING MOLECULES	FINAL PRODUCTS
TLR1/TLR2	Plasma membrane	Triacyl lipopeptides (Gram+ bacteria and mycobacteria), Neisseria porins	TIRAP, MyD88	Inflammatory cytokines
TLR2	Plasma membrane	Peptidoglycan (Gram+ bacteria), several other from Mycobacteria, Virus, Fungus and Trypanosoma	TIRAP, MyD88	Inflammatory cytokines
TLR2/TLR6	Plasma membrane	Diacyl lipopeptides (mycoplasma), lipoteichoic acid (Streptococcus), zymosan (fungus)	TIRAP, MyD88	Inflammatory cytokines
TLR3	Endosome	dsRNA (virus)	TRIF	Inflammatory cytokines, type I Interferons
TLR4	Plasma membrane	LPS, DAMPs, other PAMPs from fungus and virus	TIRAP, MyD88, TRAM, TRIF	Inflammatory cytokines, type I Interferons
TLR5	Plasma membrane	Flagellin (several flagellated bacteria)	MyD88	Inflammatory cytokines
TLR7	Endosome	ssRNA (virus)	MyD88	Inflammatory cytokines, type I Interferons
TLR8	Endosome	ssRNA (virus)	MyD88	Inflammatory cytokines, type I Interferons
TLR9	Endosome	CpG motifs from bacteria and virus, dsDNA (virus)	MyD88	Inflammatory cytokines, type I Interferons
TLR10	Unknown	Unknown	MyD88	Inflammatory cytokines

TLR signalling pathways

In order to initiate intracellular signalling pathways, after recognition of the PAMP, all TLRs have to proceed to homodimerization, the only exception being TLR2 that forms heterodimers with TLR1 and TLR6. When TLRs couple to its respective ligand, intracellular signals are transduced through a MyD88-dependent pathway (the TIR-domain adaptor molecule is myeloid differentiation factor 88 (MyD88)) or/and through a MyD88-independent pathway (adaptor molecule is TIR-domain-containing adaptor protein inducing IFN-β (TRIF)) (76, 77). With the exception of TLR3, all TLRs activate MyD88-dependent pathway, which leads to the production of several inflammatory cytokines through the early-phase of nuclear factor-kB (NF-kB) activation. On the other way, TLR3 and TLR4 (and probably TLR7, 8 and 9) signal through MyD88-independent pathway, which involves the late-phase of NF-kB activation and the production of interferons (IFN) (76). Despite similar intracellular signalling pathways, the final result of stimulating different TLRs is not exactly the same depending not only of the activated receptor but also of the cell that is stimulated (86-88). Moreover, intracellular consequences of TLR activation depend of several factors, namely the nature of the PAMPs, activation of other TLRs and PRRs, the level of cytokines, and other factors (86-88). Furthermore, current evidence suggests that when chronically activated these signalling pathways may interact and promote transcription of oncogenic factors (89, 90). This further underscores a complex and not completely understood intracellular signalization for these receptors. In Figure I.1 we can see the TLRs signalling pathways and the interaction between the several TLRs.

FIGURE I.1 – Toll-Like receptors signalling pathways



TLR stimulation through the NF-kB early activation leads to the production of several inflammatory cytokines like IL-1 and TNF-α. Some TLRs can activate the late-phase of NF-kB activation and the production of type I IFNs like IFN-β. Adapted from reference (91)

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CHAPTER II – TOLL-LIKE RECEPTORS AS THERAPEUTIC TARGETS IN GASTROINTESTINAL DISEASES

"Opportunity is missed by most people because it is dressed in overalls and looks like work!"

Thomas Edison (1847-1931)

Review

Expert Opinion

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Toll-like receptors as therapeutic targets in gastrointestinal diseases

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Importance of the field: Toll-like receptors (TLRs) are innate immunity receptors that recognize several different antigens, initiating immunological/inflammatory responses. Recent evidence associates numerous pathophysiological processes and diseases with dysregulated activation of these receptors, conferring a potential therapeutic value to their modulation.

Areas covered in this review: The aim of this systematic review that covers literature from the past 10 years is to address the role of TLRs in the pathophysiology of gastrointestinal (GI) diseases as well as the therapeutic potential of modulating TLRs' signaling pathways in GI pathology.

What the reader will gain: This review shows that TLRs play an important role in the pathophysiology of several GI diseases and that modulating TLRs signaling pathways may have an enormous therapeutic potential. Different methods for modulation of TLRs' activity in GI tract, with direct agonists/antagonists but also with non-specific substances, like antibiotics or probiotics, are presented.

Take home message: Even though TLRs modulators have been used for therapy in some GI diseases, further research, particularly in humans, is needed in order to establish the precise role of the different TLRs in the diverse GI diseases and to motivate clinical trials that consider TLRs as therapeutic targets in GI pathology.

Keywords: gastrointestinal disease, therapeutic targets, therapy, toll-like receptors

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1. Introduction

The innate immune system recognizes several components of microbes and initiates protective immunological responses. We now know that this microbiological recognition is a specific and highly coordinated process involving pattern recognition receptors (PRRs) that identify preserved structures of different pathogens, the so-called pathogen-associated molecular patterns (PAMPs) [1,2]. Moreover, this initial recognition of PAMPs by PRRs is essential, not only for the initial control of a potential infection (innate immunity), but also for triggering a late antigen-specific acquired immunity (adaptative immunity) [3,4]. Toll-like receptors (TLRs) are the most important family of PPRs, with 10 different TLRs being ubiquitously expressed in humans [5,6]. The existence of several TLRs enables the innate immune system to recognize different groups of pathogens while initiating appropriate and distinct immunological responses, according to the PAMP recognized [1,5,7]. Under normal physiological conditions TLRs do not recognize self ligands. However, after tissue lesion they may recognize endogenous antigens, the so-called damage-associated molecular patterns (DAMPs), promoting sterile inflammation [8,9].

TLRs are membrane-surface receptors consisting of a distinct leucine-rich repeat (LRR) extracellular domain that confers specificity to the receptor, and a conserved

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Article highlights.

- Regulation of TLRs activity is very important for gastrointestinal (GI) homeostasis.
- TLRs play a significant role in the pathophysiology of several gastric, hepatic, pancreatic, small bowel and colonic diseases.
- Dysregulation of TLR activation is found in several GI pathologies.
- There are several and different ways of modulating TLRs activity in GI tract.
- TLR modulation in GI pathology may be associated with beneficial therapeutic effects.
- Despite the great number of studies about the role of TLRs in some areas, like in IBD, the data is far from conclusive.
- Future translational studies and clinical trials are needed in order to clarify the potential for TLR modulation in GI pathology.

This box summarises key points contained in the article.

toll/IL-1 receptor (TIR) intracellular domain [5,6]. In general, TLR2 recognizes PAMPs mainly from Gram-positive bacteria, TLR4 is the receptor for Gram-negative bacteria lipopolysacharide (LPS), TLR5 recognizes bacteria flagellin, TLR3, TLR7 and TLR8 recognize viral components namely double- (TLR3) and single-stranded RNA (TLR7/8), TLR9 recognizes unmethylated CpG DNA from bacteria and virus, and finally, TLR1 and TLR6 form heterodimers with TLR2 in order to sense tri-acyl (mycobacterium) and di-acyl lipopeptides (mycoplasma), respectively. In addition, TLR4 and TLR2 can detect a wide range of antigens not only from bacteria but also from fungi, parasites, viruses (particularly TLR2) and DAMPs (TLR4) [2,10].

When TLRs couple to their respective ligands, intracellular signals are transduced through a myeloid differentiation factor 88 (MyD88)-dependent pathway (the TIR-domain adaptor molecule is MyD88) or/and through a MyD88-independent pathway (the adaptor molecule is TIR-domain-containing adaptor protein inducing IFN-β (TRIF)). With the exception of TLR3, all TLRs activate the MyD88-dependent pathway which leads to the production of several inflammatory cytokines through the early-phase of NF-κB activation. On the other hand, only TLR3 and TLR4 signal through the MyD88-independent pathway, which involves the late-phase of NF-κB activation and the production of IFN-β [5]. Despite similar intracellular signaling pathways, the final result of stimulating different TLRs is not exactly the same depending not only on the activated receptor but also on the cell that is stimulated [11-13]. This further underscores the complex and not completely understood intracellular signalling for these receptors.

The gastrointestinal tract is both colonized by several microorganisms and their associated PAMPs and is also exposed to other organisms, pathogenic or not, that on a daily basis come into contact with the intestinal barrier. Intestinal cells cannot initiate an inflammatory response to the commensal PAMPs but at the same time they have to be able to respond to potentially invading pathogens. So, a strict regulation of TLR activation is fundamental for maintaining gatrointestinal homeostasis. This is accomplished in several ways that involve the receptor itself, the signaling cascade and also a precise cellular and anatomic compartmentalization of TLRs [14-16]. Although gastrointestinal epithelial cells express TLRs, a marked downregulation of surface expression of these receptors exists in the colon, particularly in the mature cells, when compared with small bowel or crypt cells [14,16-19]. Numerous intracellular negative regulation mechanisms have also been described in these cells, such as decreased transcription of TLRs [20], proteolytic degradation of TLRs or its signaling molecules [21,22] and high expression of several TLR-antagonists [15]. In fact, commensal bacteria augment the expression of Toll-interacting protein (TOLLIP), a molecule that is highly expressed in normal colonic mucosa, and of PPARγ, which inhibit IL-1R-associated kinase (IRAK), a component of TLR signaling, and NF-κB activation, respectively, antagonizing in this way TLRs' activity [23-27]. It is clear that under normal physiological circumstances only invasive pathogens can overcome these mechanisms and activate TLRs.

Recent studies show that besides their essential role in immune responses and inflammation, TLRs also play a part in epithelial regeneration, wound healing, maintaining normal physiology in several organs, auto-immunity processes and even carcinogenesis [28-32].

In this review, we aim to summarize the role of TLRs in the pathophisiology of several GI diseases and to describe current findings and future clinical implications of considering TLRs as therapeutic targets in such diseases.

2. Role of TLRs in the pathophysiology and therapy of GI diseases

2.1 Esophagus

Esophageal epithelial cells express several TLR molecules and respond to TLR stimulation [33]. However, clear evidence linking pathogens, innate immunity receptors and disease is lacking.

2.2 Stomach

2.2.1 Helicobacter pylori infection and associated pre-neoplasic conditions

Helicobacter pylori is a Gram-negative bacterium that adheres to the surface of gastric mucosa, causing marked inflammation without invasion of gastric epithelial cells [34]. It is clear that TLRs have a role in *H. pylori* recognition and subsequent innate and adaptive immunity against this bacterium [35]. However, which is the principal TLR responsible for this process is a question of some controversy. TLR2 appears to be the receptor responsible for most of the inflammatory changes occurring as the result of *H. pylori* infection. Indeed, several studies showed that TLR2, but not TLR4, was required for *H. pylori* -induced NF-κB activation and cytokine

production by epithelial [36] and antigen-presenting cells [37]. Cytotoxin-associated gene A (Cag A), an important virulence factor of *H. pylori*, promotes a higher production of IL-8 by TLR2 and not TLR4 signaling [38]. Nevertheless, other studies suggest that TLR4 play an important role in *H. pylori* infection by recognizing several *H. pylori* antigens [39-41]. More recent studies demonstrate that either in epithelial or dendritic cells, TLR2 is the principal receptor for recognition of *H. pylori*, but this process depends also in a minor extent of TLR4 that acts in synergy with TLR2 [35,42-46]. TLR9 recognizes *H. pylori* DNA and appears to have a complementary and synergistic interaction with the other two receptors [43,47]. On the other hand, TLR5 appears to have no role in the detection of *H. pylori* since, despite some initial studies suggesting interaction between *H. pylori* flagellin and this receptor [36,48], more conclusive studies demonstrate that TLR5 is unresponsive to *H. pylori* flagellin [49-51].

Besides *H. pylori* recognition, these receptors also have a role in the progression of gastric lesions associated with *H. pylori* infection. Chronic *H. pylori* infection increases TLR4 and activation of NF-κB [40,41]. Intestinal metaplasia and dysplasia are associated with a more diffuse cytoplasmatic distribution of TLR4 [52]. Additionally, some studies showed an association between TLR4 polymorphisms and the severity of gastric lesions associated with *H. pylori* infection [53-55]. The role of TLR2 in the progression of lesions is not established. However, the TLR2 –196 to –174ins allele was associated with severity of intestinal metaplasia and mucosal atrophy [56]. Together with the essential role of TLR2 in the recognition of *H. pylori*, this indicates that TLR2 may have an important role in the progression of gastric lesions.

Concerning *H. pylori* infection, although more studies are needed, TLRs agonists, particularly TLR2 but also TLR4 and TLR9, may help to mount effective long-term immunity and increase the eradication rates when associated with antibiotherapy. Moreover, inhibition of TLR4 signaling may delay the progression of gastric lesions. In fact, a recent meta-analysis showed that green tea decreases the risk of gastric cancer [57]. Curiously, green tea catechins appear to interfere with TLR4 signaling conferring cytoprotective effects against *H. pylori* -induced gastric cytotoxicity [58].

2.2.2 *Gastric adenocarcinoma*

Gastric carcinoma cells express several TLRs, enabling interaction with *H. pylori* or other microorganisms [52]. As several PAMPs can induce gastric-carcinoma-promoting factors, such as IL-8, via epithelial TLRs, TLR expression by gastric carcinoma cells may have a dangerous potential [36,52,59]. In fact, *H. pylori* augments the growth of gastric cancer cells via the LPS–TLR4 pathway, promoting proliferation and progression of gastric cancers [60]. In other studies induction of COX-2 overexpression, invasivity and angiogenesis of gastric cells by *H. pylori* involved TLR2/TLR9 and NF-κB activation [61,62]. Other non-*H. pylori* PAMPs may also promote tumor growth via TLR2 signaling [63]. Confirming this role of TLRs in gastric carcinogenesis, some studies described an association of TLR4 [64,65] and TLR2 [66] polymorphisms with intestinal type gastric adenocarcinoma.

So, it appears that TLR4, TLR2 and eventually TLR9 may promote gastric carcinogenesis as well as invasivity and angiogenesis. Antagonizing these molecules may have a role in the therapy of intestinal type gastric carcinoma.

2.3 Pancreas

2.3.1 *Acute pancreatitis*

Recent research has made it apparent that TLR4, by promoting the release of many inflammatory cytokines, has an important role in the patophysiology of acute pancreatitis, contributing to the pancreatic lesions but particularly to the systemic multi-organ dysfunction associated with this disease. In fact, some studies suggest that TLR4 recognizes several DAMPs originated by pancreatic lesion, such as pancreatic elastase and heparan sulphate produced by hydrolysis of cell membrane as well as extracellular matrix, thus inducing systemic inflammation [67-69]. Other molecules, namely extracellular heat-shock protein 70 may also aggravate pancreatitis through TLR4 activation [70]. Several studies show that TLR4 deficiency or ablation, ameliorates the severity of acute pancreatitis, predominantly the extra-pancreatic organ damage and the systemic inflammatory response [71-76]. Despite some evidence that LPS may have some role in the inflammatory response originated by TLR4 activation [77,78], the majority of this process appears to be independent of LPS [72,79] and probably involves DAMPs. Other TLRs such as TLR2 [80] and TLR9 [81] may also play a part in acute pancreatitis, however, no definitive conclusions can be made.

As evidenced by several works, blocking TLR4 is a potential therapeutic target in acute pancreatitis [75,76,79]. A simple measure like L-arginine administration may decrease TLR4 expression and severity of lesions [75,76]. Still, two considerations have to be made: first, almost all evidence of the TLR4 role in acute pancreatitis came from animal studies; second, antagonizing TLR4 may augment the risk of infection in these susceptible patients [73,82].

2.3.2 *Chronic pancreatitis*

Few studies addressed the role of TLRs in chronic pancreatitis. Pancreatic stellate cells, a major profibrogenic cell type, express TLRs and respond to PAMPs by inducing production of cytokines but not proliferation or production of type I collagen [83]. A recent study showed that TLR3 signaling induces chronic pancreatitis through the Fas–Fas ligand-mediated cytotoxicity in autoimmune-prone mice [84].

2.3.3 *Pancreatic cancer*

No single study suggest that TLRs play a role in pancreatic carcinogenesis, despite pancreatic cells and pancreatic cancer cells appear to express at least some TLRs [85-87]. Nevertheless, one potential strategy treatment for pancreatic cancer is targeted immunotherapy, and use of TLRs for this effect

has been performed by some. In a model of orthotopic pancreatic cancer in mice, a tumor suppressive effect was observed after treatment with a synthetic lipopeptide (MALP-2), which signals through TLR2 and TLR6 [88]. This tumor-suppressive effect of MALP-2 was confirmed in a Phase I/II human trial, obtaining a mean survival remarkably high for pancreatic cancer [89]. In an orthotopic human pancreatic carcinoma xenograft, therapeutic synergism between gemcitabine and a TLR9 agonist, CpG-oligodeoxynucleotides (ODN), was associated with delayed development of bulky disease and significantly improved survival time [87]. Finally, a recent study showed that inhibition of TLR3 signaling in human pancreatic cancer cells with phenylmethimazole (C10), a novel TLR signaling inhibitor, can decrease cell growth and migration [86].

In conclusion, TLR2/6 and TLR9 agonists could be an option for immunotherapy in pancreatic cancer. Further studies are needed to clarify if antagonizing TLR3 is also an option for treatment of this disease.

2.4 Liver and biliar diseases

2.4.1 Alcoholic liver disease (ALD)

There is no doubt that alcohol leads to a significant elevation of portal and systemic levels of LPS (endotoxemia), the TLR4 main ligand [90,91]. Several studies demonstrate that LPS is a crucial mediator of liver injury in ALD [92-94]. Endotoxin, by activating TLR4 on Kupffer cells, cells that express several TLRs and are the first one to encounter gut-derived PAMPs, leads to the production of several pro-inflammatory and pro-fibrogenic mediators promoting liver injury [95-98]. The important role of TLR4 in ALD is further supported by the blunted inflammatory liver injury observed in mice deficient in TLR4 or its coreceptors despite significant endotoxemia [95,96,99,100]. Other TLR molecules may have a secondary role since intrahepatic expression of most of the TLRs is upregulated by chronic alcohol consumption, further potentiating cytokine production in response to LPS binding [101].

So, blocking the TLR4 liver activation appears to be a therapeutic option in ALD. However, a direct blockage of TLR4 may significantly increase the infection risk in these patients, who are particularly susceptible to infections [94,102,103]. More realistic options appear to be the use of antibiotics/probiotics that reduce endotoxemia, modulating in this way TLR4 function and attenuating liver injury [92,104].

2.4.2 Non-alcoholic steatohepatitis (NASH)

A recent review [105] and a recent study [106] suggest that the patophysiology of NASH may be similar to that of ALD, and that the same TLR4 dependent mechanisms that occur in ALD also are central in the pathogenesis of NASH. In fact, a high-fat diet and obesity increase intestinal permeability to LPS [107] as well as LPS-containing microbiota and endotoxemia [108]. Similar to what happens in ALD, endotoxin may promote Kupffer cell activation as well as insulin resistance, fat accumulation and inflammation in the liver [108,109]. Supporting the role of TLR4 signaling in this process is the fact that TLR4-deficient mice, but not TLR2-deficient mice, exposed to a methionine/choline-deficient (MCD) diet, a common experimental model of NASH, have presented decreased liver injury and lipid accumulation [110].

Once again, the use of antibiotics or probiotics in obese mice resulted in decreased LPS levels and attenuated severity of NASH and metabolic syndrome, suggesting that modulation of gut microflora may attenuate the deleterious effects of TLR4 activation [111-114].

2.4.3 Hepatitis B

There is increasing evidence to support an important role of TLRs in hepatitis B virus (HBV) infection. Besides presenting antigens that stimulate TLRs, HBV promotes downregulation of several TLR molecules as well as attenuation of activation of these receptors which may help the infection to persist [115-117]. Curiously, in HBeAg-negative but not in HBeAg-positive patients upregulation of TLR2, a TLR that does not appear to be involved in HBV recognition, and increased TNF-α production were found, which may promote the more severe hepatic lesion that is seen in these patients [118,119]. Supporting the role of TLRs in HBV infection, many recent studies show that activation of several TLRs, particularly TLR3, TLR7 and TLR9 but also TLR4 and TLR5, can block viral replication through an IFN-dependent inhibition of HBV [120-122].

The clinical value of these findings is translated in the augmented immune responses to HBsAg vaccination that is seen with TLR9 (CpG-ODN) and/or TLR7/8 agonists (Resiquimod) [123-125]. Human studies are needed to confirm the value of these TLRs agonists in prophylaxis or even in promoting erradication of HBV infection.

2.4.4 Hepatitis C

In a similar manner to HBV, existing evidence suggest that hepatitis C virus (HCV) promotes decreased activation of TLR signaling related to control of viral infection but at the same time increases activation of TLR pathways that generate liver inflammation [126-130]. After TLR3 binding to dsRNA from HCV, HCV proteins, NS3/4A, block in several ways the activation of TLR3 and the subsequent production of type I IFNs [131,132]. The activation of other TLRs, such as TLR7 and TLR9, may also be blunted by HCV proteins, with further compromise of immune virus clearance by several primary immune cells [133-136]. At the same time HCV core protein and NS3 activate TLR2, with involvement of TLR1 and TLR6, and maybe also TLR4, promoting hepatic inflammation and injury [126,128,137].

TLR3- and TLR4-stimulated non-parenchymal liver cells are able to regulate HCV replication through production of IFN-β [138]. Treatment with intravenous isatoribine, a TLR7 agonist, or with CPG 10101, a TLR9 agonist, caused a significant reduction of plasma HCV RNA in patients infected with genotype 1 as well as non-genotype 1 HCV with induction

of markers of a heightened immune antiviral state [139,140]. This data further underscores the immense potential of using TLRs as therapeutic targets in HCV infection.

2.4.5 *Hepatic fibrosis and cirrhosis*

TLR4 plays a crucial role in liver fibrosis and in the physiopathology of cirrhosis. There is no doubt that liver fibrosis and cirrhosis is associated with significant increase in plasma LPS, the TLR4 main agonist [90,141-144]. Studies using different models of fibrosis and cirrhosis and different mechanisms to block TLR4 signaling, namely with TLR4 or its coreceptors CD14 and LPS binding protein (LBP) deficient mice as well as with gut-sterilized mice, confirmed that LPS–TLR4 activation is essential for hepatic fibrogenesis [145,146]. Even though TLR4 activation in Kupffer cells promotes the production of several pro-inflammatory and also pro-fibrogenic mediators [95-98], TLR4 activation in hepatic stellate cells is the vital step for collagen production and consequently fibrosis and cirrhosis [146-148]. Recent studies confirmed that variants of TLR4 gene modulate risk of liver fibrosis [149-151] and that a TLR4–MD2 fusion protein inhibits LPS-induced pro-inflammatory signaling in hepatic stellate cells [152]. TLR9-deficient mice also have decreased fibrosis, suggesting that TLR9 activation on hepatic stellate cells may have a role in hepatic fibrogenesis [153,154].

Besides promoting fibrosis and cirrhosis, TLRs may also have a role in cirrhosis complications, namely in the incredibly high infection risk of these patients [94,102,103]. Lin *et al.* clearly demonstrated that in Child C patients monocyte stimulation with LPS was attenuated [155]. More importantly, Testro *et al.* showed that, in the same group of patients, this blunted response to LPS was dependent of decreased TLR4 expression and that antibiotic therapy may restore TLR4 levels and also the immunologic response to LPS [156]. These two works suggest that changes in TLR4 expression and signaling are important for the 'endotoxin tolerance' and that modulation of TLR4 function with antibiotics may reverse this phenomenon. Other TLR molecules may contribute to the immunodeficiency of cirrhosis. In fact our group [157] and others [158] demonstrated that a blunted TLR2 activation in immunological cells is present in cirrhotic patients, which may contribute to the infection risk. Synbiotic therapy was able to reverse this immunological defect [158].

So, a direct antagonist of TLR4 may decrease hepatic fibrosis, but at the same time, it may significantly augment the infection risk. Curcumin, a dietary component that has been shown to inhibit TLR4 activation [159-161], also appears to block activated hepatic stellate cells [159]. Modulation of TLR activity through decreasing endotoxemia by the use of probiotics/synbiotics [113,158,162] or antibiotics [92,156,163] may at the same time decrease liver fibrosis and stimulate immunity.

2.4.6 *Hepatocarcinoma*

There is no clear evidence linking TLRs and hepatocarcinoma despite the fact that in chronic HBV infection CpG DNA of HBV, through activation of TLR9, may contribute to the malignant transformation of benign liver cells [164]. TLR3 expression in hepatocarcinoma appears to have a role with regard to proapoptotic activity [165,166]. In more than 80% of the cases this tumor arises from chronic inflammation and fibrosis of the liver, with cirrhosis being considered a pre-neoplasic condition [167-169]. TLR4 has a crucial role in inflammation and in liver fibrosis, and several inflammatory factors that are upregulated by TLR4 activation, such as COX-2 and NF-κB, have been shown to be important in hepatocarcinogenesis [168-170]. Myd-88, the TLR adaptor protein, also appears to be important for the development of hepatocarcinoma [171,172]. In fact, a recent study suggests that TLR4 mediates synergism between alcohol and HCV in hepatic oncogenesis [173].

We believe that the constant activation of hepatic TLR4 by elevated LPS plasma levels that exist in cirrhosis promotes hepatocarcinogenesis and that antagonizing this effect may have an important role in prophylaxis and even therapy for this tumor. TLR3 agonists may have therapeutic potential as cytotoxic agents in hepatocarcinoma [166].

2.4.7 *Primary biliary cirrhosis (PBC)*

Despite there being no clear evidence linking TLRs with the physiopathology of PBC, it appears that TLR activation is dysregulated in this disease. An initial study suggested that TLR4 expression was higher in the liver of PBC patients [174]. This was not confirmed in the study of Shimoda *et al.*, where TLRs expression levels in biliary epithelial cells from patients and controls were found to be similar [175]. However, when stimulated with a TLR3 agonist, they secreted higher levels of chemokines, but only when co-cultured with liver-infiltrating mononuclear cells [175]. TLR-3 and type I IFN signaling pathways were active in both the portal tract and liver parenchyma of early-stage PBC but not in controls [176]. Additionally, monocytes isolated from PBC patients were hyperresponsive to TLRs agonists, and B cells when stimulated with a TLR9 ligand have been found to promote hyper-IgM levels [177-180].

Importantly, antagonizing TLRs activation using PPAR-γ ligands have been suggested to be of therapeutic benefit to attenuate biliary inflammation in PBC [181].

2.4.8 *Ischemic/reperfusion (I/R) lesion and liver allograft rejection*

Current knowledge confers a central role of TLR4 activation by DAMPs via the MyD88-independent pathway in the inflammatory process seen in I/R lesions [182-186]. On the other hand, the role of TLR2 activation is not so clearly defined, with some studies suggesting an important role [187] while others do not [184]. In fact, not only the absence of TLR4 [182] but also downregulation of TLR2 expression in the donor organ reduces I/R injury [188]. Some molecules like bicyclol or *N*-acetylcysteine have been shown to

decrease liver injury in I/R by a non-selective attenuation of TLR2 and TLR4 activation [189-191], thus emphasizing the importance of these TLRs in I/R lesion.

Other aspect concerning liver transplantation where TLRs may play a role is acute rejection. A recent work suggests that acute liver rejection is associated with significantly greater levels of immune cells TLR4 expression and function, implicating TLR4 response in the initiation of allograft rejection [192].

It is clear that TLR4 antagonists may have a therapeutic role in several aspects of liver transplantation.

2.4.9 *Other hepatic and biliary diseases*

Acetaminophen-induced hepatotoxicity is dependent on TLR9 and TLR4 activation by DAMPs [193,194]. Importantly, TLR9 antagonists reduced mortality from acetaminophen hepatotoxicity [193].

TLR2 and TLR4 may play a part in acute allograft rejection after liver transplantation [195] and an antagonist of TLR4 (E5564) had a beneficial effect in acute liver injury [196].

2.5 Small bowel

2.5.1 *Celiac disease*

Celiac disease is an autoimmune disorder provoked by gluten that affects primarily the small intestine. Its pathogenesis involves various immunological factors, however, the role of innate immunity is not determined. It was first suggested that innate immune response has some importance in celiac sprue since gliadin stimulation of macrophage inflammatory gene expression and intestinal permeability were MyD88-dependent [197]. Latter, a subset of autoantibodies against transglutaminase were found to bind TLR4, inducing activation of monocytes [198]. Finally, and more importantly, mucosal expression of TLR2 and TLR4 was found to be increased either in treated or untreated patients [199]. Further investigation is needed in this area.

2.5.2 *NSAIDs' enteropathy*

Recent studies have been focused on the importance of TLR4 in NSAIDs' intestinal lesions. In the study of Kato *et al.*, upregulation of iNOS/NO through the increased expression of TLR4 in the small intestine of arthritic rats was shown, suggesting that increased susceptibility of small intestine to NSAID-provoked ulceration involved TLR4 activation [200]. These results were confirmed using mutant mice, clearly establishing that NSAID-induced small intestinal damage is TLR4-dependent and that therapy with Gram-negative- but not Gram-positive-acting antibiotics decreased these lesions [201]. Finally, probiotic exhibited a prophylactic effect on indomethacin-induced enteropathy by suppressing the LPS/TLR4 signaling pathway [202].

Human studies are needed to confirm the potential of modulation of TLR4 activation by probiotics in NSAID enteropathy treatment.

2.5.3 *Radiation injury*

Ionizing radiation is associated with massive apoptosis in radiosensitive organs, particularly in intestinal crypt cells [203]. A polypeptide drug derived from Salmonella flagellin injected into mice before or after total body irradiation activated TLR5 and protected mice from lesion, improving survival without decreasing tumor radiosensitivity [204]. Thus, TLR5 agonists have the potential to improve the therapeutic index of cancer radiotherapy and may also be beneficial against chemotherapeutic therapies that induce significant apoptosis of intestinal cells.

2.6 Colon

2.6.1 *Inflammatory bowel disease (IBD)*

The dysregulation of innate and adaptive intestinal immune responses to bacterial microbiota is believed to be highly involved in the pathogenesis of IBD. TLRs play a key role in microbial recognition in innate immunity and control the adaptive immune responses. So, is not strange to see that a large amount of literature examines the role of TLRs in the patophysiology of IBD. However, the results have not so far been conclusive and some are even contradictory.

TLR2 activation on intestinal epithelial cells (IECs) does not appear to cause robust induction of proinflammatory gene expression, but rather provokes augmented IEC barrier function [205,206]. In fact, $TLR2^{-/-}$ mice presented increased susceptibility to chemical induced colitis and oral treatment with a TLR2 ligand prior to colitis induction significantly suppressed mucosal inflammation and apoptosis and restored epithelial integrity [207]. More recently, this increased susceptibility to colitis and impaired mucosal repair was associated with deficiency of trefoil factor (TFF3), a mucin glycoprotein of intestinal mucus that is synthesized upon TLR2 stimulation [208]. Importantly, some TLR2 or its correceptors TLR1 and TLR6 polymorphisms have been found to be associated with disease patterns in IBD. Specifically, the TLR2-R753Q variant that confers a functional deficient in the ability to induce TFF3 synthesis, leading to impaired healing, is associated with severe ulcerative colitis (UC) [208,209]. On the other hand, NOD2 mutations, important Crohn's disease (CD) susceptibly genes, appear to contribute to IBD by causing excessive TLR2 activation, dysregulation and induction of antigen-specific colitis [210,211]. Several studies do in fact contradict in some way this protective role of TLR2 in colitis. In humans with IBD, increased expression of TLR2 and TLR4 in the colonic mucosa was found, particularly during intestinal inflammation, suggesting that TLRs may contribute to the inflammatory process [212,213]. In complete contrast with the possible TLR2 anti-inflammatory action, Liu *et al.* suggested that Pam3CSK, a TLR2 agonist, may in fact aggravate colitis [214]. Others showed that improvement in patients with active IBD following leukocyte apheresis is associated with decreased numbers of TLR2-positive cells in intestinal mucosa [215] and that pro-inflammatory intestinal bacteria aggravates acute colitis and ileitis via TLR2 and

TLR4 [216,217]. Additionally, monocytes isolated from patients with active IBD, but not from patients with inactive disease, expressed higher levels of TLR2 and increased TNF-α production in response to TLR2 stimulation when compared with controls [218]. VIP appears to downregulate uncontrolled inflammation by reducing TLR2 and TLR4 expression [219,220]

Similar to TLR2, TLR4 activation in normal conditions does not appear to cause important inflammation, instead it promotes intestinal healing. $TLR4^{-/-}$ and $Myd88^{-/-}$ mice after induction of colitis present impaired mucosal regeneration with greater colonic damage and mortality [221-223]. However, in a different background, specifically on IL-10-deficient mice, the absence of MyD88 protected the mice from the development of colitis, suggesting a key pathogenic role of TLR signaling for intestinal inflammation at least in IL-10-deficient mice [224]. A recent study confirmed that in mice that lack both IL-10- and TLR4-mediated signals, aberrant regulatory T-cell function and dysregulated control of epithelial homeostasis combine to exacerbate intestinal inflammation [225]. TLR4 do appear to have a role in the initiation of intestinal inflammation, not only in $IL10^{-/-}$ colitis, since treatment with a lipid A-mimetic, CRX-526, which has antagonistic activity for TLR4, inhibits the development of moderate-to-severe disease in two mouse models of colonic inflammation [226]. In other models of colitis a non-specific inhibition of TLR4 activation obtained in different ways, for example with probiotics, was associated with significant reduction of mucosal inflammation [227-231]. However, and supporting the important role of TLR4 in epithelial regeneration, a specific TLR4 antagonist antibody ameliorated inflammation but impaired mucosal healing in murine colitis [232]. Several studies associate TLR4 polymorphisms, particularly the TLR4 Asp299Gly polymorphism, with IBD, confirming the important role of TLR4 in IBD pathogenesis [233-238].

Few studies address the role of TLR3 in IBD. Activation of TLR3 by poly(I:C) treatment protected against dextran sodium sulfate (DSS)-induced acute colitis [239]. However, other studies suggested that TLR3 signaling may have a deleterious effect on intestinal inflammation [240,241]. In humans, TLR3 expression was downregulated in inflamed and non-inflamed mucosa of CD but not of UC patients [242].

TLR5 is highly expressed in the basolateral but not in the apical membrane of IEC. The current knowledge is that intact epithelial cells do not respond to the TLR5 agonist, flagellin, but when an epithelial barrier disruption occurs flagellin via TLR5 basolateral membrane activation is able to elicit strong inflammatory responses [243,244]. In fact, CD-associated virulent *Escherichia coli* bacteria, via expression of flagella, are able to potentiate an inflammatory mucosal immune response involving increased expression of TLR5 [245]. Other studies confirmed that in CD patients, tolerance to commensal-derived flagellin is lost with enhanced flagellin reactivity [246,247]. Confirming that TLR5 activation is important at least in CD inflammation, a dominant-negative TLR5 polymorphism, that confers inactivity to TLR5, was negatively associated with CD [248]. However, TLR5 may also have a homeostatic function since deletion of TLR5 results in spontaneous colitis in mice, apparently through increased TLR4 signaling, suggesting that TLR5 signaling may have a physiological role in modulating TLR4 activation [249].

Recent studies showed that expression of TLR8 is highly upregulated in the colonic epithelium from patients with active IBD [250] and high-frequency haplotypes in the X chromosome locus TLR8 are associated with both CD and UC in females [251]. So, TLR8 have recently attracted some interest, although its role in IBD, if any, is still to be defined.

Finally, similar to most of the other TLRs, TLR9 appear to act as a double-edged sword in IBD. TLR9 activation with CpG-ODNs improved the severity of induced-models of colitis [252]. TLR9-induced type I IFN protected mice from experimental colitis [253]. CpG DNA/TLR9 interaction induced regulatory properties in $CD4^{+}CD62L^{+}$ T cells which prevented intestinal inflammation in a transfer model of colitis [254]. The beneficial anti-inflammatory effects of probiotics were shown to be dependent on TLR9 signaling [255,256]. In humans, CpG-ODNs inhibited colonic production of inflammatory cytokines in *ex vivo* mucosal biopsies of UC patients but not controls [257]. However, on the other hand, intraperitoneal administration of CpG-ODN increased the severity of DSS-induced colitis [258]. Medium-chain triglycerides decreased the incidence of spontaneous colitis by reducing TLR9 mRNA [259]. TLR9-deficient mice presented less intestinal inflammation in a induced-model of colitis and blocking CpG effects with adenoviral ODN resulted in a significant amelioration of colitis, suggesting that CpG motifs of bacterial DNA contributes to the perpetuation of chronic intestinal inflammation [260]. TLR9 polymorphisms appear to modulate susceptibility to CD [261,262].

How can we explain these apparent contradictory and opposing effects of TLRs in IBD? In a similar way to that described for TLR9 [263], we believe that apical or basolateral TLRs activation in IEC initiates distinct signaling pathways, with apical stimulation promoting anti-inflammatory and homeostatic effects and basolateral stimulation initiating defensive inflammatory reactions, which may have beneficial effects against pathogens, but in IBD will perpetuate injury. In conclusion, activating TLRs when the epithelial barrier is intact may result in a protective effect against inflammation, while when the epithelium is disrupted it may lead to aggravation of inflammation not only by stimulation of IEC but also of immune submucosal cells. In **Table 1** we list the most pertinent TLRs targets for prophylaxis and therapy in IBD.

2.6.2 *Colorectal cancer (CRC)*

TLRs signaling appears to have an important role in carcinogenesis of several tumors, by promoting either apoptosis or survival of neoplasic cells, and this potential is being increasingly used for therapeutic purposes [264]. The link between innate immunity receptors and CRC is highlighted

Table 1. Role of TLRs in GI diseases according to pathology and eventual therapeutic target for TLR intervention.

Organ	Disease	TLR	Pathophysiology	Therapeutic target	Commentary*
Stomach	*Helicobacter pylori* infection	2	TLR2 appears to be the receptor responsible for most of the inflammatory changes occurring as the result of *H. pylori* infection [36-38]	TLR2 agonists may potentiate immunity against *H. pylori* (vaccine?) or promote higher eradication rates with ATB	‡No study evaluated TLR2 agonism as a therapeutic target – studies are needed in this area
		4	Acts in synergy with TLR2 in *H. pylori* recognition but with a lesser role [35,42-46]. May provoke progression of gastric lesions associated with *H. pylori* infection [40,41,53-55]	TLR4 antagonists may decelerate progression of gastric lesions associated with *H. pylori* infection	‡,§More clinical studies needed to support therapeutic role
		9	Appears to act in synergy with TLR2 and 4 in *H. pylori* recognition [43,47]	TLR9 agonists may act in the same way as TLR2 agonists but probably with lower efficacy (?)	‡Little evidence to support therapeutic role
	Intestinal-type adenocarcinoma	2, 9	TLR2 and 9 activation promotes invasion and angiogenesis through overexpression of COX-2 [61,62]	TLR2 and/or 9 antagonists may decrease tumor growth	‡More studies are needed in this area
		4	*H. pylori* augmented the growth of gastric cancer cells via the TLR4 pathway [60]	TLR4 antagonists may decrease tumor growth	‡More studies are needed in this area
Pancreas	Acute pancreatitis	4	TLR4 activation by DAMPs promotes the release of inflammatory cytokines, contributing to the pancreatic lesion but particularly to the systemic multi-organ dysfunction [71-76]	TLR4 antagonists ameliorate severity of acute pancreatitis [75,76,79]	‡Human studies are needed. May increase infection risk
		2, 9	Activation of these receptors may also contribute to the inflammatory process [80,81]	TLR2 and/or 9 antagonists may decrease disease severity (?)	‡Little evidence to support therapeutic role
	Chronic pancreatitis	3	TLR3 signaling induces chronic pancreatitis in autoimmune-prone mice [84]	TLR3 antagonist for decreasing progression of disease (?)	‡Little evidence to support therapeutic role
	Pancreatic cancer	2/6	TLR2/6 activation on immune cells provoked a tumor suppressive effect in humans [89]	TLR 2/6 agonists appears to increase survival in humans [89]	‡,§Therapeutic potential confirmed – larger Phase III studies needed
		9	TLR9 activation can have an important immunostimulatory effect against cancer cells [87]	TLR9 agonists in synergy with chemotherapy can improve survival [87]	‡Human studies are needed
		3	May have a role in tumor progression since blocking TLR3 signaling decrease cell growth and migration [86]	TLR3 antagonists may delay development of disease [86]	‡Further studies are needed

*Commentary is related to the eventual therapeutic role of the substance cited in the previous column.
‡Data came mainly from basic studies.
§Data supported by some clinical studies.
ATB: Antibiotherapy; ALD: Alcoholic liver disease; CRC: Colorectal carcinoma; DAMPs: Damage-associated molecular patterns; HBV: Hepatitis B virus; HCV: Hepatitis C virus; I/R: Ischemic/reperfusion; IBD: Inflammatory bowel disease; NASH: Non-alcoholic steatohepatitis; NEC: Necrotizing enterocolitis.

Table 1. Role of TLRs in GI diseases according to pathology and eventual therapeutic target for TLR intervention (continued).

Organ	Disease	TLR	Pathophysiology	Therapeutic target	Commentary*
Liver	ALD	4	TLR4 activation by LPS has a crucial role in the inflammatory process of ALD [95,96,99,100]	TLR4 antagonists decrease liver injury by alcohol [92,104]	‡,§Direct blockage of TLR4 may increase infection risk
	NASH	4	TLR4 role in NASH appears to be similar to ALD [105,106]	TLR4 antagonists may decrease liver injury in this context [111-114]	‡Human clinical studies are needed
	Hepatitis B	3, 7, 8, 9	HBV chronic infection promotes down-regulation of several TLRs which may help the infection to persist [115-117]	TLR 7/8 and TLR9 agonists augment immune response to HBsAg vaccination [123-125] Potential role for treatment?	‡,§Treatment with these substances should be evaluated in clinical trials
	Hepatitis C	3, 7, 9	HCV promotes a decreased activation of TLR3, 7 and 9 signaling related to control of viral infection [126-130]	TLR7 and TLR9 agonists caused a significant reduction of HCV RNA and heightened immune state [139,140]	‡,§Therapeutic potential confirmed – larger studies are needed
	Fibrosis and cirrhosis	4	LPS-TLR4 activation of hepatic stellated cells is essential for hepatic fibrogenesis [145-148]	TLR4 antagonists decrease liver fibrosis and cirrhosis development [113,152,158,162]	‡,§Clinical trials needed – Direct blockage of TLR4 may increase infection risk
		9	TLR9-deficient mice have decreased fibrosis [153,154]	TLR9 antagonists may decrease fibrogenesis (?)	‡Little evidence to support therapeutic role
	Infection in cirrhosis	2, 4	Attenuated TLR4 [156] and TLR2 [157,158] immune cells function may contribute to the immunodeficiency of cirrhosis	TLR modulators (antibiotic/synbiotic) may restore TLR function [156,158], decreasing infection risk	§Clinical trials needed
	Hepatocarcinoma	4	Factors that are upregulated by TLR4 activation like NF-κB and COX-2, are important for hepatocarcinogenesis [168-170]	TLR4 antagonists may decrease the risk of hepatocarcinoma and/or decrease tumor burden (?)	‡Studies are needed in this area
		3	TLR3 activation may promote apoptosis of liver tumor cells [165,166]	TLR3 agonists may have an antitumor effect [166]	‡More studies are needed
	I/R lesion	2, 4	TLR4 activation by DAMPs plays a central role in I/R injury [182-186]. TLR2 activation may also have a role [187,188]	TLR2 and TLR4 antagonists appear to decrease liver injury [189-191]	‡,§More studies are needed in this area

*Commentary is related to the eventual therapeutic role of the substance cited in the previous column.

‡Data came mainly from basic studies.

§Data supported by some clinical studies.

ATB: Antibiotherapy; ALD: Alcoholic liver disease; CRC: Colorectal carcinoma; DAMPs: Damage-associated molecular patterns; HBV: Hepatitis B virus; HCV: Hepatitis C virus; I/R: Ischemic/reperfusion; IBD: Inflammatory bowel disease; NASH: Non-alcoholic steatohepatitis; NEC: Necrotizing enterocolitis.

Table 1. Role of TLRs in GI diseases according to pathology and eventual therapeutic target for TLR intervention (continued).

Organ	Disease	TLR	Pathophysiology	Therapeutic target	Commentary*
Small bowel	NSAIDs enterophaty	4	NSAID-induced small intestinal damage is TLR4-dependent [200,201]	TLR4 antagonists may have a prophylactic effect [202]	‡Human studies are needed
	Radiation injury	5	TLR5 activation protects mice from radiation injury [204]	TLR5 agonists have the potential to improve the therapeutic index of cancer radiotherapy or chemotherapy (?)	‡Little evidence to support – More studies are needed in this area
Colon	IBD prophylaxis	2	TLR2 ligands prior to colitis induction significantly suppressed mucosal inflammation and apoptosis and restored epithelial integrity [207]	TLR2 agonists may have a role in maintenance, promote mucosal healing and protect against flares (?)	‡More studies are needed in this area
		4	TLR4 activation promotes intestinal healing [221-223]	TLR4 agonists may have a role in maintenance (?)	‡May increase the risk of acute flares – Little evidence to support
		5	Deletion of TLR5 results in spontaneous colitis in mice – TLR5 activation in normal mucosa appears to modulate TLR4 activation [249]	TLR5 agonists may be used in maintenance (?)	‡More studies are needed in this area
		9	TLR9 activation may prevent beginning of inflammation [252-254]	TLR9 agonists for maintenance (?)	‡May increase the risk of acute flares – Little evidence to support
	IBD flares	2	Increased expression of TLR2 in mucosa of IBD patients [212,213] – activation of TLR2 may aggravate colitis [214,216,217]	TLR2 antagonists may decrease intestinal inflammation in acute flares (?)	‡,§More studies are needed in this area- may compromise mucosal healing
		4	TLR4 is overexpressed in mucosa of IBD patients [212,213]; Blocking of TLR4 attenuates mucosal inflammation [226]	TLR4 antagonists decrease intestinal inflammation in acute flares [226-231]	‡,§May impair mucosal healing - More studies are needed in this area
		5	TLR5 ligands potentiate the inflammatory mucosal immune response [245]	TLR5 antagonists may decrease intestinal inflammation in acute flares (?)	‡More studies are needed in this area
		9	CpG motifs of bacterial DNA apparently contribute to the perpetuation of intestinal inflammation [260]	TLR9 antagonists may decrease inflammation in acute flares [260]	‡,§More studies are needed in this area

*Commentary is related to the eventual therapeutic role of the substance cited in the previous column.

‡Data came mainly from basic studies.

§Data supported by some clinical studies.

ATB: Antibiotherapy; ALD: Alcoholic liver disease; CRC: Colorectal carcinoma; DAMPs: Damage-associated molecular patterns; HBV: Hepatitis B virus; HCV: Hepatitis C virus; I/R: Ischemic/reperfusion; IBD: Inflammatory bowel disease; NASH: Non-alcoholic steatohepatitis; NEC: Necrotizing enterocolitis.

Table 1. Role of TLRs in GI diseases according to pathology and eventual therapeutic target for TLR intervention (continued).

Organ	Disease	TLR	Pathophysiology	Therapeutic target	Commentary*
	CRC	2, 4	TLR2 but mainly TLR4 activation may be involved in carcinogenesis and tumor proliferation [269-271,276]	TLR4(TLR2?) antagonists promote apoptosis of tumor cells [272,273]	‡,§More human clinical studies are needed
		5, 9	TLR5 but mainly TLR9 activation has shown to promote antitumor immunity [278-283]	TLR5 and/or TLR9 agonists induce tumor cells necrosis/apoptosis [278-283]	‡Human studies are needed
	NEC	4	LPS-mediated TLR4 signaling is fundamental for the inflammatory process of NEC [284,285]	TLR4 antagonists may decrease severity of NEC [286]	‡Human studies are needed
		9	Activation of TLR9 inhibited LPS-mediated TLR4 signaling and significantly reduced NEC severity [288]	TLR9 agonists may decrease severity of NEC (?)	‡More animal studies as well as human studies are needed

*Commentary is related to the eventual therapeutic role of the substance cited in the previous column.
‡Data came mainly from basic studies.
§Data supported by some clinical studies.
ATB: Antibiotherapy; ALD: Alcoholic liver disease; CRC: Colorectal carcinoma; DAMPs: Damage-associated molecular patterns; HBV: Hepatitis B virus; HCV: Hepatitis C virus; I/R: Ischemic/reperfusion; IBD: Inflammatory bowel disease; NASH: Non-alcoholic steatohepatitis; NEC: Necrotizing enterocolitis.

by the fact that IBD and chronic inflammation are established risk factors for CRC [265], germ-free rats given carcinogens are protected from colonic cancer [266] and cancer and adenomas present higher bacteria levels than normal mucosa [267]. In fact, recently it has been shown that, in induced-colitis murine models, bacterial-induced inflammation through TLR/MyD88 signaling appears essential for progression of adenoma to carcinoma [268]. TLR4 signaling appears to be critical for colon carcinogenesis, at least following chronic colitis. Fukata *et al.* showed that $TLR4^{-/-}$ mice are protected against the development of colitis-associated neoplasia and the mechanisms by which TLR4 activation appears to promote the development of colitis-associated cancer includes enhanced COX-2 expression and increased EGFR signaling [269,270]. Recently, it was shown that human CRC cells overexpress TLR4 when compared with normal mucosa and that CRC cell lines when stimulated with LPS activate the PI3K–Akt signaling pathway, involved in CRC growth and progression [271]. Rapamycin cytotoxic effect appears to involve at least in part inhibition of TLR4 activation on tumor cells, in this way promoting apoptosis [272,273]. In others murine models of CRC, silencing of TLR4 decreased CRC tumor burden and metastasis [274,275]. In cultured human colon cancer cells TLR2 activation also induced production of oncogenic factors like TGFβ and HGF [276]. Additionally, a small study found association of microsatelite GT polymorphisms of the *TLR2* gene and Asp299Gly polymorphism of the *TLR4* gene with sporadic CRC [277].

Others TLRs, by promoting anticancer immunity, may have a different role in CRC carcinogenesis with antitumor activity. In mouse xenografts of human colon cancer, lack of TLR5 dramatically enhanced tumor growth and, in contrast, TLR5 activation by flagellin greatly increased tumor necrosis [278]. Several studies also suggest an important antitumor effect of TLR9 activation. Rayburn *et al.* demonstrated that human CRC cells express TLR9 and that TLR9 agonism leads to decreased cell survival and proliferation, inducing apoptosis of CRC cells *in vitro* [279]. Other authors confirmed these results, suggesting that agonists of TLR9 may even synergize with anti-angiogenic factors and reduce the growth of metastasized tumor cells [280-283].

In conclusion, it appears that blocking TLR2 but principally TLR4 may be an interesting therapeutic target for CRC. On the other hand, TLR5 but mainly TLR9 activation should be considered as potential immunotherapeutic target to modulate growth of colonic tumors.

2.6.3 *Necrotizing enterocolitis (NEC)*

NEC is an important cause of death among premature infants. Initial studies demonstrated the fundamental role of bacteria, LPS and TLR4 in experimental NEC [284,285]. In a neonatal rat model, polyunsaturated fatty acid supplementation reduced the incidence of NEC through inhibition of TLR4 gene expression [286]. Leaphart *et al.* showed that NEC in mice and humans is associated with increased expression of TLR4

in the intestinal mucosa and that TLR4 activation promotes enterocyte apoptosis, significantly compromising intestinal repair. In this study, TLR4 mutant mice were protected from NEC [287]. Gribar *et al.* confirmed the importance of TLR4 in the pathogenesis of NEC and suggested that TLR9 is also very important. In this study, activation of TLR9 with CpG-DNA inhibited LPS-mediated TLR4 signaling and significantly reduced NEC severity, whereas TLR9-deficient mice exhibited increased NEC severity. They also found that even in humans NEC was associated with increased TLR4 and decreased TLR9 intestinal expression [288].

We can say that antagonists of TLR4 or agonists of TLR9 are potential therapeutic agents for NEC.

3. Conclusion

TLRs play a fundamental role in GI homeostasis allowing colonization by commensal microflora and at the same time, when necessary, initiating an immunological response to invasive microorganisms. These receptors represent the first line of defense against pathogens and it's logical to say that when this first barrier is disrupted, a pathological process begins. In fact, in this systematic review we accumulate a great amount of evidence that support TLR dysregulation as an important factor for several GI diseases, from the stomach to the colon. More importantly, it has become clear that TLRs are potential therapeutic targets for many of the most important GI pathologies, not only inflammatory/immunological but also oncological and even vascular diseases. In some of these diseases, the potential therapeutic role for modulation of TLR activation is already confirmed and clinical trials have been done or are in progress. In other diseases further research, mainly translation research, is needed in order to confirm the role of TLRs in the pathophysiological process of the disease for, later on, considering TLRs as therapeutic targets.

4. Expert opinion

A growing body of evidence supports the hypothesis that TLRs are indeed therapeutic targets for GI diseases. Nevertheless, in our opinion, there are several points that deserve future consideration:

1. When considering gastric pathology there are few studies that evaluated therapeutic value of TLR modulation despite their role in *H. pylori* recognition and inflammatory process. We believe that future studies should evaluate gastric expression and activity of these receptors in normal gastric mucosa, pre-neoplasic and carcinoma lesions – do, in fact, activation or attenuation of one or more TLRs contribute to the development of gastric adenocarcinoma, the most serious consequence of *H. pylori* infection? Do different mucosal patterns of TLRs expression confer distinct cancer risks? We believe that gastric cancer prophylaxis and treatment will be, in the near future, targets for TLR-directed therapy.
2. As we have seen, TLR4 activation is associated with deleterious effects in acute pancreatitis. Since severe acute pancreatitis does not have an established effective medical therapy, antagonists of TLR4 should be evaluated in this context.
3. When considering liver diseases there is no doubt that TLRs, mainly TLR4, play a fundamental role in inflammation and fibrosis of the liver. Particularly, we believe that TLR4 antagonists may become an important therapy after liver transplantation. Large clinical randomized trials should be design to prove the clinical usefulness of TLR modulation in this context.
4. In the near future with the growing incidence of cardiac pathology, NSAID enteropathy may become an important disease. What are the therapeutic options for the patient that takes aspirin for ischemic cardiac disease, and presents with ferropenic anemia further increasing the cardiac risk? Since NSAID -induced small intestinal damage is TLR4-dependent, we believe that human studies with TLR4 antagonists should be initiated.
5. Probably, IBD is the pathology where TLRs have the most important pathophysiological role. However, in our opinion and despite a large number of studies in this area, we just do not know what is happening in IBD. Why is that? Probably, the different models of induced-colitis represent different stages of the disease or may not even represent what really happens in human IBD. On the other hand, CD and UC have different pathophysiological processes and the role of the different TLRs may be distinct in each of these diseases. Before considering TLRs as therapeutic targets in IBD, more studies, particularly translational studies, are needed.
6. Current evidence suggests that TLR2 or TLR4 antagonists may have a role in the treatment of CRC. Human studies are needed in this area. But, is there a role for TLRs in the development of adenoma and CRC? Few studies addressed this question that may be central for cancer prophylaxis. We believe that TLRs may be important for colon carcinogenesis and so studies in this area are urgently needed.

The GI tract is on a daily basis exposed to different microorganisms that are present in the food that we ingest, modulating our commensal intestinal microflora. These organisms and their respective PAMPs are primarily sensed by TLRs. So what we eat can in fact be a risk factor for GI pathology and TLRs may be the imperative link between food and disease.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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CHAPTER III – TOLL-LIKE RECEPTORS AND LIVER DISEASE

"Is life worth living? It all depends on the liver"

William James (1842-1910)

A) THE ROLE OF LPS/TOLL-LIKE RECEPTOR 4 SIGNALING IN CHRONIC LIVER DISEASES

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REVIEW ARTICLE

The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases

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Abstract Toll-like receptor 4 (TLR4) is a pattern recognition receptor that functions as lipopolysaccharide (LPS) sensor and whose activation results in the production of several pro-inflammatory, antiviral, and anti-bacterial cytokines. TLR4 is expressed in several cells of healthy liver. Despite the constant confrontation of hepatic TLR4 with gut-derived LPS, the normal liver does not show signs of inflammation due to its low expression of TLR4 and ability to modulate TLR4 signaling. Nevertheless, there is accumulating evidence that altered LPS/TLR4 signaling is a key player in the pathogenesis of many chronic liver diseases (CLD). In this review, we first describe TLR4 structure, ligands, and signaling. Later, we review liver expression of TLR4 and discuss the role of LPS/TLR4 signaling in the pathogenesis of CLD such as alcoholic liver disease, nonalcoholic fatty liver disease, chronic hepatitis C, chronic hepatitis B, primary sclerosing cholangitis, primary biliary cirrhosis, hepatic fibrosis, and hepatocarcinoma.

Keywords Toll-like receptor 4 · Lipopolysaccharide · Chronic liver diseases

Abbreviations

Akt	Serine/threonine protein kinase
ALD	Alcoholic liver disease
Anti-BEC-Ab	Antibiliary epithelial cell antibodies
AP-1	Activator protein 1
ATF3	Activating transcription factor-3
BAMBI	Bone morphogenetic protein and activin membrane-bound inhibitor
Bcl-3	B cell leukemia-3
BEC	Biliary epithelial cell
CCL	Chemokine
CCl_4	Carbon tetrachloride
CLD	Chronic liver diseases
CYLD	Cylindromatosis protein
DAMP	Damage-associated molecular patterns
DEN	Diethylnitrosamine
DUBA	De-ubiquitinating enzyme A
ERK	Extracellular signal-regulated kinase
GSK-3β	Glycogen synthase kinase-3β
HBV	Hepatitis B virus
HCC	Hepatocarcinoma
HCV	Hepatitis C virus
HSC	Hepatic stellate cell
ICAM	Intercellular cell adhesion molecule
IFN	Interferon
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
IκBα	Inhibitor of NF-κB
JNK	C-Jun N-terminal kinase
KC	Kupffer cells
LBP	LPS binding protein
LPS	Lipopolysaccharide
MCDD	Methionine- and choline-deficient diet
miR	MicroRNA
MyD88	Myeloid differentiation factor 88
MyD88s	Splice variant of MyD88
NAFLD	Non-alcoholic fatty liver disease
NEMO	NF-κB essential modifier
NF-κB	Nuclear factor κB

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PAMP	Pathogen-associated molecular pattern
PBC	Primary biliary cirrhosis
PI3K	Phosphatidylinositol 3-kinase
Pin	Peptidyl-prolyl isomerase
PRR	Pattern recognition receptor
PSC	Primary sclerosing cholangitis
RIP	Receptor-interacting serine–threonine kinase
ROS	Reactive oxygen species
RP105	Radioprotective 105
SARM	Sterile alpha- and armadillo-motif-containing protein
SHP	Src homology 2 domain-containing protein tyrosine phosphatase
SIGIRR	Single immunoglobulin IL-1R-related molecule
SNP	Single nucleotide polymorphism
SOCS1	Suppressor of cytokine signaling-1
ST2L	Transmembrane form of ST2
sTLR4	Soluble decoy TLR4
TAK	Transforming growth factor-β-activated kinase
TANK	TRAF family member associated NF-κB activator
TBK	TANK binding kinase
TGF	Transforming growth factor
TIRAP	Toll/IL-1 receptor domain-containing adaptor protein
TNF	Tumor necrosis factor
TIR	Toll/interleukin 1 receptor
Tollip	Toll interacting protein
TLR	Toll-like receptor
TRAF	Tumor necrosis receptor-associated factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIAD3A	Triad domain-containing protein 3 variant A
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing interferon-β
VCAM	Vascular cell adhesion molecule

Introduction

The innate immune system recognizes several components of microbes and initiates protective immunological responses. This microbiological recognition is a specific and highly coordinated process involving pattern recognition receptors (PRRs) that identify preserved structures of different pathogens, the so-called pathogen-associated molecular patterns (PAMPs) [1, 2]. Toll-like receptors (TLRs) are the most important family of PRRs, with ten different TLRs being ubiquitously expressed in humans [1, 2]. TLR4 acts as a receptor for lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria, promptly inducing the production of several pro-inflammatory, antiviral, and anti-bacterial cytokines [1, 2].

The TLR4 is expressed in several liver cells, and the liver, due to its anatomic location, is constantly confronted with gut-derived LPS [3]. Despite the constant confrontation of TLR4-expressing liver cells with gut-derived LPS, the normal liver does not show signs of inflammation, which on one hand can be explained by the relatively low expression of TLR4 and its adaptor molecules in the liver [3]. On the other hand, under normal circumstances, the liver negatively regulates TLR4 signaling at different levels, contributing to a process known as "liver tolerance" [3]. A breakdown of liver tolerance, by increased exposure of TLR4 to LPS and/or increased expression or sensitivity of TLR4, may induce an inappropriate immune response which can contribute to chronic inflammatory liver diseases [3]. Recent studies provide evidence for a role of LPS/TLR4 signaling in the pathogenesis of alcoholic liver disease, nonalcoholic fatty liver disease, chronic hepatitis C, chronic hepatitis B, primary sclerosing cholangitis, primary biliary cirrhosis, hepatic fibrosis, and hepatocarcinoma [3].

Herein we first review TLR4 structure, ligands, and signaling pathways. Later, we review liver expression of TLR4 and discuss the role of LPS/TLR4 signaling in the pathogenesis of chronic inflammatory liver diseases.

TLR family

The TLR, originally identified as homologs of *Drosophila* Toll, belong to the superfamily of interleukin-1 receptors [4]. The human TLR family currently consists of ten members, which are structurally characterized by the presence of a distinct leucine-rich repeat extracellular domain that confers specificity to the receptor, and a conserved toll/interleukin 1 (IL1) receptor (TIR) intracellular domain [5].

The existence of several TLRs enables the innate immunity system to recognize different groups of pathogens while initiating appropriate and distinct immunological responses, according to the PAMP recognized [3] (Fig. 1). TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, and TLR3, TLR7, TLR8, and TLR9 are expressed on the endosome–lysosome membrane. TLR1 and TLR6 form heterodimers with TLR2 in order to sense tri-acyl (mycobacterium) and di-acyl lipopeptides (mycoplasma), respectively. TLR4 and TLR5 are

the receptors for the Gram-negative bacterial cell wall components, lipopolysaccharide (LPS), and bacterial flagellin, respectively. Intracellular TLRs, TLR3, TLR7/8, and TLR9 detect viral-derived and synthetic double-stranded RNA, viral-related single-stranded RNA, and bacterial unmethylated CpG-DNA, respectively. The ligands for TLR10, TLR12, and TLR13 remain unidentified. TLR8 does not signal in mice. TLR10 is expressed in humans, but not in mice. TLR11, TLR12, and TLR13 are expressed in mice, but not in humans.

TLR4 ligands

The TLR4 is expressed on the cell surface and is the receptor for the Gram-negative bacteria cell-wall component, LPS [4]. LPS is composed of hydrophilic polysaccharides of the core and O-antigen and a hydrophobic lipid A component, which corresponds to the conserved molecular pattern of LPS and is the main inducer of biological responses to LPS [4]. Stimulation of TLR4 by LPS is a complex process (Fig. 1), which includes the participation of several molecules [LPS binding protein (LBP), CD14 and MD-2] [6, 7]. LBP (a soluble protein) extracts LPS from the bacterial membrane and shuttles it to CD14 (a glycosylphosphatidylinositol-anchored protein, which also exists in a soluble form). CD14 then transfers the LPS to MD-2 (a soluble protein that non-covalently associates with the extracellular domain of TLR4). Binding of LPS to MD-2 induces a conformational change in MD-2 which then allows the complex MD-2-TLR4 to bind to a second TLR4 receptor, thus achieving TLR4 homo-dimerization and signaling.

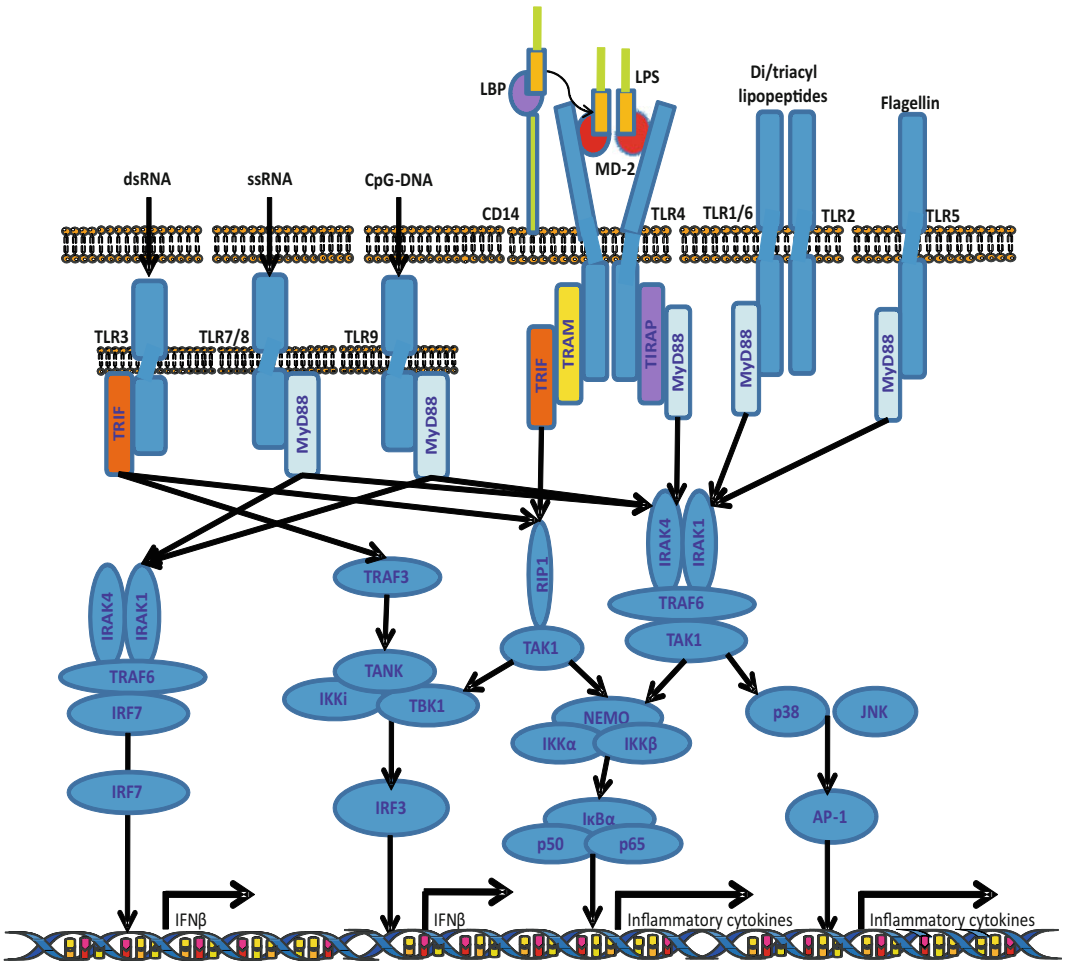

Fig. 1 Overview of signaling of LPS/TLR4 and other TLRs. LPS recognition is facilitated by LBP and CD14 and is mediated by TLR4/MD-2 receptor complex. TLR4 signaling cascade can be separated into MyD88-dependent and MyD88 independent pathways which mediate the activation of proinflammatory cytokines and IFN-β. These two pathways also mediate the intracellular signaling of other TLRs, enabling interaction between TLR4 and other TLRs at different levels. See text for abbreviations

Besides LPS, TLR4 also senses endogenous ligands initiating danger signals, such as high-mobility group box-1, hyaluronan, heat shock protein 60, and free fatty acids (C12:0, C14:0, C16:0, and C18:0) [8–10]. Recent reports demonstrated that necrotic cells stimulate TLR4 associated with MyD88 under sterile conditions, thereby pre-emptively inducing an inflammatory response in the absence of microbial challenge [11, 12]. Due to the association of many endogenous ligands with tissue injury, they are termed damage-associated molecular patterns (DAMPs). Interestingly, recent studies show that many of the proposed endogenous TLR4 ligands may also have the capacity to bind and transport LPS and/or enhance the sensitivity of cells to LPS, suggesting that many of these molecules may be more accurately described as PAMP-binding molecules or PAMP-sensitizing molecules, rather than genuine ligands of TLR4 [13].

TLR4 signaling

Binding of ligands to the extracellular domains of TLRs causes a rearrangement of the receptor complex and triggers the recruitment of specific adaptor proteins to the intracellular domain, thus initiating a signaling cascade [6, 7].

TLR4 signals through adaptor molecules such as MyD88, toll/IL-1 receptor domain-containing adaptor protein (TIRAP), toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) to activate transcription factors such as nuclear factor (NF)-κB, activator protein 1 (AP-1), and interferon regulatory factors (IRFs). These transcription factors then initiate the transcription of a specific set of genes involved in proinflammatory, anti-viral, and anti-bacterial responses and genes that control cell survival and apoptosis. TLR4 signaling has been divided into MyD88-dependent (mediated by MyD88) and MyD88-independent (mediated by TRIF) pathways (Fig. 1) [5]. These two pathways also mediate the intracellular signaling of other TLRs, enabling the interaction between TLR4 and other TLRs at different levels from adaptor molecules to transcription factors (Fig. 1). MyD88 is an essential part of the signaling cascade of all TLRs except for TLR3. In contrast, TRIF only interacts with TLR3 and TLR4.

In the MyD88-dependent pathway, TLR4, through TIRAP, recruits MyD88 to activate IL-1R-associated kinase (IRAK)-4 and IRAK-1, which then associate with tumor necrosis receptor-associated factor (TRAF)-6 and transforming growth factor-β-activated kinase 1 (TAK-1). These activate the complex inhibitor of NF-κB kinase (IKK), formed by NEMO, IKKα e IKKβ, which phosphorylates and degrades IκBα (inhibitor of NF-κB), allowing nuclear translocation of NF-κB (normally sequestered in the cytoplasm by ligation to IκBα). NF-κB leads to expression of effectors genes (TNF-α, IL-6, and IL-12). The MyD88-dependent pathway can also activate p38 and c-Jun N-terminal kinase (JNK), leading to AP-1 activation followed by transcription of genes involved in regulation of cell proliferation, morphogenesis, apoptosis, and differentiation.

In the MyD88-independent pathway, TLR4, through TRAM, recruits TRIF. This recruits TRAF3 which associates with TRAF family member associated NF-κB activator (TANK), TBK1 (TANK binding kinase 1) and IKKi with subsequent phosphorylation and nuclear translocation of IRF-3. IRF-3 leads to IFN-β transcription. In MyD88-independent pathway, TRIF also associates with the receptor-interacting serine–threonine kinase (RIP)-1 to activate NF-κB. NF-kB induction in the MyD88-dependent pathway occurs with fast kinetics, whereas NF-kB activation in the MyD88-independent pathway occurs with slower kinetics.

The significance of the two different downstream pathways and the role of distinct adapter molecules of TLR4 activation in liver diseases are largely unknown. Nonetheless, many studies suggest that the activation of the different downstream pathways may be cell- and effect-specific. This may have important implications for developing TLR4 modulators as potential therapeutic agents.

Negative regulation of TLR4 signaling

Because TLR4 stimulation can induce potent inflammatory responses, inhibitory pathways are necessary to protect the host from inflammation-induced damage [14]. The balance is maintained by multiple negative regulators, and the regulation is very precise. TLR4 signaling can be regulated at multiple levels (from receptor level to transcription factors level; Table 1), through many kinds of mechanisms (degradation, deubiquitination, and competition are the most frequently observed). Table 1 describes the targets of each inhibitor. **sTLR4** (soluble decoy TLR4), **RP105** (radioprotective 105; a homolog of TLR4), **SIGIRR** (single immunoglobulin IL-1R-related molecule), **ST2L** (transmembrane form of ST2; homolog of the IL-1 receptor), **MyD88s** (splice variant of MyD88), **SARM** (sterile alpha- and armadillo-motif-containing protein), **TRAF1**, **TRAF4**, and **IRAK-2c** (splice variant of IRAK-2) inhibit TLR4 signaling by means of competing with various adaptors and transcription factors for binding sites. **TRIAD3A** (triad domain-containing protein 3 variant A), **SOCS1** (suppressor of cytokine signaling-1), and **Pin1** (peptidyl-prolyl isomerase1) inhibit several molecules of TLR4 signaling by means of polyubiquitination and subsequent proteasome-dependent degradation. **A20**, **DUBA**

Table 1 Negative regulation of TLR4 signaling

Level	Inhibitor
TLR4	sTLR4, RP105, SIGIRR, and TRIAD3A
Adaptors molecules	
Myd88	MyD88s
TRIF	TRIAD3A, SARM, TRAF1, and TRAF4
TIRAP	ST2L, TRIAD3A, and SOCS-1
Myd88-dependent pathway	
IRAK	IRAK-M, IRAK-2c, Tollip, SHP-1, and miR-146
TRAF6	TRAF4, A20, CYLD, and miR-146
NF-KB	TRAIL, Bcl3, ATF3, and PI3K
Myd88-independent pathway	
RIP1	A20 and TRIAD3A
TRAF3	DUBA
TBK1	SHP-2
IRF3	PIN-1

Abbreviations – see text

(de-ubiquitinating enzyme A), and **CYLD** (cylindromatosis protein) inhibit several mediators of TLR4 signaling by deubiquitination.

There are many other negative regulators that use different mechanisms to control TLRs signaling pathways. **Bcl-3** (B cell leukemia-3) and **TRAIL** (tumor necrosis factor-related apoptosis-inducing ligand) inhibit activation of NF-κB by stabilization of NF-κBp50 and IκBα, respectively. **IRAK-M** (a member of IRAK family without kinase activity) inhibits MyD88-mediated signaling by preventing the dissociation of IRAKs from MyD88. **ATF3** (activating transcription factor-3) binds to the promoters and recruits histone deacetylase, resulting in altered chromatin structure to limit access to transcription factors (such as NF-κB). Both the Src homology 2 domain-containing protein tyrosine phosphatase (**SHP**)-**1** and **SHP-2** are intracellular tyrosine phosphatases, which inhibit IRAK-1 and TBK1, respectively. **Tollip** constitutively suppresses IRAK by forming a complex that is dissociated after TLR4 activation. MicroRNAs are 21–22-nucleotide, non-coding small RNAs that have been shown to be centrally involved in immune system development and function. Very recently, it was shown that **miR-146** expression was increased by LPS stimulation [15], and miR-146 may inhibit IRAK-1 and TRAF6. **PI3K** (phosphatidylinositol 3-kinase) is a member of the lipid kinase family. Recognition of PAMP by TLRs can activate PI3K, which leads to activation Akt (serine/threonine protein kinase) and subsequent inactivation of glycogen synthase kinase-3β (GSK-3β). Inhibition of GSK-3β decreases NF-kB-dependent production of proinflammatory cytokines.

The expression of most negative regulators (including PI3K, A20, IRAK-M, and miR-146) can be induced by the activation of TLR4 and uses a mode of negative feedback to terminate TLR4 activation. However, there are also some constitutively expressed factors (including Tollip) that could possibly exert their functions only when TLRs are overactivated.

TLR4 expression in the liver

The healthy liver contains low mRNA levels of TLR4 and signaling molecules such as MD-2 and MyD88 in comparison to other organs [16, 17], suggesting that the low expression of TLR4 and signaling molecules may contribute to the high tolerance of the liver to LPS from the intestinal microbiota to which the liver is constantly exposed.

Because of the unique anatomical link between the liver and intestines, Kupffer cells (KC) are the first cell to encounter gut-derived toxins including LPS. Accordingly, Kupffer cells express TLR4 and are responsive to LPS [18]. Upon triggering, TLR4 signaling drives Kupffer cells to produce TNF-α, IL-1β, IL-6, IL-12, IL-18, and anti-inflammatory cytokine IL-10 [19].

Hepatocytes may uptake and eliminate LPS from portal and systemic circulation [20]. Hepatocytes express mRNA for TLR4 and respond to TLR4 ligands although there are contradictory data about the amount of TLR4 mRNA expression and the level of responsiveness to LPS [21, 22].

Activated human hepatic stellate cells (HSCs) express TLR4 and CD14 and respond to LPS [23]. TLR4 directly stimulates HSC to induce proinflammatory features, such as upregulation of chemokines (CCL2, CCL3, and CCL4) and adhesion molecules [vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), and E-selectin] and profibrogenic features including the enhancement of TGF-β signaling by the downregulation of TGF-β pseudoreceptor, bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) [21, 23].

Other liver cells, such as biliary epithelial cells, sinusoidal endothelial cells, and hepatic dendritic cells, express TLR4 and are responsive to LPS, but this expression and response have not been studied in detail [20].

The role of LPS/TLR4 signaling in CLD

There is increasing evidence for a role of LPS/TLR4 signaling in the pathogenesis of alcoholic liver disease, nonalcoholic fatty liver disease, chronic hepatitis C, chronic hepatitis B, primary sclerosing cholangitis, primary biliary cirrhosis, hepatic fibrosis, and hepatocarcinoma (Table 2). The evidence for a role of LPS and TLR4 in these diseases comes from two kinds of studies:

Table 2 Evidence for the role of LPS/TLR4 signaling in chronic hepatic diseases

Disease	LPS expression	TLR4 expression	TLR4 sensitivity	Effects of modulation of TLR4	Cellular and intracellular pathways
Alcoholic liver disease	↑ [24–26]	↑ [29]	↑ [29, 30]	Suppression of TLR4 protects against ALD [30–32]	KC, Myd88-independent, TNF-α and IL-6 and ROS [34, 35]
Non-alcoholic fatty liver disease	↑ [37–41]	↑ [42]	↑ [42]	Suppression of TLR4 protects against NAFLD [39–41, 43–45]	KC, Myd88-dependent, TNF-α and IL-6 and ROS [39, 45]
HCV infection	↑ [49]	↑ (in B cells and hepatocytes) [22, 50] ↑ (in DC and macrophages) [51, 52]	↑ (in B cells and hepatocytes) [22, 50] ↓ (DC and macrophages) [51, 52]	Activation of TLR4 suppresses HCV replication [53]	KC and LSEC, Myd88-independent, IFN-β [53]
HBV infection	↑ [56]	↓ [57]	↓ [57]	Activation of TLR4 suppresses HBV replication [58]	KC, Myd88-independent, IFN-β [59]
Primary biliary cirrhosis	↑ (in BEC) [60, 61]	↑ (in BEC) [62]	↑ [63]	nd	BEC, TNF-α, IL-1 [63]
Primary sclerosing cholangitis	↑ (in BEC) [60]	↑ (in BEC) [64]	↑ [64]	nd	BEC, TNF-α, IL-1 [64]
Hepatic fibrosis	↑ [21, 67]	BEC ↑ [62, 64] Hepatocytes ↑/= [62, 69] PBMC ↓/= [70, 71]	BEC ↑ [62, 64] Hepatocytes ↑/= [62, 69] PBMC ↓/= [70, 71]	Suppression of TLR4 protects against hepatic fibrosis [21, 55, 72–76]	HSC, Myd88-dependent, Chemokines, adhesion molecules, BAMBI [21]
Hepatocarcinoma	↑ [22]	↑ [22]	↑ [22]	Suppression of TLR4 protects against hepatocarcinoma [12, 22]	KC and hepatocytes, Myd88-dependent, Nanog; IL-6 [12, 22]

↑ increased, ↓ decreased, = maintained, *LPS* lipopolysaccharide, *BEC* biliary epithelial cells, *DC* dendritic cells, *KC* Kupffer cells, *LSEC* liver sinusoidal endothelial cells, *PBMC* peripheral blood mononuclear cells, *ROS* reactive oxygen species, *nd* not determined

1. Studies showing that LPS/TLR4 signaling is altered as a result of altered portal LPS levels and/or hepatic TLR4 expression in these diseases.
2. Studies showing that modulation of LPS/TLR4 signaling (by suppressing/attenuating LPS production or TLR4 gene expression) influences the pathogenesis of these diseases.

Alcoholic liver disease

Alcoholic liver disease (ALD) is characterized by a spectrum of liver pathology ranging from fatty liver, steatohepatitis, to cirrhosis.

The LPS and TLR4 have been proposed as key players in the pathogenesis of ALD. Chronic ingestion of alcohol leads to a strong elevation of portal and systemic levels of LPS in animal models and humans [24–26]. The elevation of LPS appears to be predominantly caused by two mechanisms. First, alcohol exposure can promote the growth of Gram-negative bacteria in the intestine, which leads to enhanced production of LPS [27]. In addition, alcohol metabolism by Gram-negative bacteria and intestinal epithelial cells can result in accumulation of acetaldehyde, which in turn can increase intestinal permeability by opening intestinal tight junctions. Increased intestinal permeability can lead to increased transfer of LPS from the intestine to portal and systemic circulation [28]. Furthermore, chronic alcohol consumption upregulates hepatic TLR4 and sensitizes it to LPS to enhance TNF-α production [29]. Exposure to LPS during chronic alcohol consumption results in increased production of inflammatory mediators as well as in induction of reactive oxygen species (ROS) [30]. Finally, inhibition of LPS/TLR4 signaling by altering intestinal microbiota and LPS production (antibiotics or probiotics) or suppressing TLR4 gene expression protects against ALD. Indeed, treatment with lactobacillus or antibiotics suppresses alcohol-induced liver injury by reducing LPS circulating levels [31, 32]. TLR4-mutant mice have a strong reduction of alcohol-induced liver injury despite elevated LPS circulating levels [33].

Recent studies have clarified the cellular and molecular pathways by which LPS/TLR4 signaling promotes ALD. Kupffer cells have been established as a crucial cellular target of LPS in alcohol-induced liver injury as demonstrated by a strong reduction of alcoholic liver injury following depletion of Kupffer cells with gadolinium chloride [34]. Moreover, Hritz et al. [35] demonstrated that TLR4-mediated signal in ALD is mediated through a MyD88-independent pathway, most likely through the adapter molecule TRIF. Hepatic alcohol-induced production of inflammatory mediators (TNF-α and IL-6) and TLR4 co-receptors (CD14 and MD2) was prevented by TLR4 deficiency [35]. In addition, ROS production by cytochrome P450 and the nicotinamide adenine dinucleotide phosphate complexes was also prevented by TLR4 deficiency [35]. These data suggest that TLR4-mediated alcoholic liver injury is carried out by increased inflammatory mediators (TNF-α and IL-6) and ROS production. Taken together, these data suggest that activation of TLR4 in Kupffer cells by LPS is a key pathogenetic mediator of ALD through production of inflammatory cytokines and ROS.

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) includes a continuum of disease ranging from steatosis to steatohepatitis and cirrhosis and usually develops in the setting of obesity and insulin resistance [36]. Mechanisms involved in the development of NAFLD are not yet fully clarified, and therapeutic options are still limited.

There is accumulating evidence that LPS/TLR4 signaling plays an essential role in the pathogenesis of NAFLD. In different human and animal studies, NAFLD was associated with increased portal LPS levels, through mechanisms involving bacterial overgrowth, and increased intestinal permeability and bacterial translocation [37–41]. Wigg et al. [37] found that bacterial overgrowth was prevalent among 22 patients with NAFLD. Bergheim et al. [38] showed that even the early stages of fructose-induced NAFLD are associated with an increased intestinal translocation of bacterial LPS. NAFLD has also been associated with increased sensitivity to LPS, mainly by increased hepatic TLR4 expression. Leptin or leptin receptor-deficient animals that are genetically obese are highly susceptible to LPS and develop NAFLD after low dose of LPS [42]. Finally, suppression of LPS/TLR4 signaling by alteration of intestinal microbiota (antibiotics or probiotics) or genetic manipulation protects against NAFLD. Selective intestinal decontamination results in decreased LPS levels in mice in a high-fat diet and reduced hepatic triglycerides in mice with diet-induced obesity as well as in leptin-deficient mice [40, 41]. Probiotics diminish non-alcoholic steatohepatitis in leptin-deficient mice [43, 44]. The crucial role for TLR4 signaling in NAFLD was further confirmed in TLR4-mutant mice that display decreased liver injury and lipid accumulation following a methionine- and choline-deficient diet (MCDD) and fructose-induced NAFLD [39, 45].

Besides the role of LPS/TLR4 signaling in the pathogenesis of NAFLD, there is also accumulating evidence showing a bidirectional connection between TLR4 signaling and insulin resistance (to which NALFD is intimately associated). There are several studies showing that LPS/TLR4 activation induces inflammatory signaling pathways

which mediate insulin resistance and studies showing that insulin resistance may lead to activation of LPS/TLR4 signaling. Cani et al. [46] demonstrated that subcutaneous infusion of a low dose of LPS resulted in liver insulin resistance in a CD-14-dependent manner. On the other hand, it was shown that free fatty acids, which are often elevated in insulin resistance states, due to increased release from adipose tissue, can induce insulin resistance through activation of TLR4 [10]. Notably, a recent human study demonstrated that TLR4 expression and its ligands (high-mobility group box-1, hyaluronan, heat shock protein 60, and LPS), signaling, and functional activation are increased in recently diagnosed type-2 diabetes and contribute to a proinflammatory state [47].

Recent studies have clarified the mechanisms by which increased LPS/TLR4 signaling promotes NAFLD. Destruction of Kupffer cells with clodronate liposomes blunted histological evidence of non-alcoholic steatohepatitis in a model of MCDD and prevented increasing of TLR4 expression, underscoring a direct link between TLR4 and Kupffer cells within pathogenesis of NAFLD [39]. Hepatic lipid peroxidation, Myd88, and TNF-α levels were significantly decreased in fructose-fed TLR4 mutant mice in comparison to fructose-fed wild-type mice, suggesting that MyD88 may be critical in mediating the effects of TLR4 activation in the promotion of NAFLD, through enhanced ROS and induction of TNF-α [45]. Taken together, these data suggest a major role of TLR4 signaling in the pathogenesis of NAFLD through activation of Kupffer cells and enhanced ROS and TNF-α production.

HCV infection

About 30% of patients chronically infected with hepatitis C virus (HCV) show signs of active hepatic inflammation and are at risk of developing fibrosis, cirrhosis, and HCC [48].

There is an accumulating evidence that LPS and TLR4 play a key role in the pathogenesis of HCV infection. Patients with chronic HCV infection display increased serum levels of LPS even in the absence of significant hepatic fibrosis [49].

Interaction of HCV and TLR4 expression and signaling is robust although complex and may be cell-specific. Machida et al. [50] found that HCV, through the action of its NS5A protein, induces expression of TLR4 on the surface of B cells, leading to enhanced IFN-β and IL-6 production and secretion, particularly in response to LPS. Machida et al. [22] also provided evidence that hepatocyte-specific transgenic expression of the HCV nonstructural protein NS5A upregulates TLR4 expression and signaling. They demonstrated enhanced TAK-1–TRAF-6 and TAK-1–IRAK-1 interactions and phosphorylation of JNK and Iκ-Bα (downstream mediators of TLR4 signaling) in NS5A mice given LPS. Miyazaki et al. [51] found that myeloid dendritic cells from patients with chronic HCV display an increased expression of TLR4, but a decrease in the cytokine production secondary to activation of TLR4 by LPS, thus suggesting the impairment of TLR4 signaling by HCV in myeloid dendritic cells. Abe et al. [52] demonstrated that murine macrophages overexpressing NS3, NS3/4A, NS4B, or NS5A showed a strong suppression of TLR4 signaling. NS5A interacts with MyD88 to prevent IRAK-1 recruitment and cytokine production, such as IL-1, IL-6, and IFN-β response to the ligands for TLR4 [53].

TLR4 signaling itself may regulate HCV replication. Broering et al. [53] found that supernatants from TLR4-stimulated non-parenchymal liver cells (Kupffer cells and sinusoidal epithelial cells) led to potent suppression of HCV replication in murine HCV replicon bearing MH1 cells through IFN-β and induction of IFN-stimulated genes. These novel findings are of particular relevance for the control of HCV replication by the innate immune system of the liver.

Finally, TLR4 has also been associated with many clinical consequences of HCV infection. Machida et al. [22] demonstrated that, in a murine model, synergism between alcohol and HCV in liver damage and tumor formation is mediated by sustained activation of LPS/TLR4 signaling, which results from HCV NS5A-induced hepatic TLR4 expression and alcohol-induced endotoxemia. Recently, in a gene centric functional genome scan in patients with chronic hepatitis C virus, a major CC allele of TLR4 encoding a threonine at amino acid 399 (p.T399I) emerged as the second single nucleotide polymorphism (SNP) with highest ability to predict the risk of developing cirrhosis, indicating a protective role in fibrosis progression of its c.1196C_T (rs4986791) variant at this location (p.T399I), along with another highly cosegregated c.896A_G (rs4986790) SNP located at coding position 299 (p.D299G) [54]. Interestingly, later on, it was shown that these two SNP are associated with reduced TLR4-mediated inflammatory and fibrogenic signaling and lower apoptotic threshold of activated HSCs [55].

Taken together, these data support the hypothesis that HCV selectively influences TLR4 signaling, impairing it in cells that limit HCV replication (dendritic cells and macrophages), while at the same time enhancing it in cells (hepatocytes and B cells) that generate a chronic inflammatory state. Thus, it is likely that the interaction between HCV and TLR4 promotes virus expansion, inflammation, and potentially the progression to fibrosis and cirrhosis.

HBV infection

Hepatitis B virus (HBV) causes a chronic infection in about 10% of adults that may result in cirrhosis and HCC [45].

Recent studies have shown that LPS/TLR4 signaling may have an important role in the pathogenesis of HBV infection. One study reported a 72-fold induction of LPS levels in chronic HBV infection [56]. Moreover, a significant correlation was revealed between systemic LPS levels with virus replication and the degree of basic clinical and laboratory signs in patients with chronic viral hepatitis B [56].

Interaction of HBV and TLR4 expression and signaling is complex. One study demonstrated that TLR4 was downregulated in HBV-infected peripheral blood monocytes, and these cells also had a decreased cytokine response to TLR4 ligands [57]. On the other hand, TLR4 was shown to block HBV replication through its ability to upregulate IFNs. The injection of LPS into HBV transgenic mice reduced HBV replication in an IFN-α/β-dependent manner [58]. These antiviral effects of TLR4 activation are directed at nonparenchymal cells, but not hepatocytes that express low level of TLR4. Further experiments demonstrated that nonparenchymal cell-derived mediators inhibit HBV replication in HBV-Met cells. The supernatants from TLR4-stimulated Kupffer cells inhibit HBV replication independently of MyD88 in vitro, suggesting that TRIF-dependent IFN-β plays a role [59].

Taken together, these data suggest that TLR4 signaling is impaired in HBV infection and that TLR4 agonists can block HBV replication through activation of TRIF-dependent pathway in Kupffer cells.

Hepatic autoimmune disorders

The pathogenesis of hepatic autoimmune disorders remains still largely unknown. It is believed that autoimmunity may develop from genetic predispositions, but the onset of autoimmune tissue injury or disease flare is often triggered by microbial infection. Aberrant innate immune response to infections, providing the necessary inflammatory milieu to activate pre-existing autoreactive cellular repertoire, has the potential to initiate the development of autoimmunity. There is increasing evidence for LPS/TLR4 signaling in the pathogenesis of primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).

Primary biliary cirrhosis (PBC) is a chronic inflammatory cholestatic disease of unknown origin that affects small and medium intrahepatic bile ducts. Recent studies have demonstrated that significant amounts of LPS accumulate in biliary epithelia of PBC patients [60]. Ballot et al. [61] reported that 64% of PBC sera were positive for IgM antibodies against lipid A, an immunogenic and toxic component of LPS. TLR4 expression is significantly elevated in biliary epithelial cells and periportal hepatocytes of PBC patients [62]. Monocytes from PBC patients appear more sensitive to the ligand for TLR4 (LPS), producing higher levels of proinflammatory cytokines, particularly IL-1β, IL-6, IL-8, and TNF-α [63].

The PSC is characterized by the destruction of hepatic bile duct and a high frequency of antibiliary epithelial cell antibodies (anti-BEC-Ab). One study revealed that, in primary sclerosing cholangitis, LPS gets accumulated abnormally in biliary epithelial cells [60]. Anti-BEC-Ab-stimulated BECs or PSC patient-derived BECs express higher levels of TLR4 and respond to ligands for TLR4 to produce higher levels of inflammatory cytokines (IL-1β, IL-8, IFN-γ, TNF-α, granulocyte–macrophage colony-stimulating factor, and TGF-β) [64].

These data suggest that in CBP and PSC increased accumulation of LPS and TLR4 expression in biliary epithelial cells enhances secretion of selective pro-inflammatory cytokines integral to the inflammatory response that may be critical in the breakdown of self-tolerance and initiation and perpetuation of bile duct injury.

Hepatic fibrosis

The development of hepatic fibrosis and cirrhosis occurs in virtually any type of chronic hepatic injury [65]. In terms of chronic liver injury, several studies have highlighted the role of transforming growth factor-β (TGF-β) in activating hepatic stellate cells (HSC), the main producers of extracellular matrix in the fibrotic liver and the promotion of a fibrogenic phenotype [65, 66]. On the other hand, chronic liver inflammation is a key prerequisite for triggering liver fibrosis [65, 66]. However, until now, the cell-type specific molecular mechanisms linking pathways driving inflammation on one hand and liver fibrogenesis on the other hand have not been defined yet. Recently, there is accumulating evidence that TLR4-induced activation and sensibilization of HSC may constitute the molecular link between hepatic inflammation and fibrogenesis.

The LPS is elevated in experimental models of hepatic fibrosis and in patients with cirrhosis [21, 67]. It is believed that changes in intestinal motility, subsequent alterations of the intestinal microbiota, decreased mucosal integrity, and suppressed immunity in hepatic fibrosis contribute to a failure of the intestinal mucosal barrier, and causes increases in bacterial translocation and LPS levels in later stages of hepatic fibrosis and cirrhosis [68].

Data regarding expression of TLR4 in cirrhotic patients are conflicting, with studies showing increased expression on BEC, maintained or increased expression on hepatocytes, and maintained or decreased expression on PBMC [62–64, 69–71].

Several studies have demonstrated that modulation of the intestinal microbiota in advanced cirrhosis by probiotics or antibiotics is beneficial for the prevention of bacterial translocation and spontaneous bacterial peritonitis

[72, 73]. It has been shown that antibiotics prevent hepatic injury and fibrosis induced by CCl4 treatment or a choline-deficient diet, and that LPS enhances hepatic fibrosis induced by a MCCD [74, 75]. Treatment of mice with nonabsorbable broad-spectrum antibiotics also resulted in a clear reduction in the fibrotic response of mice, upon bile duct ligation [21]. Recently, Velayudham et al. showed that VSL#3 (a probiotic) protects against MCDD-induced liver fibrosis, through modulation of collagen expression and inhibition of TGF-β expression and signaling [76].

Recent studies, using TLR4 mutant as well as gut-sterilized, CD14- and LBP-deficient mice, have demonstrated the crucial role for the LPS–TLR4 pathway in hepatic fibrogenesis [21, 77]. TLR4-mutant mice display a profound reduction in hepatic fibrogenesis in three different experimental models of biliary and toxic fibrosis [77].

In a recent study, Seki et al. [21] analyzed the cell-specific molecular mechanism underlying the role of LPS/TLR4 on liver fibrosis. They showed that chimeric mice that contain TLR4-mutant Kupffer cells and TLR4-intact HSCs developed significant fibrosis and the mice that contain TLR4-intact Kupffer cells and TLR4-mutant HSCs developed minimal fibrosis after bile duct ligation, indicating that TLR4 on HSCs, but not on Kupffer cells, is crucial for hepatic fibrosis. Notably, Kupffer cells are essential for fibrosis by producing TGF-β independent of TLR4. TLR4-activated HSCs produce chemokines (CCL2, CCL3, and CCL4) and express adhesion molecules (ICAM-1 and VCAM-1) that recruit Kupffer cells to the site of injury. Simultaneously, TLR4 signaling downregulates the TGF-β decoy receptor (BAMBI) to boost TGF-β signaling and allow for unrestricted activation of HSCs by Kupffer cells, leading to hepatic fibrosis. Finally, by using adenoviral vectors expressing an inhibitor of NF-κB kinase (IκB)-superrepressor and knockout mice for MyD88 and the adapter molecule TRIF, the authors demonstrated that TLR4-dependent downregulation of BAMBI is mediated via a pathway involving MyD88 and NF-κB, but not TRIF. In summary, they demonstrated that LPS/TLR4 signaling acts in a profibrogenic manner via two independent mechanisms: it induces the secretion of chemokines from HSCs and chemotaxis of Kupffer cells which secrete the profibrogenic cytokine TGF-β; additionally, TLR4-dependent signals augment TGF-β signaling on HSCs via downregulation of the TGF-β pseudoreceptor BAMBI.

Recently, Huang et al. [54] conducted a gene centric functional genome scan in patients with chronic hepatitis C virus, which yielded a Cirrhosis Risk Score signature consisting of seven single nucleotide polymorphisms (SNPs) that may predict the risk of developing cirrhosis. Among these, a major CC allele of TLR4 encoding a threonine at amino acid 399 (p.T399I) was the second most predictive SNP among the seven, indicating a protective role in fibrosis progression of its c.1196C>T (rs4986791) variant at this location (p.T399I), along with another highly cosegregated c.896A>G (rs4986790) SNP located at coding position 299 (p.D299G). In a subsequent study, the same group examined the functional linkage of these SNPs to hepatic stellate cell (HSC) responses [55]. They showed both HSCs from TLR4-deficient mice, and a human HSC line (LX-2) reconstituted with either TLR4 D299G and/or T399I complementary DNAs were hyporesponsive to LPS stimulation compared to those expressing wild-type TLR4 as assessed by the expression and secretion of LPS-induced inflammatory and chemotactic cytokines (i.e., monocyte chemoattractant protein-1, IL-6), downregulation of BAMBI expression, and activation of NF-κB-responsive luciferase reporter. In addition, spontaneous apoptosis, as well as apoptosis induced by pathway inhibitors of NF-κB, extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase were greatly increased in HSCs from either TLR4-deficient or Myd88-deficient mice, as well as in murine HSCs expressing D299G and/or T399I SNPs [55]. Recently, Li et al. expanded the list of TLR4 SNPs that are independently associated with the risk of liver fibrosis progression and the development of cirrhosis [78].

Taken together, these data suggest that LPS/TLR4 signaling in HSC is essential for liver fibrosis development, by stimulating production chemokines that recruit Kupffer cells and at the same time allowing for unrestricted activation of HSCs by Kupffer cells-derived TGF-β.

Hepatocarcinoma

During recent years, evidence has been accumulating to show that inflammation has an important role in initiation, promotion, and progression of tumors [79, 80]. The generation of pro-inflammatory cytokines in the tumor microenvironment provokes activation of NF-κB in cancer cells, leading to protection against pro-apoptotic host immune defense mechanisms [79, 80]. It has been shown that cytokines and growth factors produced by tumor-infiltrating macrophages, lymphocytes, and other cell types in the inflammatory tumor microenvironment influence cell differentiation and exert antiapoptotic and proangiogenic effects which stimulate the growth of cancer cells, tumor invasiveness, and metastasis [79, 80].

Hepatocarcinoma (HCC), a prominent example for inflammation-associated cancer, is a major complication in the end-stage of cirrhosis [81]. In most cases, HCC in humans is the outcome of continuous injury and chronic inflammation; thus, it provides a good and realistic inflammatory-related cancer model to gain insight about the role of TLR4 in the carcinogenesis [81]. Two studies have revealed TLRs, in particular TLR4, as major factors linking hepatic chronic inflammation and hepatocarcinoma.

Diethylnitrosamine (DEN) is a chemical carcinogen used to create a mouse model of HCC [82]. The pathogenesis of HCC in this mouse model differs from that in humans and thus may not be directly comparable to human HCC. Nevertheless, the mouse model of DEN-induced HCC has a histology and genetic signature similar to that of human HCCs with poor prognosis and recapitulates a dependence on inflammation and gender disparity seen in human HCC [83]. Naugler et al. [12] showed in a model of DEN-induced HCC in mice that the tumor appears in 100% of males but only in 13% females. This is correlated with increased liver injury and a higher production of IL-6 in males after toxicant administration. They also showed that IL-6 production after DEN-induced liver injury occurs through TLR4 stimulation and demonstrated the implication of the innate immune response in the hepatocarcinogenic process. They observed that the accumulation of IL-6 mRNA in Kupffer cells incubated with LPS or necrotic hepatocytes was markedly reduced in MyD88 null mice. They also found that liver damage and hepatic IL-6 levels were significantly diminished after DEN administration in MyD88-deficient mice. Importantly, these mice also showed a significant reduction in the number and size of DEN-induced liver tumors.

Clinical and epidemiological evidence implicates long-term alcohol consumption in accelerating HCV-mediated tumorigenesis [84]. A recent study provided evidence that TLR4 mediates the synergism between alcohol and HCV in hepatic oncogenesis. Machida et al. [22] studied the molecular mechanism of synergism between alcohol and HCV, using mice with hepatocyte-specific transgenic expression of the HCV nonstructural protein NS5A, which is known to have a cryptic *trans*-acting activity for cellular gene promoters. They demonstrated that NS5A and alcohol synergistically induce hepatocellular damage and transformation via accentuated and/or sustained activation of TLR4 signaling, which results from HCV NS5A-induced hepatic TLR4 expression and alcohol-induced endotoxemia. Additionally, Nanog, a stem cell marker, was identified as a novel downstream gene transcriptionally induced by activated TLR4 signaling that is largely responsible for TLR4-mediated liver tumor development.

Taken together, these data suggest that TLR4 signaling in Kupffer cells and hepatocyte may constitute the link between hepatic chronic inflammation and hepatocarcinoma.

Conclusion

TLR4, as the other members of toll-like receptors family, is an essential player of innate immune system. It is activated by LPS, a Gram-negative bacterial cell wall component, as well as endogenous components derived from dying host cell. Activation of TLR4 results in the production of several pro-inflammatory, anti-viral, and anti-bacterial cytokines, which mount a rapid protective response against invading pathogens. Nevertheless, these cytokines may also trigger harmful responses such as cell death, fibrosis, and cancer.

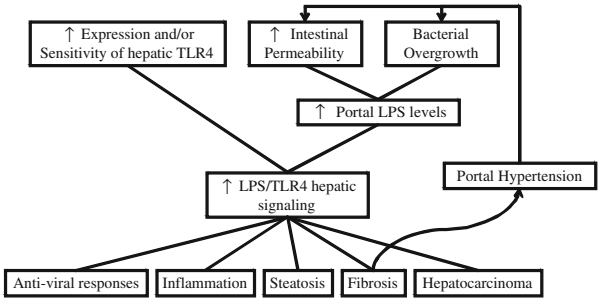

Fig. 2 Overview of the role of LPS/TLR4 signaling in chronic liver diseases. Increased expression and/or sensitivity of hepatic TLR4 and increased portal LPS levels (resulting from bacterial overgrowth and intestinal permeability) can lead to enhanced LPS/TLR4 signaling. This can induce anti-viral responses, inflammation, steatosis, fibrosis, and hepatocarcinoma. Hepatic fibrosis contributes to portal hypertension development which further increases bacterial overgrowth and intestinal permeability, creating a positive feedback process

Despite the constant confrontation of hepatic TLR4 with gut-derived LPS, the normal liver does not show signs of inflammation due to its low expression of TLR4 and ability to inhibiting TLR4 signals. Enhanced signaling of TLR4 may lead to persistently elevated inflammatory cytokines, resulting in chronic liver injury (Fig. 2). Indeed, in CLD, such as ALD, NAFLD, PSC, CBP, and fibrosis, it has been shown that LPS/TLR4 signaling is enhanced and is essential for liver injury. Enhanced LPS/TLR4 signaling may result from increased expression and/or sensitivity of TLR4 and, mainly, from increased exposure to LPS. Increased portal levels of LPS have been documented in many CLD and result mainly from increased intestinal permeability. In initial stages of CLD this increase of intestinal permeability may be dependent on etiology of CLD (i.e., alcohol, diet), but later on liver fibrosis and subsequent portal hypertension can become the main inducers of this alteration.

In many of CLD, inhibition of TLR4 has been shown to decrease liver injury, reinforcing the importance of LPS/TLR4 signaling in the pathogenesis of those diseases. Of the many possibilities to suppress TLR4 signaling (modulation of LPS production, TLR and co-receptors expression and downstream signaling molecules), the first appear to be the best as the others may result in systemic suppression of TLR4 disabling it to respond to invading pathogens. Modulation of the intestinal microbiota can be achieved by antibiotics, probiotics, and symbiotics. Probiotics and symbiotics, which already proved to have positive effects

in patients with CLD, should be preferred due to their high tolerability and limited side effects.

TLR4 plays also a role in chronic viral hepatitis. Chronic hepatitis B and C viruses lead to a downregulation of antiviral TLR4 signaling pathways. On the other hand, TLR4 was shown to block HBV and HCV replication through its ability to upregulate IFNs. This suggests that TLR4 agonists may boost anti-viral immunity and therefore represent a novel treatment approach for chronic viral hepatitis.

Although we need more studies, mainly in human patients, to translate TLR4 pathogenesis into clinical practice in CLD, we can anticipate that with further research on LPS/TLR4 signaling, this pathway will become an important pharmacological target in CLD.

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B) ATTENUATION OF TLR2-MEDIATED INNATE IMMUNE RESPONSE IN PATIENTS WITH ALCHOLIC CHRONIC LIVER DISEASE

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CLINICAL STUDIES

Attenuation of toll-like receptor 2-mediated innate immune response in patients with alcoholic chronic liver disease

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Abstract

Background: Alcoholic chronic liver disease (ACLD) is a common form of acquired immunodeficiency. *Aim:* To evaluate *ex vivo* toll-like receptor (TLR) 2 and TLR4 innate immune response in stable ACLD. *Methods:* Blood was collected from 26 males with stable ACLD and from 17 controls. Serum was used for lipopolysaccharide (LPS), sCD14, LPS-binding protein (LBP), tumour necrosis factor-alpha (TNF-α) and interleukin 10 (IL-10) quantification. Peripheral blood monocytes (PBM) protein expression of TLR2 and TLR4 was determined by flow cytometry. Primary cultures of anti-CD11b positive selected PBM were stimulated with the TLR2/TLR6 ligand zymosan (Zym), with TLR2/TLR1 ligand lipopeptide (Lp) and with TLR4 ligand LPS. PBM TLR1, TLR2, TLR4, TLR6, MD2, CD14, TNF-α and IL-10 gene expression was evaluated by reverse transcription-polymerase chain reaction. *Results:* Stable ACLD patients showed increased circulating LPS ($+22.5 \pm 4.1\%$), LBP ($+60.6 \pm 12.2\%$) and sCD14 ($+23.5 \pm 4.6\%$), with no differences in TNF-α and IL-10. Zym and Lp, but not LPS, induced TNF-α production by monocytes was blunted in ACLD ($-66 \pm 20.4\%$ Zym; $-40.1 \pm 13.5\%$ Lp; $P < 0.05$). Basal TNF-α mRNA expression was decreased in PBM from ACLD patients ($-50.1 \pm 21.0\%$; $P < 0.05$), with no significant differences in the other studied genes. Results were similar in Child–Pugh A and B/C patients. *Conclusions:* Patients with stable ACLD show an attenuation of TLR2-mediated innate immune response in PBM, which may represent an important mechanism for acquired immunodeficiency. This was neither related with decreased TLR2 or its co-receptors expression nor with impaired TLR4 activation, being already present in the early stages of disease.

Patients with chronic liver disease are particularly susceptible to infections, with increased morbidity and mortality from sepsis, mainly in the presence of cirrhosis (1–4). In fact, this disease is considered to be one of the most common forms of acquired immunodeficiency (1–4). Ascites, hypoalbuminaemia, intestinal bacterial overgrowth, increased intestinal permeability, bacterial translocation and increased endotoxaemia are important factors for this susceptibility (2–6). However, several immunological factors have also shown to be contributory. Namely, defects in polymorphonuclear leukocytes recruitment and activation (7, 8), deficiencies in the complement system (9) as well as defects in macrophage activation and adherence have been described (10, 11).

Alcoholic chronic liver disease (ACLD) is one of the most common forms of chronic liver disease worldwide, being the leading cause of death from liver disease in the western world (1, 12, 13). Excessive alcohol consumption has been shown to impair both the cellular and the humoral immune response, even in the absence of chronic liver disease (1, 14, 15). Moreover, in patients with ACLD, active alcohol consumption has a negative impact in the prognosis, potentiating the risk of infection associated with chronic liver disease (1, 12, 14, 16, 17).

Innate immunity is the first line of defense against infection, its activation being critical for the acute inflammatory response and subsequent adaptative immunity. Recently, receptors for highly conserved

molecular structures of microorganisms have been described (18). The toll-like receptors (TLRs) are one of the most representative class of pathogen-associated molecular patterns (PAMPs) receptors that play a critical role in innate immunity activation (19, 20). Of the several identified TLRs described, the TLR2 and TLR4 subtypes are critically involved in the innate immune response to bacterial infections, being abundantly expressed in immune cells such as polymorphonuclear leukocytes and monocytes/macrophages (18, 21). TLR4, in association with CD14 and MD2 co-receptors, is essential for innate immune activation in response to the lipopolysaccharide (LPS) of Gram-negative bacteria (18, 22–24). Differently, TLR2 is essential for the innate immune response to Gram-positive bacteria, being activated by bacterial lipoproteins and peptidoglycan (24, 25). Moreover, TLR2 also recognizes several microbial components from Gram-negative bacteria, fungus and even virus (20, 23, 26–28). In order to recognize different antigens, TLR2 may homodimerize and heterodimerize with other TLRs, such as TLR1 and TLR6, or with non-TLR molecules (29–31). Stimulation of TLR2 and TLR4 by microbial ligands initiates a signaling cascade that promotes NF-κB activation and subsequent production of pro-inflammatory mediators such as tumour necrosis factor-alpha (TNF-α) (20, 32–34).

Despite the central role of TLRs activation in the innate immune response to infection, its role in the pathophysiology in ACLD remains undefined. In the present study, TLR2- and TLR4-mediated innate immune response was evaluated *ex vivo* in stable ACLD. Namely, peripheral blood monocytes (PBM) primary cultures stimulation with the TLR2/TLR6 ligand zymosan (Zym) and the TLR2/TLR1 ligand lipopeptide (Lp), as well as with the TLR4 ligand LPS, were performed, and the PBM gene expression profile was evaluated.

Material and methods

Participants

The study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki S. and was approved by the Ethic Committee of Hospital João, Porto. Informed consent was obtained from each patient and control.

The study sample was composed of 26 male patients with ACLD followed in the hepatology outpatient clinic of Hospital de S. João. Patients were considered to have alcohol-related liver disease if alcohol intake had been in excess of 60 g/day for more than 10 years and if tests for viral and immune causes of disease were negative and other etiologies of liver disease were clinically excluded. The diagnosis of cirrhosis was performed clinically and confirmed by histology, endoscopy (e.g. oesophageal varices) and/or ultrasonography (e.g. nodular liver; ascites). At least 3 months of abstinence was necessary for inclusion in study. Any patient with infection, gastrointestinal haemorrhage or hospital admission within the previous 6 weeks was excluded from the study. Hepatocarcinoma or other significant comorbidities (e.g. congestive heart failure; renal insufficiency) were also exclusion criteria. In order to compare and confirm the stability of the selected ACLD patients, five additional patients with ACLD admitted to the gastroenterology ward of Hospital S. João with acute encephalopathy and/or gastrointestinal haemorrhage, but no clinical or analytical evidence of infection, were included for TNF-α serum levels and PBM TNF-α production evaluation.

Seventeen male age-matched blood donors (mean age of 54 years) from the same area of residence with alcohol intake <20 g/day served as controls.

Blood sampling

Peripheral blood was collected using sterilized needles, syringes and containers. Three tubes of 3 ml were used for routine analysis (complete blood count, glucose, electrolytes, ethanol, renal and liver function tests, as well as coagulation study) in order to complete clinical severity of disease and for stratification of patients according to the classification of Child–Turcotte–Pugh (35). Another 3 ml blood tube was used for serum separation after centrifugation at 2370 *g* for 15 min at 4 °C with posterior storage at -80 °C in 1.5 ml sterilized aliquots. Whole blood (8–10 ml) was used for PBM isolation.

Endotoxaemia, sCD14, lipopolysaccharide-binding protein, tumour necrosis factor-α and interleukin10 assays in peripheral blood

Serum endotoxin was measured using the chromogenic limulus amoebocyte lysate assay (Cambrex Corporation, East Rutherford, NJ, USA; sensitivity 0.1 EU/ml).

Serum levels of soluble CD14 (sCD14; R&D Systems, Minneapolis, MN, USA; sensitivity 125 pg/ml), LBP (Hycult Biotechnology, Uden, the Netherlands; sensitivity 4.4 ng/ml), TNF-α (Biosource, Nivelles, Belgium; sensitivity 0.7 pg/ml) and IL-10 (Biosource; sensitivity 1.6 pg/ml) were determined by enzyme-linked immunosorbent assays.

All assays were done in duplicate, in accordance to the manufacturer's instructions.

Isolation, culture and activation of peripheral blood monocyte

Peripheral blood monocytes were isolated from whole blood by density-gradient centrifugation with Ficoll-Paque (GE Heathcare Lifesciences, Buckinghamshire, UK) followed by positive selection isolation with anti-CD11b microbeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the cell layer containing mononuclear cells of the blood was collected and washed three times in phosphate-buffered saline (PBS – pH 7.2), containing bovine serum albumine (0.5%) and EDTA (2 mM). The cell suspension was resuspended with CD11b microbeads (MACS, Miltenyi Biotec) and incubated at 4 °C for 15 min. Then, the solution was applied to a column placed in a suitable magnetic separator (MACS, Miltenyi Biotec).

After the column had been rinsed with buffer, the magnetic labeled cell faction was collected and counted in a Neubauer chamber (the average of freshly isolated monocytes was 1×10^6 cells). Cell viability was shown by the exclusion of trypan blue (approximately 98%). Immediately after separation and isolation of PBM, 1×10^5 cells were collected, centrifuged at 4 °C, 400 g during 5 min and the final cell pellet was used for mRNA isolation with TriPure Isolation reagent (Roche, Germany), according to the manufacturer's instructions.

Afterwards, PBM primary culture was performed. The monocyte samples were adjust to 1×10^5 cells per well and cultured in triplicate in RPMI-1640 medium (GE Healthcare Lifesciences), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L glutamine and 12% fetal bovine serum (GE Healthcare Lifesciences) at 37 °C and 5% of CO_2. After 36 h incubation (period of maximum adherence for monocytes with our protocol), nonadherent cells and supernatants were removed and fresh medium was added (time 0 h). PBMs from patients and controls were incubated separately in three different wells with Zym [2 μg/ml] for TLR2/TLR6 stimulation, with LPS [1 μg/ml] for TLR4 stimulation and 0,9% NaCl as an internal control. In a subset of patients ($n = 16$; seven stable patients Child A, four stable Child B and five unstable patients) and controls ($n = 7$), PBM were also stimulated with bacterial Lp Pam3Cys-SK4 [40 μg/ml] in order to evaluate the TLR2/TLR1 dependent activation. The supernatants were collected and the medium was replaced at 3, 6, 12 and 24 h. After collection, supernatants were frozen at − 80 °C until analysis of TNF-α levels (R&D Systems; sensitivity 1.6 pg/ml). At the end of the experimental protocol, the cultured PBM were lysed and resuspended for mRNA isolation.

mRNA isolation and quantification

Total mRNA was extracted from PBMs using the TriPure isolation reagent according to the manufacturer's instructions (Roche). Concentration and purity were assayed by spectrophotometry (Eppendorf 6131000.012, Hamburg, Germany). Two-step real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to perform mRNA relative quantification. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle of graded dilutions from a randomly selected sample from the control group. For the relative quantification of specific mRNA levels, 50 ng of total mRNA from each sample underwent two-step real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all experimental groups, which led to the use of this gene as the internal control.

Reverse transcription (20 μl; 10 min at 22 °C, 50 min at 50 °C and 10 min at 95 °C) was performed in a standard thermocycler (Whatman Biometra 050-901). Ten percent of the cDNA yield was used as a template for real-time PCR (LightCycler II, Roche) using SYBR green (Qiagen 204143) according to the manufacturer's instructions.

Specific PCR primers pairs for the studied genes were: **GAPDH** – fw (P1) 5′– TTG GCC AGG GGT GCT AAG -3′ and rev (P2) 5′- AGC CAA AAG GGT CAT CAT CTC -3′; **CD14** – fw 5′- TGA GGT TCG GAG AAG TTG CAG ACG -3′ and rev 5′-TCG TGC TTG TTG CTG CTG CTG C -3′; **Md2** – fw 5′- TGT TGT ATT CAC AGT CTC TCC -3′ and rev 5′- ACA CCA TGA ATC TTC CAA AGC GCA – 3′; **TLR1** - fw 5′- ATG GTG GCA ACG ATG GTG AC– 3′ and rev 5′ GGG CTG GCC TGA TTC TTA T– 3′; **TLR2** – fw 5′- GAT CCC AAC TAG ACA AAG ACT -3′ and rev 5′- CTG CGG AAG ATA ATG AAC ACC -3′; **TLR4** – fw 5′- CTA AAC CAG CCA GAC CTT GAA -3′ and rev 5′- ACC TGT CCC TGA ACC CTA TGA -3′; **TLR6** - fw 5′- GAT GGG CAA AAT AGA GTT CGT AAT -3′ and rev 5′- TGT CCC TGG CAA GAG CA - 3′; **TNF-α** – fw 5′- GGT TTG CTA CAA CAT GGG CTA -3′ and rev 5′-AAG AGT TCC CCA GGG ACC TCT C -3′; **IL-10** – fw 5′- CAG GTA ACC CTT AAA GTC CTC CAG -3′ and rev 5′- TCC GAG ATG CCT TCA GCA GAG TG -3′.

Results of mRNA quantification were expressed in two ways: (i) nonstimulated PBM (basal expression): Values were expressed as an arbitrary unit (AU) set as the average value of control group, after normalization for GAPDH; (ii) stimulated PBM: Values were expressed as the ratio gene/GAPDH in order to compare the change in thr mRNA expression (% of variation).

Toll-like receptor 2 and 4 protein quantification in peripheral blood monocyte

Cell surface staining was performed on whole blood using the following anti-human monoclonal antibodies: anti-TLR2 (Alexa Fluor® 488 Mouse Anti-Human CD282; BD Pharmingen™, San Diego, CA, USA), anti-TLR4 (Biotin Mouse Anti-Human Toll-Like Receptor 4 conjugated with PE Streptavidin, BD Pharmingen™), anti-CD45 (CD45 Per CP-CY5.5, BD Pharmingen TM) and anti-CD14 (CD14 APC, BD Pharmingen™). A total of 100 000 cells were acquired for each sample, and dead cells were gated out based on their light scatter properties. PBM were gated based on the positivity for CD45 and CD14. Data acquisition was performed in FACSCanto II using FACSDiva™ Software (BD Pharmingen TM) and analysed using INFINICYT 1.2™ Software (Cytognos, Salamanca, Spain). TLR2 and TLR4 values were expressed as a ratio of the geometric mean fluorescence of individual study patients to mean control values for that session.

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS for Windows version 17.0; SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of mean (SEM) and the proportion of variation compared with controls (mean difference of the proportions and SEM of

the difference). One-way ANOVA and Student's *t* test for paired and unpaired data (or correspondent nonparametric test) were used, when appropriate, for comparison between groups. χ^2-test was used for comparison of proportions. When necessary, the test was preceded by a natural logarithm transform to obtain a normal distribution. The statistical significance was set at $P<0.05$.

Results

Baseline characteristics of patients

The baseline characteristics of patients are shown in Table 1. None of the patients had leukocyte levels higher than 10 000 cells/mm^3 and Plasma C-reactive protein was lower than 10 mg/L in all patients. Ethanol levels were null in all patients. None of controls had serious infections in the previous year against 23% (6/26) hospital admissions because of infection in the stable ACLD patient group.

Table 1. Baseline characteristics of patients ($n=26$)

	Child-A ($n=14$)	Child-B/C ($n=12$)	Total ($n=26$)
Age	54 (2.5)	58 (3.7)	56 (2.2)
Serum bilirubin (mg/dl)	1.3 (0.16)	2.8 (0.6)	1.99 (0.3)
Serum albumin (g/L)	41 (1.1)	33 (1.2)	37 (1.1)
Presence of ascites	2 (8%)	9 (35%)	11 (42%)
INR	1.2 (0.04)	1.4 (0.06)	1.3 (0.05)
Serum creatinine (mg/dl)	1.0 (0.05)	1.05 (0.09)	1.03 (0.05)
MELD	9 (0.7)	13 (0.6)	10.8 (0.6)
Serum leukocytes (mm^3)	5435 (567)	4930 (620)	5202 (413)
Plasma C-reactive protein (mg/L)	3.8 (0.8)	5.1 (0.9)	4.4 (0.6)
Comorbilities	6 (23%)	5 (19%)	11 (42%)
DMNID	5 (19%)	3 (12%)	8 (31%)
HTA	1 (4%)	1 (4%)	2 (8%)
Heart disease	1 (4%)	0 (0%)	1 (4%)
Renal disease	1 (4%)	1 (4%)	2 (8%)
Lung disease	1 (4%)	0 (0%)	1 (4%)
Concomitant treatments			
Norfloxacin	1 (4%)	3 (12%)	4 (15%)
Beta-blockers	7 (27%)	7 (27%)	14 (54%)
Lactulose	1 (4%)	6 (23%)	7 (27%)

Values are presented as mean (SEM) or number of patients (with % from total population).
INR, international normalized ratio; MELD, model for end-stage liver disease.

Serum levels of tumour necrosis factor-α, interleukin-10, lipopolysaccharide, LPS-binding protein, sCD14, toll-like receptor2 and toll-like receptor4

Serum levels of TNF-α, IL-10, LPS, LBP and sCD14 from patients and controls are shown in Table 2. In patients, there was a statistically significant elevation of LPS when compared with controls (a mean increase of $22.5\pm4.1\%$; $P=0.04$). This was associated with a significant elevation of serum levels of sCD14 ($+23.5\pm4.6\%$; $P=0.02$) and LBP ($+60.6\pm12.2\%$; $P=0.03$). However, there were no differences in the serum levels of TNF-α (7.48 vs 8.3 pg/ml; $P=0.4$) or IL-10 (14.9 vs 14.7; $P=0.6$). When comparing patients with or without ascites, the values were similar to Child A and B/C, respectively; however, TNF-α serum levels were significantly higher in stable patients with ascites (8.8 ± 1 vs 6.7 ± 0.5 pg/ml; $P=0.03$), despite both groups being within the normal range of controls TNF-α (5.0–11.5 pg/ml).

Toll-like receptor 2 and toll-like receptor 4 stimulation in peripheral blood monocyte primary culture

The constitutional production of TNF-α (measured TNF-α at 0 h) from stable ACLD patients' PBM was significantly lower than that from controls (36 h production of 388 ± 52 vs 693 ± 152 pg/ml; $P=0.01$; patients vs controls, respectively). When PBM were stimulated with LPS, no differences were found in TNF-α production between stable ACLD patients and controls (Fig. 1). However, when PBM were stimulated with Zym, there was a significantly lower production of TNF-α at all studied time points in the stable ACLD patient group (Fig. 1). Globally (all studied time points considered), Zym TNF-α production by PBM was reduced by $66\pm20.4\%$. Regarding PBM stimulation with Lp, there was a significantly lower production in the stable ACLD patient group at 3 h ($-62\pm23\%$; $P=0.03$) and at 6 h ($-64\pm29\%$; $P=0.04$), but not at 12 or 24 h (Fig. 1), compared with control. Globally, Lp-induced TNF-α production was reduced by $40.1\pm13.5\%$ ($P=0.03$). Of note, no significant differences were detected between patients with or without ascites or between stable ACLD Child–Pugh A and B/C patients, for LPS, Zym and Lp.

Table 2. Serum levels of TNF-α, IL-10, LPS, LBP and sCD14 from patients ($n=26$) and controls ($n=17$)

	Controls ($n=17$)	Total patients ($n=26$)	*P*	Child-A ($n=14$)	Child-B/C ($n=12$)	*P*
LPS (EU/ml)	0.40 (0.02)	0.49 (0.02)	**0.04**	0.49 (0.03)	0.48 (0.03)	0.77
LBP (ng/ml)	12 279 (1290)	20 774 (2403)	**0.03**	18 810 (2980)	23 095 (3930)	0.4
sCD14 (ng/ml)	1363 (75)	1686 (74)	**0.02**	1590 (83)	1800 (125)	0.18
TNF-α (pg/ml)	8.3 (1.0)	7.48 (0.5)	0.4	6.54 (0.4)	8.57 (0.9)	0.06
IL-10 (pg/ml)	14.9 (0.6)	14.2 (0.7)	0.6	14.5 (1.0)	13.8 (0.9)	0.6

Values are presented as mean (SEM). Patients had significant higher levels of LPS, LBP and sCD14 (bold) but not TNF-α or IL-10 when comparing with controls. There were no differences between Child-A and Child-B/C.
IL, interleukin; LBP, LPS binding protein; LPS, lipopolysaccharide; TNF, tumour necrosis factor.

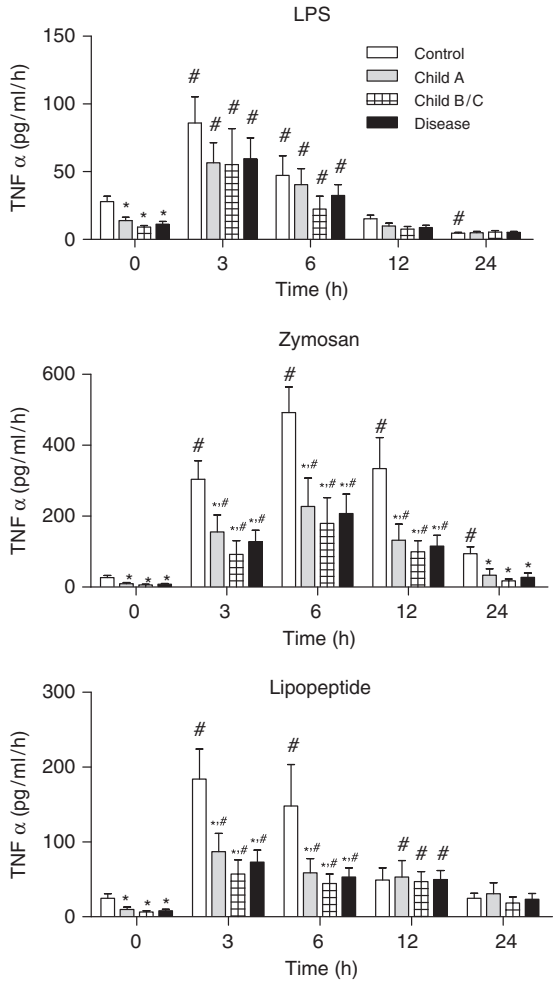

Fig. 1. Monocyte production of tumour necrosis factor (TNF)-α after stimulation with lipopolysaccharide (LPS) (up), zymosan (middle) or lipopeptide (down). *$P < 0.05$ vs control group; #$P < 0.05$ vs production at time 0 h. There were no significant differences in TNF-α production when monocytes were stimulated with LPS. However, when stimulated with zymosan or lipopeptide, the production of TNF-α per hour was significantly higher in controls.

Peripheral blood monocyte gene expression profile

There was no difference in PBM TLR4 or TLR2 protein levels between patients and controls (0.98 vs 1.00; $P = 0{,}8$).

No significant differences were detected in PBM mRNA basal expression of TLR1, TLR2, TLR4, TLR6, CD14, MD2 and IL-10 between controls and stable ACLD patients (Table 3). However, a nonstatistically significant trend for lower TLR2 (0.74 ± 0.05 vs 1.0 ± 0.25 AU; $P = 0.10$) and TLR1 expression (0.5 ± 0.1 vs 1.0 ± 0.48 AU; $P = 0.10$) was observed in patients with stable ACLD. Interestingly, the basal expression of TNF-α mRNA in patients PBM was half from the expression in controls (0.5 ± 0.08 vs 1.0 ± 0.25 AU; $-50.1 \pm 21\%$; $P = 0.02$).

mRNA expression of toll-like receptor 2 and toll-like receptor 4 after peripheral blood monocyte stimulation

Zym or LPS stimulation of PBM induced different patterns of TLR2 and TLR4 expressions in both patients and controls, as shown in Fig. 2. When stimulated with Zym, there was a $78 \pm 4\%$ and $67 \pm 8\%$ reduction of TLR4 and TLR2 in PBM of stable ACLD patients respectively. However, in the control group, there was also a significant reduction in the expressions of TLR4 and TLR2 (85 ± 4 and $55 \pm 14\%$ respectively). After stimulation with Zym, the expression of TLR2 was lower in stable ACLD patients' PBM in comparison with controls, although this difference did not reach statistical significance (0.28 ± 0.08 vs 0.59 ± 0.28 ratio TLR2/GAPDH; $P = 0.1$). Differently to Zym, LPS stimulation resulted in TLR4 downregulation but TLR2 upregulation in both groups (Fig. 2).

Decompensate alcoholic chronic liver disease patients

Tumour necrosis factor-α serum levels and PBM TNF-α production were measured in five cirrhotic patients hospitalized for gastrointestinal bleeding and/or severe encephalopathy (80% Child C, mean model for end-stage liver disease 20 ± 0.9, all patients with ascites). In patients with decompensated ACLD, TNF-α serum levels were 20 ± 4 pg/ml, significantly higher ($+141 \pm 48.2\%$)

Table 3. Monocyte mRNA basal expression of Toll-like receptor 1, TLR2, TLR4, TLR6, CD14, Md2, tumour necrosis factor-α and interleukin-10 from patients ($n = 26$) and controls ($n = 17$)

	Controls ($n = 17$)	Total patients ($n = 26$)	*P*	Child-A ($n = 14$)	Child-B/C ($n = 12$)	*P*
TLR1 (AU)	1 (0.46)	0.47 (0.08)	0.1	0.42 (0.05)	0.54 (0.15)	0.5
TLR2 (AU)	1 (0.24)	0.74 (0.06)	0.1	0.71 (0.07)	0.77 (0.08)	0.6
TLR4 (AU)	1 (0.08)	0.96 (0.07)	0.7	0.97 (0.09)	0.93 (0.11)	0.8
TLR6 (AU)	1 (0.22)	0.73 (0.11)	0.2	0.77 (0.13)	0.67 (0.2)	0.6
CD14 (AU)	1 (0.13)	0.98 (0.07)	0.8	0.98 (0.09)	0.97 (0.12)	0.9
Md2 (AU)	1 (0.21)	1 (0.08)	0.9	0.98 (0.11)	1 (0.12)	0.7
TNF- α (AU)	1 (0.27)	0.49 (0.08)	**0.02**	0.53 (0.11)	0.45 (0.12)	0.6
IL-10 (AU)	1 (0.7)	1.3 (0.6)	0.8	1.5 (1.0)	1.1 (0.5)	0.7

Values are presented as mean (SEM). Only the basal expression of TNF-α (bold) was different in patients when compared with controls (half the expression).

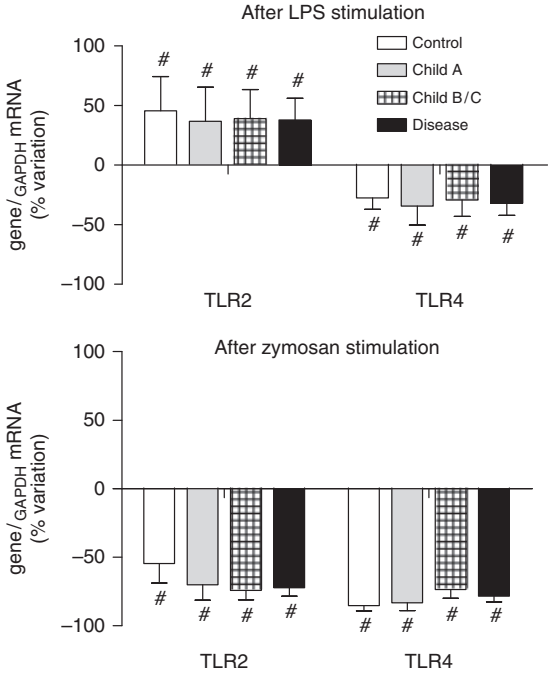

Fig. 2. Percentage (%) of variation in the gene expression of toll-like receptor (TLR)2 and TLR4 after monocyte stimulation either with lipopolysaccharide (LPS) (up) or with zymosan (down). #$P < 0.05$ vs the initial gene expression. The pattern of genic expression variation was similar between patients and controls.

when compared with controls or with stable ACLD patients ($P = 0.02$). The PBM basal production from patients with decompensated ACLD was $25 \pm 16\%$ higher than that from controls ($P = 0.12$) and $70 \pm 17\%$ higher than that from stable patients ($P < 0.001$). In this subset of patients, all stimulations in PBM primary cultures were blunted when compared with controls (LPS: $-55 \pm 30.3\%$, $P = 0.05$; Zym: $-81 \pm 29\%$, $P = 0.002$; Lp: $-67 \pm 28\%$, $P = 0.04$).

Discussion

In the present study, TLR2- and TLR4-mediated innate immune responses were analysed *ex vivo* in PBM of patients with stable ACLD. A selective attenuation of TLR2-mediated innate immune response was found in these patients, which was not dependent on decreased TLR2 expression or impaired TLR4 signaling pathways.

Previous reports studied the innate immunity receptors in chronic liver disease (17, 36–42). However, the results are difficult to extrapolate, given the differences in the studied populations in terms of distinct liver disease aetiologies (36, 37, 41, 42), various alcohol-consumption status (acute ingestion, chronic ingestion and abstinence) (17) and distinct liver disease stages (hepatitis, established cirrhosis and decompensated disease) (38, 42, 43). In our study, TLR2 and TLR4 innate immune responses were analysed in a group of patients with stable ACLD in different stages (Child–Pugh A and B/C). In fact, our patients were selected from an outpatient hepatology clinic and presented no recent history of infection, gastrointestinal bleeding, hospital admission or evidence of recent alcohol ingestion. Moreover, we compared TNF-α serum levels and basal TNF-α production in PBM primary culture with a group of decompensated ACLD patients with clearly distinct results, further supporting the stability of our group of patients.

In agreement with previous studies (16, 39, 44–46), increased LPS circulating levels were found in ACLD. In this disease, endotoxaemia has been attributed to several factors such as intestinal bacterial overgrowth, and structural and functional alterations of intestinal mucosal barrier that promote bacterial translocation (6). Increased LPS circulating levels have been proposed to underlie a low-grade systemic pro-inflammatory state associated with chronic liver disease (1, 12, 47–50). In our study, however, increased TNF-α circulating levels supporting a systemic pro-inflammatory state was restricted to patients with decompensated ACLD. This might be because of the parallel increase in the serum levels of the LPS-binding proteins LBP and sCD14. In fact, LPS transfer to lipoproteins by LBP and sCD14 may neutralize the immune response to LPS (51–53).

We did not find differences either in basal TLR4 levels or in LPS-induced TNF-α production in PBM from stable ACLD patients, compared with controls. Accordingly, other studies (17, 37, 54) found similar patterns of cytokine production in LPS-stimulated immune cells of abstemic/stable cirrhotic patients. In advance or unstable disease, however, TLR4 response may be compromised. Tazi *et al.* (38) describe enhanced LPS-induced TNF-α production in hospitalized Child-C patients, the majority active drinkers, on the contrary, Lin *et al.* (39) and Wasmuth *et al.* (42) found a decreased production of TNF-α after LPS activation of immune cells, suggesting the existence of immune paralysis in both advanced (39) and unstable cirrhosis (42). A recent study (41) involving Child-C patients listed for transplantation appears to confirm a diminished TLR4 function in these patients. Interestingly, this immune defect was reversible with antibiotic therapy. Similarly, and despite the fact that our study was not designed to evaluate unstable disease, in our patients with decompensated ACLD, a significant attenuation in TLR4-mediated innate immune response was also observed. These results suggest a significant compromise of TLR4 signaling pathways. However, this may be present only in advance and/or unstable disease.

In our study, TLR2-mediated innate immune response was blunted in PBM of stable ACLD patients. Similarly, Riordan *et al.* (37) described blunted TLR2-mediated innate immune response *ex vivo* in peripheral blood mononuclear cells from patients with different cirrhosis etiologies. Interestingly, in both studies, the attenuation of TLR2-mediated innate immune response was not

related with decreased TLR2 expression. In fact, no significant differences in TLR2 mRNA or protein levels were observed between ACLD and control groups, while in the study of Riordan and colleagues, TLR2 levels were even increased. Staldbauer *et al.* (40) also found neutrophil dysfunction in the presence of TLR2 overexpression. Importantly, using two different TLR2 agonists (Lp and zymosan), we have shown for the first time that this immune deficiency involves TLR2/1 and TLR2/6 signaling pathways, potentially affecting immunologic response to a large variety of antigens. Moreover, no differences in the expression levels of TLR2-heterodimers (TLR1 and TLR6) or TLR2/TLR4 co-receptor CD14 (55–57) were detected between ACLD and control PBM. Taken together, these results suggest an impairment of TLR2-mediated intracellular signaling pathways in ACLD.

To further clarify the molecular mechanisms underlying the selective attenuation of TLR2-mediated innate immune response in patients with stable ACLD, the differential effect of Zym and LPS in PBM stimulation on TLR2 and TLR4 gene expression was analyzed. In fact, Zym and LPS stimulation has distinct effects on TLR2 and TLR4 expression levels. Whereas Zym-mediated TLR2 stimulation induced a downregulation of both TLR2 and TLR4, LPS-mediated TLR4 stimulation was accompanied by a selective upregulation of TLR2 and a downregulation of TLR4. These differences could be related to a distinct intracellular pathway activation. In fact, although TLR2 and TLR4 share most of its intracellular pathways, TLR4 also activates MyD88-independent pathways (30, 31).

In conclusion, PBM of stable ACLD patients demonstrate an attenuation of TLR2- but not TLR4-mediated innate immune response. Given that TLR2 recognize several different microbial molecules, this may constitute an important mechanism of acquired immunodeficiency. Further investigation is required to study the impact of blunted TLR2 innate immunity in the infection risk and the prognosis of this disease.

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C) ROLE OF TOLL-LIKE RECEPTOR IMPAIRMENT IN CIRRHOSIS INFECTION RISK: ARE WE MAKING PROGRESS?

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Role of toll-like receptor impairment in cirrhosis infection risk: are we making progress?

To the Editor:

Given our recent paper on toll-like receptors (TLR) in alcoholic liver disease (1), we now review the role of TLR impairment in cirrhosis infection risk. In our study (1), patients with stable alcoholic chronic liver disease showed an attenuated TLR2-mediated innate immune response. We found an impaired TLR4 response only in our unstable patients. A recent study by Testro *et al*. (2) observed an impairment of TLR4 function in advanced cirrhosis, but dependent on decreased TLR4 levels. This process was reversible with antibiotics.

In our review of the literature of this subject, we found some contradictory results (Table 1). If we consider only the studies that evaluated advanced disease, we can see that Testro's results are partially in agreement with our study (1), the study from Wasmuth *et al*. (3) and also with the Child-C patients from Lin *et al*.'s study (4). Nevertheless, Testro's results are completely in disagreement with the study from Tazi *et al*. (5), which found augmented TNF-α production with decreased TLR4 levels. Also in contradiction with Testro are the results from Stadlbauer *et al*. (6). In this last study, despite studying stable patients, decreased phagocytic capacity was found in association with increased TLR4 levels. The normalization of function with probiotic was associated not with an elevation of TLR4 levels but with a reduction towards normal. Probably, methodological differences (e.g. population, cells, quantification method, other) can help to explain some but not all the differences.

Analysing all the studies, we conclude that decreased TLR levels are insufficient to alter TLR function. In fact, some found decreased function with increased (6, 7),

Table 1. Review of the studies about the role of toll-like receptor 2 and toll-like receptor 4 in cirrhotic patients according to toll-like receptor expression and function (considered as tumour necrosis factor-α production in culture)

Study	Cirrhotic population	Cell	TLR2 expression	TLR4 expression	TLR function	Therapeutic intervention
Manigold *et al*. (9)*	Stable ($n = 21$) viral and alcohol	PBMC	= ; ↑ only if endotoxaemia	= ; ↓ only in Child-A	NE	NE
Riordan *et al*. (7)†	Stable ($n = 36$) several aetiologies	PBMC	↑	=	TLR4 = ; TLR2 ↓	Symbiotic ↑ TLR2 levels and ↓ function
Wasmuth *et al*. (3)	Advanced ($n = 27$) alcohol	PM	NE	NE	TLR4 ↓	NE
Tazi *et al*. (5)‡	Advanced ($n = 48$) alcohol	PM	NE	↓	TLR4 ↑	NE
Laso *et al*. (8)	Stable ($n = 21$) alcohol	PM	NE	NE	TLR4 = ; ↓ only in active drinkers	NE
Lin *et al*. (4)	Stable ($n = 64$) several aetiologies	PM	NE	NE	TLR4 ↓ only in Child C	NE
Stadlbauer *et al*. (6)†	Stable ($n = 12$) alcohol	PN	↑	↑	TLR4 = §	Probiotic decreased TLR4 levels to normal§
Pimentel-Nunes *et al*. (1)*,†	Stable ($n = 26$) and advanced ($n = 5$) alcohol	PM	=	=	TLR4 = ; ↓ only in unstable; TLR2 ↓	NE
Testro *et al*. (2)†	Advanced ($n = 41$) alcohol	PBMC	=	↓ only in patients without ATB	TLR4 apparently ↓ in patients without ATB TLR2 apparently =	ATB increased TLR4 levels to normal with increase of function

*TLRs quantified by RNA.

†TLRs quantified by flow cytometry.

‡TLR4 quantified by Western blotting.

§Despite presenting decrease phagocytic capacity, stimulated TNF-α in culture was not different to controls and probiotic restored phagocytic capacity.

ATB, antibiotics; PBMC, peripheral blood mononuclear cell; PM, peripheral monocytes; PN, peripheral neutrophils; NE, not evaluated; =, equal to controls or equal to control group; ↓, decrease when compared with controls; ↑, increase when compared to controls.

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normal (1) and decreased levels (2). This probably implies dysfunction in intracellular signalling pathways. Actually, in our study, we found blunted TLR2 activation that was independent not only of TLR2 levels but also of TLR1, TLR6 and CD14, important membrane activation factors for TLR2 signalling pathways, thus further supporting intracellular dysfunction. We have shown *in vitro* that TLR2 and/or TLR4 agonists change the expression levels of these receptors (1). Hence, we believe that the frequent episodes of bacteraemia that occur in cirrhosis, by changing TLR expression on immune cells, can help explain these discrepancies concerning TLR expression. This also might be the reason why Stadlbauer *et al.* (6), using probiotics, promoted the decrease, and Testro *et al.* (2), using antibiotics, the increase in TLR4 levels, both trending towards normal levels of expression. Possibly, these two different therapeutic agents decrease episodes of bacteraemia, consequently with less fluctuation of TLR levels. Why they restored TLR4 function remains unclear because expression levels cannot explain the results from these two studies.

Several conclusions can be made. Firstly, TLR2 and TLR4, the most important innate immune receptors for bacteria recognition, appear to play a significant role in the infection risk of cirrhotic patients. Secondly, our study (1) and others (7) clearly suggest a blunted TLR2 function even in the early stages of cirrhosis, which may help explain the growing risk of Gram-positive bacteria infection in these patients. Thirdly, at least in advanced cirrhosis, TLR4 impairment is also present (1–4, 6). Fourthly, taking together the discrepancies in the expression levels of TLRs, it appears that other factors, probably intracellular, are fundamental to this immunodeficiency. Finally, this process may be reversible with antibiotics and/or probiotics (2, 6). However, further studies are needed before generalization since Riordan *et al.* (7) showed that the use of a symbiotic further compromised TLR2 function, in contrast to the positive immunological effects obtained by Stadlbauer *et al.* (6) and Testro *et al.* (2).

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D) INCREASED HEPATIC EXPRESSION OF TLR2 AND TLR4 IN THE HEPATIC INFLAMMATION-FIBROSIS-CARCINOMA SEQUENCE

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Increased hepatic expression of TLR2 and TLR4 in the hepatic inflammation-fibrosis-carcinoma sequence

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Abstract

We evaluated expression of TLR2, TLR4 and proinflammatory genes [NF-κB, TNF-α, cyclooxygenase-2 (COX-2)] in liver samples of patients in different stages of liver disease. Fifteen patients with unexplained transaminases elevation (reference group), 22 with viral chronic hepatitis (hepatitis group), 14 with virus-induced severe fibrosis/cirrhosis (cirrhosis group) and 10 with hepatocarcinoma (hepatocarcinoma group) were consecutively included in the study. Quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2 mRNA was done by real-time RT-PCR and TLR2 and TLR4 protein expression was evaluated by immunohistochemistry. Compared with reference, TLR2 and TLR4 mRNA was increased in hepatitis (TLR2: 2.66 ± 0.69; TLR4: 3.11 ± 0.79; $P < 0.05$) and cirrhosis (TLR2: 2.14 ± 0.5; TLR4: 1.74 ± 0.27; $P < 0.05$) and decreased in hepatocarcinoma (TLR2: 0.48 ± 0.15; TLR4: 0.54 ± 0.10; $P < 0.05$). This associated with increased TNF-α and COX-2 mRNA in hepatitis (TNF-α: 3.24 ± 0.79; COX-2: 2.47 ± 0.36; $P < 0.05$) and cirrhosis (TNF-α: 1.73 ± 0.28; COX-2: 1.8 ± 0.35, $P < 0.05$), whereas NF-κB mRNA was increased in hepatitis (2.42 ± 0.31; $P < 0.05$) and unchanged in cirrhosis (1.34 ± 0.17; $P = 0.3$). Hepatocarcinoma presented increased COX-2 mRNA (1.63 ± 0.15; $P < 0.05$) and maintained (at decreased levels) mRNA of NF-κB (0.52 ± 0.12) and TNF-α (0.52 ± 0.12; $P < 0.05$, all genes). Immunohistochemistry confirmed increased expression of TLR2 and TLR4 in hepatitis and cirrhosis and maintained expression in hepatocarcinoma. Upregulation of TLR2, TLR4 and their proinflammatory mediators is associated with virus-induced hepatic IFC sequence.

Keywords

Chronic hepatitis, cirrhosis, hepatocarcinoma, TLR2, TLR4

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Introduction

Chronic liver inflammation, irrespective of the underlying cause (metabolic, immune-driven or virus-induced), leads to fibrosis and/or cirrhosis, which are precancerous states in which the development of hepatocarcinoma is more likely. Some authors call this sequence the hepatic inflammation-fibrosis-carcinoma (IFC) sequence.[1] Nevertheless, the cellular and molecular effectors mediating the interplay between the components of hepatic IFC sequence continue to be largely unknown.

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Recently, several studies implicated TLRs as potential key orchestrators of the hepatic IFC sequence.[2–5] TLRs are one of the most representative classes of pathogen-associated molecular patterns (PAMPs) receptors that play a critical role in innate immunity activation.[6,7] The human TLR family consists of 10 members that enable the innate immunity system to recognize different groups of pathogens while initiating appropriate and distinct immunological responses according to the recognized PAMP.[6,7] Besides immune cells, most liver cells (hepatocytes, Kupffer cells and stellate cells) also express TLRs and respond to their ligands.[8,9] TLR2 and TLR4 have been the most studied TLRs in liver diseases as they sense bacterial components and, thus, may mediate liver injury associated with increased bacterial translocation that is present in many liver diseases.[10–12] TLR2 is essential for the innate immune response to Gram-positive bacteria, being activated by bacterial lipoproteins and peptidoglycan.[6,7] TLR4 acts as a receptor for LPS, a cell wall component of Gram-negative bacteria.[6,7] Besides exogenous ligands, TLR2 and TLR4 may also sense endogenous ligands initiating danger signals, such as high mobility group box 1, hyaluronan and heat shock protein 60, inducing an inflammatory response in the absence of microbial challenge.[13] Stimulation of these two receptors initiates a signaling cascade that promotes activation of NF-κB and MAPK and, consequently, production of different pro-inflammatory mediators, such as TNF-α and cyclooxygenase-2 (COX-2).[14–18]

Recent animal studies and *in vitro* hepatocyte culture models suggest that TLR2 and TLR4 may play a key role in the hepatic IFC sequence. Modulation of TLR2 and/or TLR4 function was shown to influence liver inflammation in chronic liver diseases, such as alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), chronic hepatitis C and chronic hepatitis B.[2,3]

There is also accumulating evidence that TLR4-induced activation and sensitization of hepatic stellate cells (HSCs) may constitute an important molecular link between hepatic inflammation and fibrogenesis.[19–22] Moreover, a recent study has revealed TLRs, in particular TLR4, as major factors linking hepatic chronic inflammation and hepatocarcinoma.[23]

However, to date, the suggested implication of TLR2 and TLR4 in the pathogenesis of hepatic IFC sequence is principally based on evidence obtained from animal studies or *in vitro* hepatocyte culture models. Studies using diseased human liver tissue to confirm or refute the *in vitro* and animal findings are scarce and have evaluated TLR2 and TLR4 in each stage of IFC sequence separately.

Therefore, in the present study, we evaluated the expression of TLR2 and TLR4 in liver samples from patients in each stage of virus-induced hepatic IFC sequence. The expression of NF-κB, TNF-α and COX-2 was also evaluated in order to characterize their association with TLR2 and TLR4 expression.

Materials and methods

Patients and biological samples

This study included patients from two hospitals of the North of Portugal (Braga Hospital and Portuguese Oncology Institute of Porto). The study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Braga Hospital and Portuguese Oncology Institute of Porto. Informed consent was obtained from each patient.

Patients were recruited consecutively during 2009. We defined four groups: reference, hepatitis, cirrhosis and hepatocarcinoma. Reference group included patients followed in the Hepatology Outpatient Clinic of Braga Hospital who underwent liver biopsy because of chronic unexplained transaminase elevation. We excluded from this group patients with alcohol abuse (>30 g/d in males; >20 g/d in females); analytical or histologic findings favoring hemocromatosis; autoimmune hepatitis; primary biliary cirrhosis; primary sclerosing cholangitis; and HIV infection or clinical, analytical, imagiological or histologic evidence of severe fibrosis/cirrhosis (METAVIR F3–4). The hepatitis group included chronic hepatitis B or C patients followed in the Hepatology Outpatient Clinic of Braga Hospital who underwent staging liver biopsy. The cirrhosis group was selected from the same group of patients but with histologic evidence of severe fibrosis/cirrhosis (METAVIR F3–4). The hepatocarcinoma group included chronic hepatitis B or C patients with diagnosis of hepatocarcinoma (according to the EASL 2000 Barcelona Guidelines[24]) followed in the outpatient clinic of Portuguese Oncology Institute of Porto who underwent surgical resection of hepatocarcinoma. In these groups, patients must have had >18 years serological evidence of chronic hepatitis B (HBsAg+) or C (HCVAb+) and clinical stability. Histologic evidence of cirrhosis and hepatocarcinoma was required in the hepatocarcinoma group. Patients with HIV infection or analytical or histologic findings suggestive of liver disease other than viral chronic hepatitis were excluded.

Before liver biopsy or surgical intervention, blood samples were drawn from fasting patients for routine analysis (complete blood count, glucose, electrolytes, renal and liver function tests, and coagulation study) and viral load quantification. Liver tissue was obtained by percutaneous biopsy using a 16-gauge Menghini needle or by transjugular biopsy. In the hepatocarcinoma group, we obtained hepatocarcinoma tissue [for mRNA quantification (in 6 patients) and

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immunohistochemical evaluation (in 10 patients)] and adjacent liver tissue (for cirrhosis confirmation) from the surgical specimen. The collected tissue was divided into two fragments: one was immediately placed in RNAlater (Ambion) and stored at −80°C for mRNA isolation and quantification; the other was fixed in 10% buffered formalin and embedded in paraffin for histologic and immunhistochemical analyses.

mRNA isolation and quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2

Total mRNA was extracted from tissue samples using the TriPure isolation reagent according to the manufacturer's instructions (Roche, Germany). Concentration and purity were assayed by spectrophotometry (Eppendorf 6131000.012). Two-step real-time RT-PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and the PCR threshold cycle of graded dilutions from a randomly-selected sample from reference group. For relative quantification of specific mRNA levels, 100 ng of total mRNA from each sample underwent two-step real-time RT-PCR. GAPDH mRNA levels were similar in all experimental groups, which enabled the use of this gene as an internal control. RT (20 μl; 10 min at 22°C, 50 min at 50°C and 10 min at 95°C) was performed in a standard thermocycler (Whatman Biometra 050-901). Five percent of the cDNA yield was used as a template for real-time PCR (LightCycler II, Roche) using SYBR green (Qiagen 204143), according to the manufacturer's instructions. Specific PCR primers pairs for the studied genes (GAPDH, TLR2, TLR4, NF-κB, TNF-α and COX-2) are presented in Table 1. Results of mRNA quantification were expressed as an arbitrary unit (AU) set as the average value of reference group, after normalization for GAPDH.

Immunohistochemical evaluation of TLR2 and TLR4

Tissue specimens were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. De-paraffinized tissue slides were submitted to antigen retrieval using a high temperature antigen unmasking technique in a water bath, 95°C in citrate buffer pH 6.0, for 20 min. Endogenous peroxidase activity was blocked by incubating the slides with freshly prepared 0.5% H_2O_2 in distilled water for 20 min. After washing the slides in distilled water and PBS/0.05% Tween 20 solution, immunostaining was performed using an immunoperoxidase method according to the manufacturer's instructions. The slides were incubated with normal horse serum (Vector Laboratories, Burlingame, CA, USA) 1/50 in PBS-BSA 1% at room temperature (21–23°C) for 20 min in humid chamber. Sections were then incubated with primary Ab at 4°C 16–18 h. The following primary Abs were used: rabbit polyclonal Ab anti-TLR2 (H-175, 1 : 50 dilution, Santa Cruz Biotechnology, CA, USA) and rabbit polyclonal anti-TLR4 (H-80, 1 : 100 dilution, Santa Cruz Biotechnology). The slides were then rinsed in PBS/0.05% Tween 20 solution and bound Ab was detected by applying biotinylated secondary Ab (Vectastain Universal Elite ABC Kit) for 30 min. After washing the slides with PBS/0.05% Tween 20 solution the slides were incubated with ABC reagent (Vectastain Universal Elite ABC Kit) for 30 min. The slides were washed in PBS and incubated for 7 min in 3,3-diaminobenzidine (DAB; Sigma-Aldrich, St Louis, MO, USA) 0.05 g/PBS, 0.03% H_2O_2. Following counterstaining with hematoxylin for 20 s, the slides were washed for 4 min in water, dehydrated and mounted with Entellan (Merck KGaA, Darmstadt, Germany). Normal gastric mucosa and lymph node tissue were used as negative and positive controls, respectively. An Ab diluent (non-immune IgG, TA-125-UD; Thermo Scientific) was used in some samples as additional negative control,

Table 1. Specific PCR primers pairs for the studied genes

Gene	Primers
GAPDH	F: 5′ – GGT GGT CTC CTC TGA CTT CAA CA – 3′
	R: 5′ – GTT GCT GTA GCC AAA TTC GTT GT – 3′
TLR-2	F: 5′ – GAT CCC AAC TAG ACA AAG ACT – 3′
	R: 5′ – CTG CGG AAG ATA ATG AAC ACC – 3′
TLR-4	F: 5′ – CCA TAA AAG CCG AAA GGT GAT TGT – 3′
	R: 5′ – AGA TGT GCC GCC CCA GGA C – 3′
NF-κB	F: 5′ – CCT GGA TGA CTC TTG GGA AA – 3′
	R: 5′ – TCA GCC AGC TGT TTG ATG TC – 3′
COX-2	F: 5′ – ACC GGG GGT ATA CTA CGG TC – 3′
	R: 5′ – ACG GGC CCT ATT TCA AAG AT – 3′
TNF-α	F: 5′ – GGT TTG CTA CAA CAT GGG CTA – 3′
	R: 5′ – AAG AGT TCC CCA GGG ACC TCT C – 3′

F = Forward primer, R = Reverse primer.

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Table 2. Baseline characteristics of patients

Group parameter	Reference	Hepatitis	Cirrhosis	Hepatocarcinoma
n	15	22	14	10
Age	48 ± 5	41 ± 2	48 ± 3	72 ± 2
Male/female	8/7	10/12	9/5	7/3
AST (U/l) (10-36 U/l)[1]	50 ± 7[#]	33 ± 4[#]	80 ± 14	35 ± 6[#]
ALT (U/l) (10-30 U/l)[1]	81 ± 10	44 ± 6[*,#]	108 ± 26	29 ± 5[*,#]
Bilirubin (mg/dl) (0.2-1.0 mg/dl)[1]	0.57 ± 0.15	0.59 ± 0.07	1.20 ± 0.18[§]	1.43 ± 0.10[§]
Albumin (g/dl) (3.5–5.2 g/dl)[1]	4.4 ± 0.1	4.5 ± 0.1	3.9 ± 0.2[§]	3.6 ± 0.3[§]
INR	1.04 ± 0.02	1.08 ± 0.02	1.20 ± 0.03[§]	1.22 ± 0.02[§]
HBsAg+	–	10	7	4
HBeAg+/HBeAg-	–	1/9	0/7	0/4
HBV DNA load (IU/ml)	–	3091 (<200–> 20000000)	2325340 (3300–> 20000000)	4300340 (2900–> 20000000)
HCVAb+	–	12	7	6
HCV genotype 1/2/3/4	–	7/3/0/2	4/1/1/1	5/0/0/1
HCV RNA load (IU/ml)	–	834037 (9232 –19907580)	706025 (174044 –4803266)	804029 (182055- –4803266)
METAVIR Grade A0/A1/A2/A3	–	0/13/8/1	0/0/8/6	–
METAVIR Stage F0/F1/F2/F3/F4	–	6/10/6/0/0	0/0/6/8	–

Values are presented as mean ± SEM or as median and range according to the type of distribution. [1]Normal range. $^{*}P < 0.05$ vs reference group; $^{\#}P < 0.05$ vs cirrhosis group; $^{\S}P < 0.05$ vs reference and hepatitis groups.
ALT = alanine aminotransferase, AST = aspartate transaminase, INR = XXX, HBV = hepatitis B virus, HCV = hepatitis C virus.

confirming the specificity of our protocol. We evaluated immunostaining of hepatocytes for TLR2 and TLR4 all the samples. In order to quantify TLR expression in tissue samples, three parameters were considered: (i) *sample positivity*—a sample was considered positive if hepatocytes were clearly marked by the Ab; (ii) *grade of expression*—a score of 0–3 was considered according to the number of epithelial cells marked (0 = no cells; 1 = less than 10% of epithelial cells; 2 = 10–75% cells; 3 = more than 75% cells); and (iii) *intensity of expression*—a score of 0–3 was considered according to a subjective evaluation of the intensity of marked cells (0 = no immunostaining; 1 = weak positive staining; 2 = moderate positive staining; 3 = strong positive staining). Immunohistochemical evaluation was performed independently by two experienced pathologists.

Statistical analysis

Data analysis was performed using the computer software SPSS for Windows (version 17.0; Chicago, IL, USA). Data are presented as mean ± standard error of mean (SEM) or as median and range, according to the type of distribution. Student's *t*-test was used for comparison between groups. When necessary, the test was preceded by a natural logarithm transform to obtain a normal distribution. Correlation between TLR2 and TLR4 mRNA expression and viral load, necroinflammatory activity or transaminases levels was evaluated by univariate analysis. Statistical significance was set at $P < 0.05$.

Results

Baseline characteristics of patients

The baseline characteristics of patients are shown in Table 2. A total of 61 patients were included in the study: 15 patients in reference group, 22 (10 HBsAg+ and 12 HCVAb+) patients in hepatitis group, 14 (7 HBsAg+ and 7 HCVAb+) patients in cirrhosis group and 10 (4 HBsAg+ and 6 HCVAb+) patients in hepatocarcinoma group. Histologic findings in reference group included: steatohepatitis (seven patients); macrovesicular steatosis (three patients); perivenular cholestasis (two patients); granulomatous hepatitis (two patients); normal findings (one patient). Patients with steatohepatitis or macrovesicular steatosis were considered to have NAFLD. As expected, the cirrhosis and hepatocarcinoma groups had significantly higher levels of bilirubin and INR and lower levels of albumin.

mRNA expression of TLR2, TLR4, NF-κB, TNF-α and COX-2

Quantifications of TLR2, TLR4, NF-κB, TNF-α and COX-2 mRNA are shown in Figure 1. In the hepatitis group, expression of TLR2 (2.66 ± 0.69, $P = 0.04$) and TLR4 (3.11 ± 0.79, $P = 0.03$) were greatly increased.

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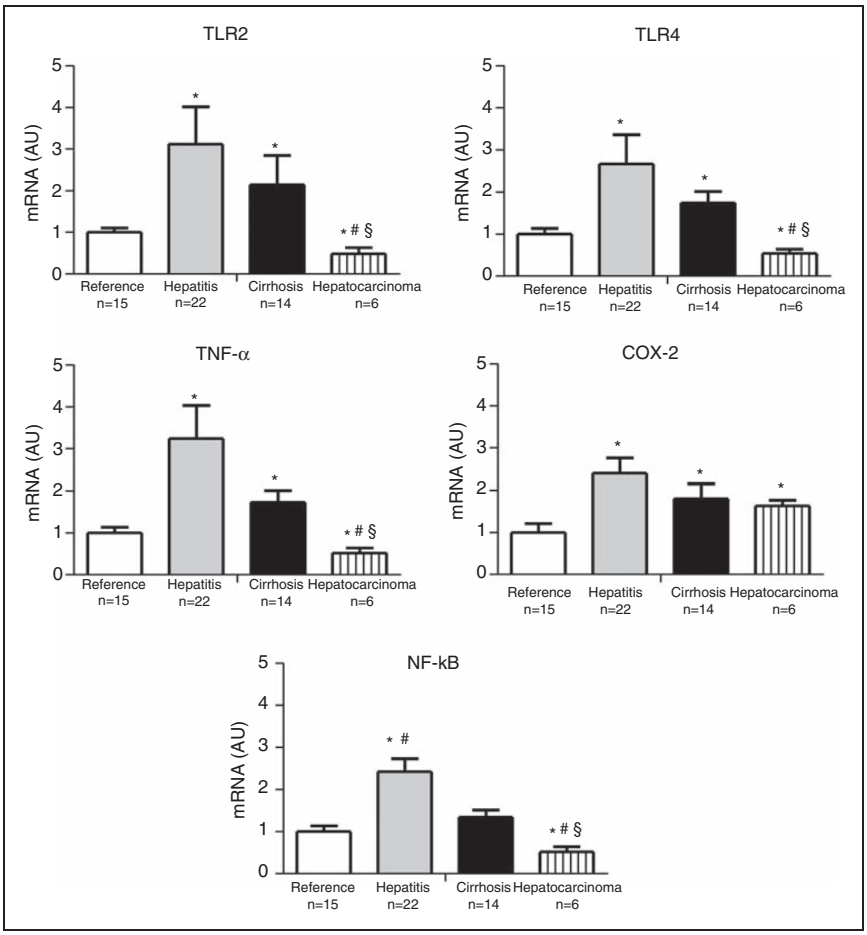

Figure 1. mRNA quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2, in the reference (mainly composed of patients with NAFLD), hepatitis (composed of patients with chronic hepatitis B or C), cirrhosis (composed of patients with post-chronic hepatitis B or C cirrhosis) and hepatocarcinoma (composed of patients with post chronic hepatitis B or C cirrhosis-related hepatocarcinoma) groups. Levels of mRNA are expressed as arbitrary unit (AU) set as the average value of control group after normalization for GAPDH. Results are presented as mean ± standard error of mean (SEM). $^{*}P < 0.05$ vs reference group; $^{\#}P < 0.05$ vs cirrhosis group; $^{\S}P < 0.05$ vs hepatitis group.

This was associated with increased expression of NF-κB (2.42 ± 0.31, $P = 0.0003$), TNF-α (3.24 ± 0.79, $P = 0.02$) and COX-2 (2.47 ± 0.36, $P = 0.003$). Compared to the reference group, this increased inflammatory profile (with exception of NF-κB) persisted in the cirrhosis group (TLR2: 2.14 ± 0.5, $P = 0.04$; TLR4: 1.74 ± 0.27, $P = 0.008$; NF-κB: 1.34 ± 0.17, $P = 0.3$; TNF-α: 1.73 ± 0.28, $P = 0.009$; COX-2: 1.8 ± 0.35, $P = 0.04$), despite a global, but not significant (except for NF-κB), decrease in expression of all the genes when compared with the hepatitis group. In the hepatocarcinoma group, all samples were positive for all studied genes. Compared with the reference group, the hepatocarcinoma group presented a higher mRNA expression of COX-2 (1.63 ± 0.15; $P = 0.02$), similar to the hepatitis and cirrhosis groups, and lower mRNA expression of TLR2 (0.48 ± 0.15), TLR4 (0.54 ± 0.10), NF-κB (0.52 ± 0.12) and TNF-α (0.52 ± 0.12; $P = 0.01$, all genes). There were no differences between chronic hepatitis B or C patients ($P > 0.05$, all genes). We did not find any difference or tendency when comparing the genetic profile between F0, F1 or F2 patients ($P > 0.05$, all genes). No correlation was seen between mRNA expression of any gene and viral load, necroinflammatory activity or transaminases levels.

Immunohistochemical evaluation of TLR2 and TLR4

All the samples, including those from the hepatocarcinoma group, were positive for TLR2 and TLR4. Compared with the reference group, TLR2 and TLR4

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expression grade was maintained in the hepatitis group and reduced in the cirrhosis and hepatocarcinoma groups, while TLR2 and TLR4 expression intensity was increased in the hepatitis and cirrhosis groups. Moreover, in the reference group, cytoplasmic staining of hepatocytes for TLR2 and TLR4 was very heterogeneous, while in the hepatitis and cirrhosis groups, hepatocytes showed a diffuse cytoplasmic staining for TLR2 and TLR4. Compared with the hepatitis and cirrhosis groups, TLR2 expression grade and intensity and TLR4 expression grade were reduced, while TLR4 expression intensity was maintained in the hepatocarcinoma group. In most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining alternating with poorly differentiated areas with low staining. There were no differences between chronic hepatitis B or C patients ($P > 0.05$, all proteins). These data are presented in Figure 2 and Table 3.

Discussion

In the present study, we evaluated the expression of TLR2 and TLR4 in liver samples from patients in each stage of virus-induced hepatic IFC sequence. We found increased TLR2 and TLR4 mRNA and protein expression in virus-induced chronic hepatitis and cirrhosis, and a maintained TLR2 and TLR4 protein expression in virus-induced hepatocarcinoma.

Although there are several animal and *in vitro* studies implicating TLRs in the pathogenesis of hepatic IFC sequence, studies using diseased human liver tissue are scarce. Hepatic expression of TLR2 was shown to be maintained in early- and late-stage biliary atresia[25] and HCV cirrhosis,[26,27] increased in primary biliary cirrhosis (PBC) and non-alcoholic steatohepatitis (NASH),[28] and decreased[27] or maintained[26] in alcoholic cirrhosis. Hepatic expression of TLR4 was shown to be maintained in early- and late-stage biliary atresia[25], alcoholic and HCV cirrhosis,[26,27] and increased in PBC[28] and NASH.[28,29] Besides contradictory results, none of these studies have evaluated simultaneously TLR expression in different stages of liver disease.

To our knowledge the present study is the first to evaluate hepatic TLR2 and TLR4 expression at different stages of the virus-induced hepatic IFC sequence. We found increased TLR2 and TLR4 mRNA and protein expression in virus-induced chronic hepatitis and cirrhosis and a maintained TLR2 and TLR4 protein expression in virus-induced hepatocarcinoma. Thus, upregulation of TLR2 and TLR4 is an early, and persistent, event in the virus-induced hepatic IFC sequence.

Regarding TLR2 and TLR4 protein expression, the differences between the groups were more evident in terms of intensity of expression (which reflects the level of expression per cell) than in terms of grade of expression (which reflects the number of cells expressing the protein). The intensity of TLR2 and TLR4 proteins expression was in line with TLR2 and TLR4 mRNA expression, while the grade of TLR expression changed little between the groups, not accompanying the changes in mRNA expression. This finding suggests that the virus-induced hepatic IFC sequence is associated with changes in the level of TLR2 and TLR4 protein expression per cell and not with changes in the number of cells expressing these proteins.

Interestingly, we found reduced TLR2 and TLR4 mRNA and protein expression in the hepatocarcinoma group when compared with the hepatitis and cirrhosis groups. Although we have no definite explanation for the reduced expression of TLR2 and TLR4 in hepatocarcinoma cells, we believe it is likely a consequence of loss of differentiation of hepatocarcinoma cells. This is suggested by the finding that in most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining alternating with poorly differentiated areas with low staining. This finding likely means that at late stages of the hepatic IFC sequence, the role of these receptors in this sequence becomes smaller.

However, our study has some limitations. Firstly, most patients included in the reference group have evidence of NAFLD and it was demonstrated that NAFLD is associated with increased hepatic TLR2 and TLR4 mRNA expression.[28,29] This suggests that the increase in hepatic expression of TLR2 and TLR4 in chronic hepatitis, cirrhosis and hepatocarcinoma may, in fact, be underestimated. Moreover, the hepatitis, cirrhosis and hepatocarcinoma groups included both patients with HBV infection or HCV infection. Nonetheless, statistical analysis revealed no difference between HBV and HCV patients. Moreover, as we included only patients with virus-induced chronic hepatitis in this study, our data cannot be generalized to other chronic hepatic diseases that follow IFC sequence. Another limitation of our study is that the method we used for quantification of protein expression was semi-quantitative. Although this could contribute to the lack of difference in terms of grade of protein expression, we were able to obtain significant differences in terms of intensity of protein expression. The validity of the protein quantification by this method is supported by the overall agreement between protein (grade of expression) and mRNA data. Furthermore, the method was validated in a previous study with consistent results.[30]

In the present study we have not explored the mechanisms underlying increased hepatic expression of TLR2 and TLR4. Nonetheless, previous studies have shown that HBV and HCV may upregulate TLR2 and TLR4 through direct and indirect mechanisms. *In vitro* studies have shown that HCV nonstructural protein NS5A upregulates TLR4 expression and that HBeAg upregulates TLR2 expression.[23,31] In chronic hepatitis

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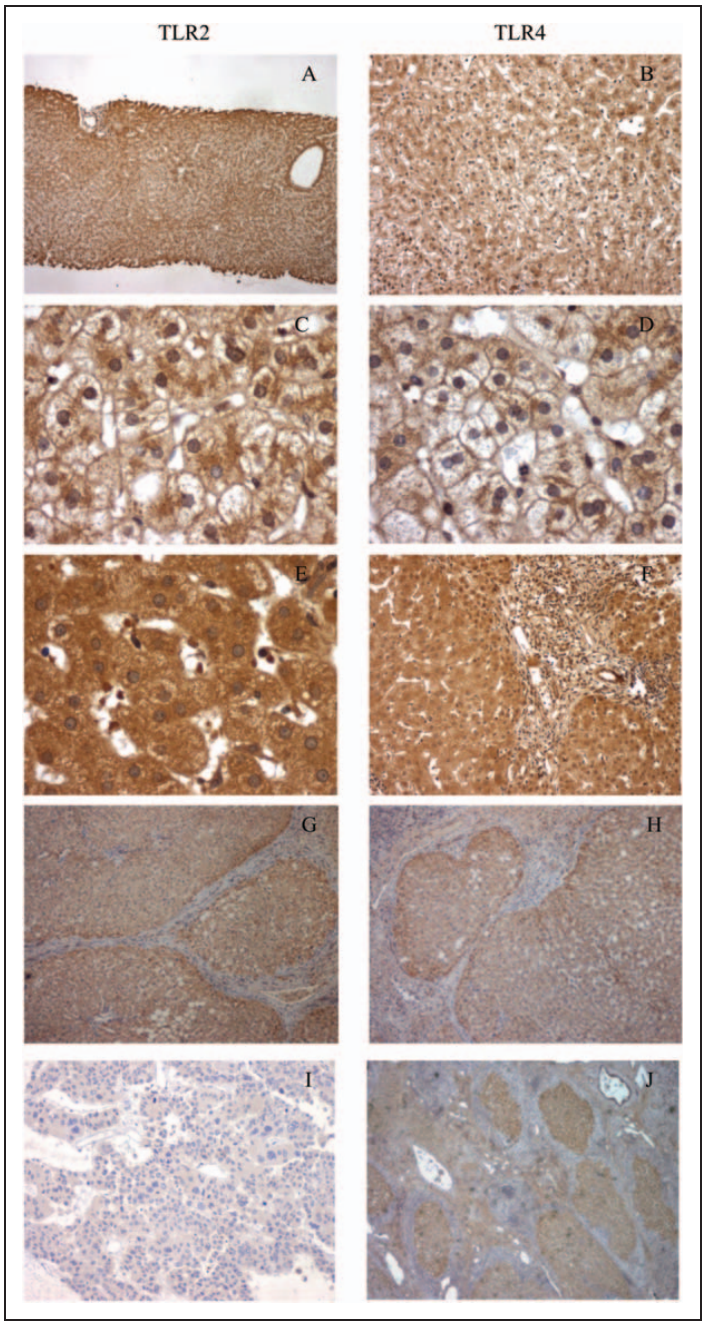

Figure 2. Immunohistochemical evaluation of TLR2 (left) and TLR4 (right). (A, B) Low power field magnification for TLR2 and TLR4 in the reference group (mainly composed of patients with NAFLD): there is a higher intensity staining in acinar zones 1 and 3 than in acinar zone 2. (C, D) High power field magnification for acinar zone 2 in the reference group showing sparse cytoplasmatic staining. (E, F) High and low power field magnification, respectively, in chronic hepatitis B or C (acinar zones 1 and 2) showing diffuse cytoplasmatic staining (in contrast with A–D images). (G, H) Low power field magnification in post-chronic hepatitis B or C cirrhosis: all hepatocytes are stained with a diffuse cytoplasmic staining. (I, J) Low power field magnification in post-chronic hepatitis B or C cirrhosis-related hepatocarcinoma: low intensity staining in poorly-differentiated areas of hepatocarcinoma with multiple bizarre cells (I) in contrast to nodular well-differentiated areas with high intensity staining in hepatocarcinoma cells (J). In most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining (J) alternating with poorly-differentiated areas with low staining (I).

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Table 3. Immunohistochemichal evaluation of TLR2 and TLR4: grade and intensity of expression

Group parameter	Reference ($n = 15$)	Hepatitis ($n = 22$)	Cirrhosis ($n = 14$)	Hepatocarcinoma ($n = 10$)
TLR2 grade	3.0	3.0	2.87 (2.80–2.94)	2.2 (2.00–2.40)
TLR2 intensity	1.73 (1.55–1.91)	2.27 (2.17–2.37)	2.37 (2.25–2.49)	1.9 (1.73–2.07)
TLR4 grade	3.0	3.0	2.87 (2.80–2.94)	2.6 (2.44–2.76)
TLR4 intensity	1.8 (1.63–1.97)	2.41 (2.31–2.51)	2.44 (2.22–2.36)	2.1 (1.87–2.33)

Values are presented as mean (95%CI).

B and C, besides up-regulated expression of TLR2 and TLR4 by the virus, other factors, such as augmented exposure to their ligands, can also contribute to increased activation of these TLRs, especially in later stages of hepatic fibrosis and cirrhosis. In fact, several studies have demonstrated that bacterial translocation is increased in patients with cirrhosis, resulting in augmented exposure of hepatic TLRs to their ligands.[10,11]

Herein, we did not search for liver cell-specific expression of TLR2 and TLR4, but instead we have focused on total hepatic mRNA expression and protein expression of hepatocytes. This may be an important issue as it has been demonstrated that HCV and HBV may affect TLRs expression in a cell-specific manner.[2–5] Nevertheless, the immunohistochemistry that we performed in this study suggests that, at least in part, this increase in TLRs expression occurs significantly in hepatocytes.

Previous studies on human samples have shown that hepatic expression of TLR2 and TLR4 in HCV cirrhosis was unchanged compared with the reference group, which is in disagreement with our results.[26,27] The reasons for this disagreement are unclear, but our data are consistent with a previous *in vitro* study showing that hepatocyte-specific transgenic expression of the HCV nonstructural protein NS5A upregulates TLR4 expression.[23] Regarding chronic hepatitis B, Visvanathan et al.[31] have shown that expression of TLR2 on hepatocytes and Kupffer cells was significantly reduced in patients with HBeAg-positive chronic hepatitis B in comparison with HBeAg-negative chronic hepatitis B and controls, whereas it was significantly increased in HBeAg-negative chronic hepatitis B compared with controls. The level of TLR4 expression did not differ significantly among the groups. Downregulation of TLR2 was also demonstrated in HepG-2 cells transduced with wild-type HBV (HBeAg-positive) but not in cells transduced with pre-core mutant HBV (HBeAg-negative). Regarding TLR2, our data are consistent with the study by Visvanathan et al.[31] as most of our chronic hepatitis B patients were HBeAg-negative and have increased TLR2. We could not compare HBeAg-positive and HBeAg-negative patients owing to the limited number of HBeAg-positive patients in our study.

In order to clarify TLR2- and TLR4-induced proinflammatory genes expression we also studied NF-κB, TNF-α and COX-2 mRNA expression. We found that hepatic TNF-α and COX-2 mRNA expressions are increased in virus-induced chronic hepatitis and cirrhosis, whereas hepatic NF-κB mRNA is increased in virus-induced chronic hepatitis, but maintained in virus-induced cirrhosis. This is an interesting finding as these proinflammatory genes have been implicated in hepatic inflammation, fibrogenesis and carcinogenesis interplay.[1,32,33] Interestingly, hepatocarcinoma samples presented increased COX-2 expression, despite lower expression of other studied genes, suggesting, in line with previous studies, that this enzyme may have an important role in hepatocarcinogenesis.[33] Although we have not investigated the functionality of TLR2 and TLR4, increased expression of TLR2 and TLR4 proteins and of NF-κB, TNF-α and COX-2 (key mediators of TLR2 and TLR4 signaling pathway) mRNA expression suggest augmented signaling of TLR2 and TLR4. When comparing hepatitis with cirrhosis we found a tendency towards lower expression of inflammatory genes mRNA; however, we cannot dismiss the possibility that this tendency is not related to a higher inflammatory cell infiltrate observed in the hepatitis group. In fact, we did not find any clear tendency in the hepatocyte immunohistochemistry results, suggesting that the difference, if any, between the two groups is not significant.

In summary, in patients with HCV or HBV chronic infection, hepatic expression of TLR2 and TLR4 is increased in chronic hepatitis and cirrhosis and is maintained in hepatocarcinoma. This is associated with increased TLR2- and TLR4-induced proinflammatory gene expression. Overall, this study suggests that TLR2 and TLR4 may be key players in the human hepatic IFC sequence associated with viral chronic hepatitis.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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CHAPTER IV – TOLL-LIKE RECEPTORS AND GASTRIC DISEASE

"An army marches on its stomach"

Napoleon Bonaparte (1769-1821)

A) INCREASED EXPRESSION OF TOLL-LIKE RECEPTORS 2, 4 AND 5 IN GASTRIC DYSPLASIA

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RESEARCH

Increased Expression of Toll-like Receptors (TLR) 2, 4 and 5 in Gastric Dysplasia

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Abstract TLRs are important innate immunity receptors. Even though TLR2, 4 and 5 appear to be important for *Helicobacter pylori* (HP) recognition, their role in the evolution of gastritis to more advanced lesions is still unknown. To compare the expression of TLR2, 4 and 5 in normal gastric mucosa, HP+ gastritis, intestinal metaplasia, dysplasia and adenocarcinoma. Immunohistochemistry for TLR2, 4 and 5 was performed with anti-TLR2-TLR4-TLR5 antibodies in 117 histological samples of normal gastric mucosa (n=22), HP+ gastritis (n=20), intestinal metaplasia (n=33), dysplasia (mucosectomy specimens, n=20) and intestinal type adenocarcinoma (surgery specimens,n=22); quantification of expression was performed independently by two pathologists taking into account the percentage of positive epithelial cells and the degree of expression (zero to three score). A statistically significant trend for progressive increase of TLRs expression from normal mucosa to gastric dysplasia was found (mean expression: normal mucosa 0.1; gastritis 1.0; metaplasia 2.2; dysplasia 2.8, $p<0.01$). All dysplasia samples presented more than 90% positive epithelial cells with strong expression (2.8;95%CI2.7–3). There was less TLRs expression in carcinomas (TLR2:1.0; TLR4:2.0 and TLR5:1.2, $p<0.05$) when compared with dysplasia, with TLR4 being more expressed than TLR2 and 5 in these lesions (p=0.03). A score of all markers' expression of eight leads to a low (4%) false positive rate in patients with precancerous conditions. Progression of gastric lesions associated with gastric carcinogenesis is associated with increased TLRs expression. Gastric dysplasia presents a high level of TLRs expression, suggesting that these receptors may play a role in adenocarcinoma development.

Keywords Gastric pathology · Dysplasia · Innate immunity receptors · TLRs

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Introduction

The innate immune system by recognizing several conserved microbial antigens is the first line of defense against infection, initiating in this way protective immunological responses [1, 2]. The toll-like receptors (TLRs) are the most important class of pathogen-associated molecular patterns (PAMPs) receptors, with ten different TLRs being ubiquitously expressed in humans [2–5]. TLRs are membrane-surface receptors consisting of a distinct leucine-rich repeat (LRR) extracellular domain that confers specificity to the receptor, and a conserved toll/interleukin 1 (IL1) receptor

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(TIR) intracellular domain [3, 4]. Of the several identified TLRs described, the TLR2, TLR4 and TLR5 subtypes are critically involved in immune responses to bacterial infections, being abundantly expressed in immune cells [6, 7]. In general, TLR2 recognizes PAMPs mainly from Gram positive bacteria, TLR4 is the receptor for Gram negative bacteria lipopolysacharide (LPS) and TLR5 recognizes bacterial flagellin [2, 8].

Helicobacter pylori (HP) is a Gram-negative bacterium that adheres to the surface of gastric mucosa, causing marked inflammation without invasion of gastric epithelial cells [9]. It is believed that HP is a major risk factor for intestinal-type gastric cancer. By promoting a chronic gastric inflammatory state, HP appears to initiate a carcinogenesis sequence that involves chronic gastritis, intestinal metaplasia (IM), gastric dysplasia and, finally, intestinal-type gastric adenocarcinoma [10–15]. However, it appears that once this process begins it could be independent of HP status since premalignant lesions such as IM present irreversible genetic alterations that can promote progression to cancer without HP presence [16–18].

It is clear that TLRs are essential for HP recognition and subsequent innate and adaptive immunity against this bacterium [19]. Several TLRs may play a role in gastric immunologic response to HP [19]. TLR2 appears to be the receptor responsible for most of the inflammatory process that occurs as the result of HP infection [19–21]. However, other studies suggest that TLR4 also play an important part in HP infection by recognizing several HP antigens [22–24]. Concerning TLR5, the data are contradictory with some studies suggesting interaction between HP flagellin and this receptor[25, 26], and others demonstrating that TLR5 is unresponsive to HP flagellin [27–29]. Nevertheless, the role of these receptors in gastric carcinogenesis may go beyond HP infection, since they have been associated to different cancers [19].

Despite the importance of TLR in the inflammatory activation to HP infection and in several oncogenic lines, its role in the progression of the lesions associated with gastric carcinogenesis remains largely unknown [19]. In the present study, TLR2, TLR4 and TLR5 expression was evaluated by immunohistochemistry in normal gastric mucosa, chronic gastritis, intestinal metaplasia, gastric dysplasia and in intestinal-type gastric adenocarcinoma in an attempt to better understand the potential role of those receptors in gastric carcinogenesis.

Material and Methods

Participants and Histological Samples

Data base of the institution, year 2004, was searched for all the gastric lesions to be studied. A total of 20 samples per lesion were estimated to be necessary. Samples of normal gastric mucosa (n=22), chronic active HP gastritis (n=20), complete (n=16) and incomplete IM (n=17) were obtained by endoscopy biopsy. Endoscopic mucosectomy tissue specimens (n=20) were considered for investigation of gastric dysplasia and surgical tissue specimens (n=22) were considered for intestinal-type gastric adenocarcinoma. HP was present in 13 IM (39%) samples and in 5 (20%) mucosectomy samples. After selection, all the samples (n=117) were reevaluated and, whenever necessary, reclassified by an independent pathologist.

The study protocol was approved by the Ethics Committee of Portuguese Oncology Institute, Porto.

Immunohistochemistry

Tissue specimens were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. Deparaffinized tissue slides were submitted to antigen retrieval using a high temperature antigen unmasking technique in a water bath, 95° in citrate buffer pH6.0, for 20 min. Endogenous peroxidase activity was blocked by incubating the slides with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 min. After washing the slides in distilled water and PBS/0.05% Tween 20 solution, immunostaining was performed using an immunoperoxidase method according to de manufacturer's instructions. The slides were incubated with normal horse serum (Vector Laboratories, Burlingame, CA,USA) 1/50 in PBS-bovine serum albumin (BSA) 1% at room temperature for 20 min in humid chamber. Sections were then incubated with primary antibody at 4°C overnight. The following primary antibodies were used: rabbit polyclonal antibody anti-TLR2 (H-175,1:50 dilution, Santa Cruz Biotechnology, California, USA), rabbit polyclonal anti-TLR4 (H-80, 1:100 dilution, Santa Cruz Biotechnology, California, USA) and rabbit polyclonal anti-TLR5 (H-127, 1:100 dilution, Santa Cruz Biotechnology, California, USA). The slides were then rinsed in PBS/0.05% Tween 20 solution, and bound antibody was detected by applying biotinylade secondary antibody (Vectastain Universal Elite ABC Kit) for 30 min. After wash the slides with PBS/0.05% Tween 20 solution the slides were incubated with ABC reagent (Vectastain Universal Elite ABC Kit) for 30 min. The slides were washed in PBS and incubated for 7 min in 3,3-diaminobenzidine (DAB; Sigma-Aldrich, USA) 0.05 g/PBS, 0.03%H_2O_2. Following counterstaining with hematoxylin for 20 s, the slides were washed for 4 min in water, dehydrated and mounted with Entellan (Merck KGaA, Darmstadt, Germany). Normal gastric mucosa and lymph node tissue were used as negative and positive controls, respectively.

Immunohistochemical Evaluation and TLRs Expression Quantification

In order to quantify TLRs expression in tissue samples three parameters were considered: 1. *Sample positivity*: A sample was considered positive if gastric epithelial cells were clearly stained by the antibody. The results were presented as a proportion (positive samples/total samples of a specific lesion); 2. *Grade of expression*: A score of 0 to 3 was considered according to the number of epithelial cells stained (0—no cells; 1—less than 10% of epithelial cells; 2—10–75% cells; 3—more than 75% cells); 3. *Intensity of expression*: A score of 0 to 3 was considered according to a subjective evaluation of the intensity of stained cells (0—no staining; 1—weak positive staining; 2—moderate positive staining; 3—intense positive staining). The mean of the grade with the intensity of expression was considered as the *final expression score*.

All the samples were evaluated and quantified by two independents pathologists.

Statistical Analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences-SPSS for Windows (version 17.0). Data was presented as mean ± 95% confidence interval (95%CI) or as a proportion of positive samples. One way ANOVA and Student's t test for paired and unpaired data (or correspondent non-parametric test) were used, when appropriate, for comparison between groups. To test the difference of positivity among groups a linear-by-linear association for the binary values was used. In order to evaluate the tendency for increase or decrease expression, t test for trend was used. Statistical significance was set at $p<0.05$. Hypothesizing the use of relative expression of TLR2, 4 and 5 to help in the diagnosis of dysplasia or invasiveness, a score was then calculated by the sum of the mean score for each marker, varying between 0 and 9. The best cutoff for the diagnosis of lesions as severe as dysplasia, for the diagnosis of dysplasia and for the diagnosis of invasive cancer were described and estimates of sensitivity and specificity for each outcome calculated.

Results

Positivity of the Samples for TLRs Expression

Figure 1 depict the results of TLR2, 4 and 5 immunoexpression for the different tissue samples. The proportion of positive samples in normal gastric mucosa was very low for all TLRs (5–14%). When HP was present these values were significantly higher (50–75%, $p<0.01$ Vs normal mucosa). Almost all the metaplasia and all dysplasia samples were TLRs positive ($p<0.01$ Vs normal or HP gastritis). When carcinoma was considered, we found a significant decrease of positive samples for TLR2 and TLR5 (55% and 64%, respectively, $p<0.05$ Vs metaplasia or dysplasia) but not for TLR4 (86%, $p=0.08$). This occurred because carcinoma were more frequently positive for TLR4 than for TLR2 or TLR5 ($p<0.05$).

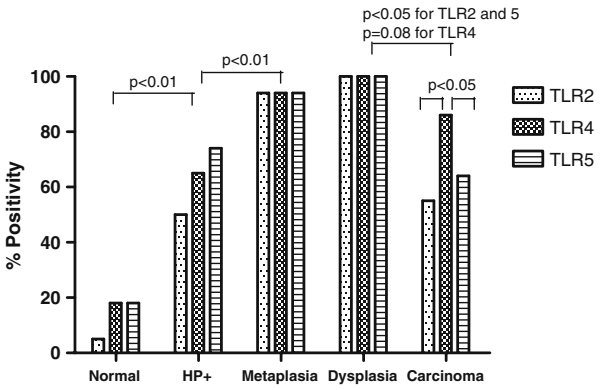

Fig. 1 Proportion of TLRs positive samples (gastric epithelium) in the different gastric lesions

TLRs Expression in the Different Gastric Lesions

In Fig. 2, TLRs expression in the different gastric lesions is shown. Normal gastric mucosa weakly expressed all TLRs (0.15; 95%CI 0.0–0.3). HP gastritis had increased TLRs expression (five to ten fold higher expression for all TLRs, $p<0.001$), still, with a weak expression (mean expression of 10% gastric epithelial cells and weak intensity of expression in the

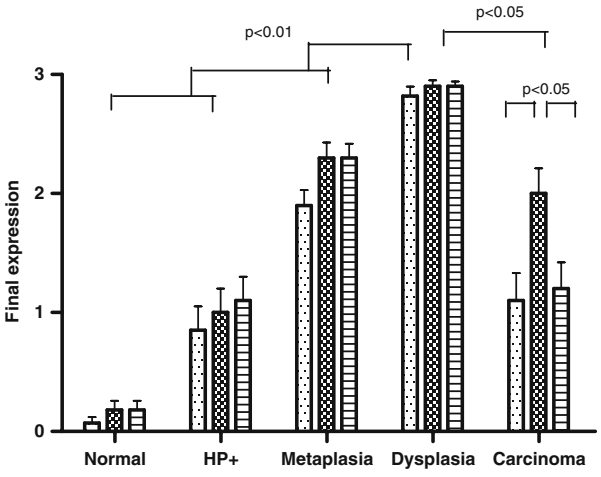

Fig. 2 TLRs final expression (mean and 95%CI) in the different gastric lesions

majority of the samples (1.0; 95%CI 0.6–1.4)). TLRs were strongly expressed in almost all areas of intestinal metaplasia (>75% gastric epithelial cells and moderate intensity of expression (2.2; 95%CI 1.8–2.6)), with no differences between complete or incomplete metaplasia (2.1 vs 2.2, p=0.8). There were also no differences between IM with or without HP (2.2 vs 2.0, p=0.5). More important, in dysplasia, TLRs expression was maximum in all areas (>90% gastric epithelial cells, strong intensity of expression (2.8; 95%CI 2.7–3)) and clearly superior to all the other gastric lesions (p<0.01). These results in dysplasia lesions were completely independent of HP status (p=0.9). In intestinal-type adenocarcinoma, some tumors had a high level of TLRs expression in almost all the cells with a strong intensity. Others, however, showed a very weak expression for one or all TLRs. Nevertheless, TLR4 expression in tumors was higher than TLR2 or TLR5 (2.0 Vs 1.0 or 1.2, respectively, p<0.05). There was a statistical significant trend for increase of TLRs expression from normal mucosa to gastric dysplasia (p<0.01).

Cellular Distribution of TLRs

Gastric epithelium of normal mucosa, with or without HP, expressed all TLRs in a polarized manner, particularly at the basolateral membrane but also at the apical membrane (Fig. 3a). On the other hand, metaplasia, dysplastic and neoplastic epithelial cells expressed all TLRs diffusely and homogeneously throughout the cytoplasm with no apparent polarization (Fig. 3b–f).

Score for the Diagnosis of Dysplasia/Cancer Using TLRs Relative Expression

When adding the relative expression of TLR 2,4 and 5, the presence of a score of 1 seems to leads to a very low rate of

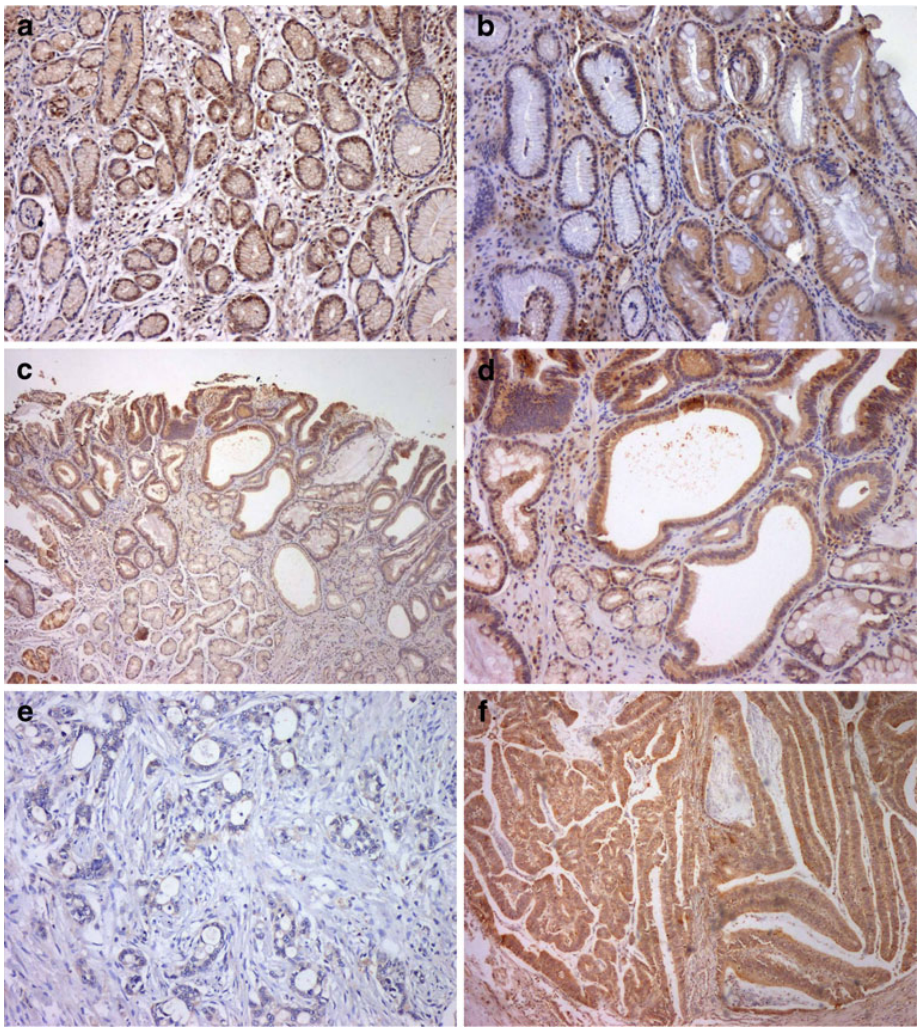

Fig. 3 Immunohistochemistry images of the different lesions. **a** HP+ gastritis—Weak to moderate and polarized expression in this case for TLR2 (similar to the others TLRs); **b** Normal and metaplasia—In the left, normal mucosa with polarized and very weak TLR5 expression, with the transition in the right for intestinal metaplasia with diffusely and moderate to strong TLRs expression; **c** (low power field) and **d** (high power field) Gastric dysplasia—In this mucosectomy specimen we can see the rising levels of TLR4 expression from normal mucosa (*down*), metaplasia (*right*), to dysplasia (*up*) that presents a very strong, diffuse, expression in almost all epithelial cells; **e** and **f** adenocarcinoma—Some tumors presented very weak expression (**e**) and others presented a very strong, diffuse expression (**f**), in this case for TLR2

Table 1 Score for the diagnosis of dysplasia and cancer obtained by adding the relative expression of TLR 2,4 and 5

Diagnosis	N	AUC (95%CI)	Cutoffs	Sensibility	Specificity
At least dysplasia	117	0,75 (0,65-0,85)	1[c]	100	30
			8[c]	40	96
Dysplasia vs other lesions[a]	95	0,95 (0,91-0,99)	1[c]	100	30
			8[c]	75	96
Invasive cancer[b]	42	0,94 (0,00-1,00)	1[d]	100	9
			8[d]	91	75

[a] Patients with invasive cancer were excluded

[b] Among patients with neoplasia

[c] The cutoff presented means that individuals with less than that value in the Cumulative score would be considered as having no outcome (dysplasia), whereas those with the cutoff value or more, would be considered with lesions as severe as dysplasia

[d] The cutoff value means that individuals with less than that value would have invasive cancer

false negative for lesions as severe as dysplasia. To adequately identify dysplasia a score of 8 seems to be very useful as it leads to a very low false positive rate (4%) in patients with precancerous conditions and also to a low false positive rate (missing invasiveness) when distinguishing dysplasia from invasive cancer (Table 1). Figure 4 shows the respective Receiver Operating Curves.

Discussion

In the present study, TLR2, TLR4 and TLR5 immunoexpression was evaluated in gastric lesions associated with gastric carcinogenesis. A significant increase of TLRs expression from normal mucosa to gastric dysplasia was found. Intestinal-type adenocarcinoma also presented significant expression of these receptors, particularly for TLR4.

Previous reports described TLRs expression in gastric lesions [23, 24, 30, 31]. Similar to our study, Schmausser et al. [31] suggested that HP augments TLRs expression in gastric mucosa and that metaplasia and carcinoma had more TLRs expression than normal mucosa. However, owing to a low number of histologic samples, they were unable to quantitatively compare TLRs expression between the different gastric lesions. Our study clearly showed that TLRs present a gradual increase of expression from normal mucosa to gastric dysplasia, with these lesions presenting more than 90% of epithelial cells with strong positivity for these receptors. Moreover, contrarily to normal mucosa, IM and dysplasia lesions presented diffuse positivity of these receptors, which may suggest an easier activation of these receptors.

Some limitations can be pointed to our study. First, quantification of expression was done only by immunohistochemistry. Second, the scores for TLRs expression were

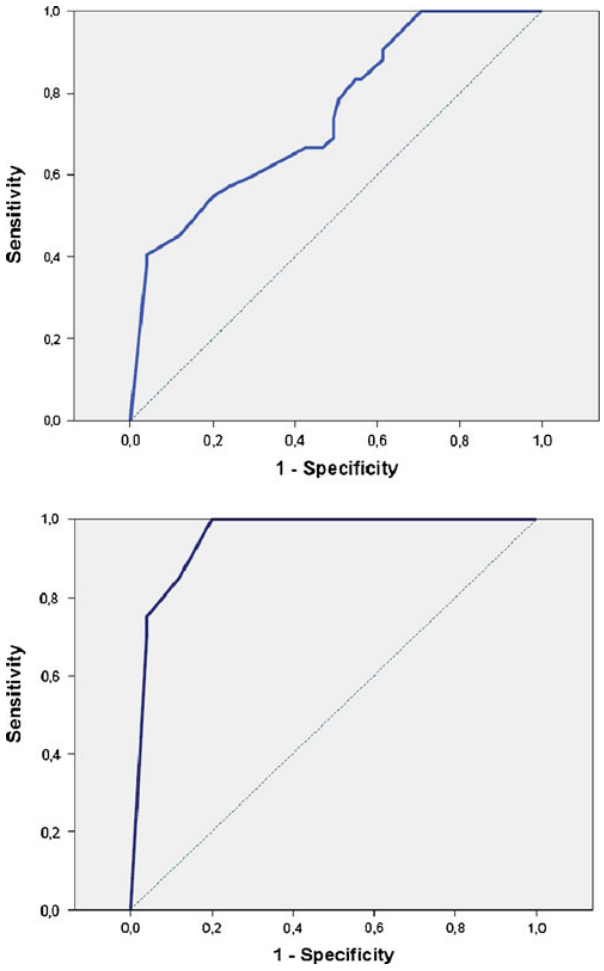

Fig. 4 Receiver operating curves for the diagnosis of neoplastic lesions (*up*) or Dysplasia versus other lesions excluding invasive adenocarcinoma (*down*) using the cumulative score of TLRs expression

subjective. However, samples were evaluated by two independent and expert gastrointestinal pathologists, that come to similar results. On the other way, a strong aspect of our study was the inclusion of mucosectomy samples. In fact, in these samples we clearly observed all the spectrum of gastric lesions, and consistently distinguished the different levels of expression in these lesions, supporting our results. Moreover, we have shown that a score obtained by adding the relative expression of TLR 2,4 and 5 may have diagnostic value since to adequately identify dysplasia a score of 8 was very useful leading to a very low false positive rate (4%) in patients with precancerous conditions and also to a low false positive rate (missing invasiveness) when distinguishing dysplasia from invasive cancer.

What can we learn from these results? In order to maintain gastrointestinal homeostasis it appears that gastric epithelium, similar to colonic epithelium, has a very low expression of TLRs, fundamentally confined to the basolateral membrane [32–34]. HP appears to initiate a cascade that leads to chronic infection and increase TLRs expression in gastric epithelial cells. Chronic infection promotes phenotypic change to gastric IM, which, as we have seen, has a high and diffuse TLRs expression. We can speculate that at this phase the presence of HP is not absolutely necessary for epithelial stimulation. Actually, gastric epithelium is exposed daily to innumerous bacteria that, despite not being able to initiate a gastric infection like HP, have the potential to stimulate these diffusely overexpressed receptors. In fact, some studies show that, when stimulated, these receptors lead to the production of several cytokines and growth factors as well as to an increase in COX-2 expression, conferring an important oncogenic potential to these receptors [35, 36]. Dysplasia, by presenting even more TLRs expression, can accelerate these processes, leading to the development of gastric adenocarcinoma. Confirming the potential role of these receptors in the progression of gastric lesions, some studies associated TLR4 and TLR2 polymorphisms with the severity of gastric lesions [37–41]. Future studies should evaluate if blockage of TLRs can delay progression of lesions and carcinoma development.

Concerning adenocarcinoma, we found that a large number of tumors significantly express these receptors, particularly TLR4. Others showed that TLRs stimulation in gastric tumor cells can induce several gastric carcinoma promoting factors leading to proliferation and progression of gastric cancers [42–44]. Taking together these observations, it is possible that TLRs expression in gastric tumors can influence prognosis and that antagonists of these receptors can have therapeutic value. Future studies should have these aspects in consideration.

In conclusion, progression of gastric lesions associated to gastric carcinogenesis is accompanied by a progressive increase of TLRs expression in gastric epithelial cells. Gastric dysplasia presents a very high level of TLRs expression, suggesting that these receptors may have a role in carcinoma development. Adenocarcinomas also present a significant expression of these receptors, which may influence tumoral progression. Molecular and functional studies are necessary to clarify the role of these receptors in gastric carcinogenesis.

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None of the authors have any disclosure.

Competing interest None to declare.

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B) HELICOBACTER PYLORI INDUCES INCREASED EXPRESSION OF TOLL LIKE RECEPTORS AND DECREASED TOLL-INTERACTING PROTEIN IN GASTRIC MUCOSA THAT PERSISTS THROUGHOUT GASTRIC CARCINOGENESIS

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Helicobacter pylori Induces Increased Expression of Toll-Like Receptors and Decreased Toll-Interacting Protein in Gastric Mucosa that Persists Throughout Gastric Carcinogenesis

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Keywords
Gastric cancer, *Helicobacter pylori*, PPARγ, TLR2, TLR4, Toll-interacting protein.

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Abstract

Background: Toll-like receptors (TLR) are essential for *Helicobacter pylori* (HP) recognition. Their role in the progression of gastric lesions leading to cancer is not established.

Aim: To evaluate for the first time in humans the expression of TLR2, TLR4, and TLR5, as well as the expression of other related molecules in the entire sequence of gastric lesions.

Methods: Biopsy samples (n = 80, 48% HP+) from normal mucosa, HP gastritis, metaplasia, dysplasia or adenocarcinoma were obtained from 44 patients. mRNA quantification of TLR2, TLR4, TLR5, Toll-interacting protein (TOLLIP), PPAR-γ, NF-κB, TNF-α, COX-1, COX-2, and CDX-2 was performed by real-time RT-PCR. TLR2, TLR4, and TLR5 protein expression was quantified by immunohistochemistry.

Results: When compared to normal mucosa (1.0 arbitrary unit (AU)), HP gastritis presented higher expression of TLR2 (2.23 ± 0.36 AU), TLR4 (1.92 ± 0.40 AU) and TNF-α (2.14 ± 0.50 AU) and lower TOLLIP and PPARγ expression (0.72 ± 0.12 AU, $p < .05$ all genes). Metaplasia and dysplasia/carcinoma presented higher expression of TLR2 (1.66 ± 0.46 and 1.48 ± 0.20 AU, respectively, $p < .05$), lower expression of TOLLIP (0.66 ± 0.09 and 0.52 ± 0.04 AU, $p < .05$) and PPARγ (0.73 ± 0.12 and 0.63 ± 0.10 AU, $p < .05$). The significant trend for decrease in TOLLIP and PPARγ was associated with increasing levels of CDX-2 from normal mucosa to carcinoma ($p < .05$), translating that in diffuse and higher TLRs protein expression ($p < .05$).

Conclusion: Gastric carcinogenesis is associated with decreasing levels of TLRs inhibitors and elevated TLRs levels throughout all the spectrum of lesions. Future studies should investigate if modulation of these receptors activity may influence gastric carcinogenesis and tumor progression.

Gastric cancer is still one of the most common cancers in the world being the fourth most common in men and the fifth in women. It remains a high lethal cancer representing 10% of all deaths for cancer [1,2]. Although multiple factors may play a role in cancer development, gastric cancer is considered the number one infection-related cancer in the world with almost 75% of all gastric cancers being attributable to *Helicobacter pylori* (H. pylori) infection [3]. Indeed, in developed countries, gastric cancer rates have decreased substantially probably because chronic *H. pylori* infection is being actively controlled, confirming *H. pylori* as the main risk factor for this tumor [4,5].

H. pylori is a Gram-negative bacterium that adheres to the surface of gastric mucosa, interacting with several innate immunity receptors such as Toll-like receptors (TLRs), and without invasion of gastric epithelial cells, it causes marked inflammation of the mucosa that

perpetuates as a chronic gastric inflammatory state [6,7]. In that way, *H. pylori* is considered to be the initiator of the Correa cascade of gastric carcinogenesis that involves chronic gastritis, intestinal metaplasia, gastric dysplasia and, finally, intestinal-type gastric adenocarcinoma [8,9].

Toll-like receptors have an important role in gastric carcinogenesis not only because they are essential for the bacteria recognition, mainly TLR2 and TLR4, but also because they appear to be overexpressed in the late stages of gastric carcinogenesis [7,10–15]. Indeed, once activated these receptors initiate inflammatory pathways that when chronically activated, such as in chronic gastritis, may acquire oncogenic potential [16–22]. For that reason, under normal physiologic state the expression of these receptors in the gastrointestinal mucosa appears to be low and the expression of several TLR-antagonists, like Toll-interacting protein (TOLLIP) and PPARγ appears to be high in order to prevent inadequate inflammatory responses to nonpathogenic antigens [7,23–35]. Confirming the role of TLRs in gastric cancer, some studies associate polymorphisms of these receptors to gastric preneoplasic and neoplasic lesions [36–39].

Although our group and others have shown that at late stages of gastric carcinogenesis TLRs are overexpressed, no single study has simultaneously studied TLRs and their interacting molecules expression throughout the entire cascade of gastric carcinogenesis [11–13,40]. Therefore, in this study, we evaluated for the first time in humans the expression of TLR2, TLR4, and TLR5 in all the lesions of gastric carcinogenesis, from normal mucosa to adenocarcinoma. The expression of NF-κB, TNF-α, COX-1, COX-2, CDX-2 (factors induced by TLRs activation) as well as TOLLIP and PPAR-γ (TLRs antagonists) was also evaluated to characterize their association with TLRs expression.

Materials and Methods

Patients and Biologic Samples

This cross-sectional study included patients from Portuguese Oncology Institute of Porto. The study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Portuguese Oncology Institute of Porto. Informed consent was obtained from each patient.

Our institution is a tertiary center to which patients with preneoplastic or superficial gastric lesions are referred and treated with minimally invasive techniques such as endoscopic submucosal dissection [41]. Patients referred for upper gastrointestinal endoscopy with 40 years or more were recruited consecutively during 2011. Hereditary syndromes (confirmed or suspicion of), upper gastrointestinal bleeding as indication for the procedure, oral anticoagulation or hematologic diseases, other active oncologic disease, pernicious anemia or other known benign (e.g., Ménétrier disease) or malign (e.g., lymphoma) gastric pathologies were exclusion criteria. Six different lesions were considered: normal gastric mucosa; normal mucosa with *H. pylori* gastritis; gastric intestinal metaplasia with or without *H. pylori* gastritis; dysplastic lesions; and adenocarcinoma of the intestinal type. Gastric atrophy by itself was not considered because the reproducibility and interobserver correlation for the histopathologic diagnosis of these lesions is low and there is no endoscopic method to accurately identify atrophy [42]. To correctly identify the different lesions, all patients underwent upper gastrointestinal endoscopy using a high-resolution (HR) Olympus endoscope with narrow band imaging (NBI) (EVIS EXERA II video system center GIF-180; Olympus, Tokyo, Japan) and a new recently developed NBI endoscopy classification with high accuracy for gastric lesions was used, with the specimen being collected with biopsy forceps under direct NBI visualization [43]. The diagnosis was always confirmed with histology. If there were discrepancies between NBI endoscopy and histology (with the exception of *H. pylori* status when histology was always considered the gold standard), the sample was not considered for analysis to prevent false positives or false negatives. Moreover, samples were considered as normal mucosa both at NBI and histology but positive for CDX-2 expression were also excluded to prevent that microscopic areas of intestinal metaplasia would be included as normal mucosa. In every patient, at least two biopsy samples of the same area (one for molecular analysis and the other for histologic/immunohistochemical evaluation) were obtained. Whenever possible, a pair of samples was obtained from each different lesion observed. One of the biologic samples was immediately placed in RNAlater (Qiagen, Valencia, Santa Clarita; California, USA) and stored at −80 °C for mRNA isolation and quantification; the other was fixed in 10% buffered formalin and embedded in paraffin for histologic/immunohistochemical evaluation. Gastric specimens were evaluated for *H. pylori* infection using modified Giemsa (2%) stain. Two expert gastrointestinal pathologists made the final histologic diagnosis according to the Sydney–Vienna classification [42,44].

mRNA Isolation and Quantification of TLR2, TLR4, TLR5, NF-κB, TNF-α, TOLLIP, PPARγ, COX-1, COX-2, and CDX-2

These methods were described elsewhere [45–47]. Briefly, total mRNA was extracted using the TriPure

isolation reagent (Roche, Grenzach, Germany) and gene quantification made by two-step real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all experimental groups, which enabled the use of this gene as internal control. Specific PCR primers pairs for the studied genes (GAPDH, TLR2, TLR4, TLR5, NF-κB, TNF-α, TOLLIP, PPARγ, COX-1, COX-2, and CDX-2) are presented as Supporting information. Results of mRNA quantification were expressed as an arbitrary unit (AU) set as the average value of control group (normal mucosa), after normalization for GAPDH, or as the ratio gene/GAPDH.

Immunohistochemical Evaluation of TLR2, TLR4, and TLR5

Our immunohistochemical protocol was previously described [13,42,45]. Briefly, tissue specimens were fixed in 10% neutral buffered formalin for 24 hours and paraffin embedded. Deparaffinized tissue slides were submitted to antigen retrieval using a high-temperature antigen-unmasking technique. The following primary antibodies were used: rabbit polyclonal antibody anti-TLR2 (H-175, 1 : 50 dilution; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-TLR4 (H-80, 1 : 100 dilution; Santa Cruz Biotechnology), and rabbit polyclonal anti-TLR5 (H-80, 1 : 100 dilution; Santa Cruz Biotechnology). Bound antibody was detected by applying biotilynated secondary antibody (Vectastain Universal Elite ABC Kit, Peterborough, United Kingdom) for 30 minutes. Samples without the primary antibodies were considered as negative controls. Normal colon mucosa and lymph node tissue were additionally used as negative and positive controls, respectively (controls images presented as Supporting information). To quantify TLRs expression in tissue samples, two parameters were considered: 1, *Grade of expression*: A score of 0–3 was considered according to the number of epithelial cells marked (0: no cells; 1: <30% of epithelial cells; 2: 30–75% cells; 3: more than 75% cells); 2, *Intensity of expression*: A score of 0–3 was considered according to a subjective evaluation of the intensity of marked cells (0: no immunostaining; 1: weak positive staining; 2: moderate positive staining; 3: strong positive staining). All the samples were evaluated and quantified by two independent pathologists.

Statistical Analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS, IBM Corporation, Armonk, New York, USA) for Windows (version 17.0). Data are presented as mean ± standard error of mean (SEM), as median and range or as a proportion, according to the type of distribution. One-way ANOVA and Student's *t*-test for paired and unpaired data (or correspondent nonparametric test) were used for comparison between groups and lesions. When justified chi-square test was used for the comparison of proportions. Stratified analysis according to gender, age, and gastric area was carried out for all the genes and data presented separately when appropriate. To evaluate the tendency for increase or decrease expression, *t*-test for trend (ANOVA with polynomial function analysis) was used. Statistical significance was set at $p < .05$.

Results

Baseline Characteristics of Patients and Specimens Included in the Study

The baseline characteristics of patients are shown in Table 1. A total of 44 patients were included in the study. A median of two pairs of biopsies samples (range 1–6) for analysis was obtained per patient. In agreement with previous report, the endoscopic NBI patterns

Table 1 Baseline characteristics of patients (n = 44) and endoscopic and histologic diagnosis

Patients	Total n = 44
Male sex, n (%)	26 (59)
Age, median (range), years	67 (41–88)
Number of biopsies, median (range)	4 (2–12)
Indications for upper gastrointestinal endoscopy, n	
Dyspepsia	12
Follow-up/previous diagnosis of dysplasia	8
Follow-up after precursors conditions (metaplasia)	6
Follow-up after gastric mucosectomy	4
For gastric mucosectomy	6
For cancer biopsy	4
Other (e.g., gastroesophageal reflux disease)	4
Main endoscopic findings, n	
Gastric superficial lesions	10
Normal	13
Papular erythematous gastritis	8
Gastric cancer	6
Gastric irregularity or scar	5
Erosive gastritis	2
Histologic diagnosis per patient, n	
Normal mucosa (antrum and body)	12
Intestinal metaplasia antrum (normal body)	10
Intestinal metaplasia corpus and antrum	6
Dysplasia (one or more areas)	10
Adenocarcinoma (one or more areas)	6
Helicobacter pylori infection	21 (48%)

strongly correlated with histology (>90% agreement for normal, metaplasia or dysplasia/cancer) but only moderate correlation with the *H. pylori* status (62% of accuracy) [43]. Only two samples considered as normal mucosa expressed CDX-2 and they were not included in the analysis (curiously, NBI patterns suggested the possibility of small areas of intestinal metaplasia in those samples). At the end, a total of 22 pairs of fragments of normal mucosa, 17 pairs of normal mucosa with *H. pylori* gastritis, 18 of intestinal metaplasia (eight with and 10 without *H. pylori*), 15 pairs of gastric dysplasia, and eight of adenocarcinoma were collected for molecular analysis.

mRNA Expression of TLR2, TLR4, TLR5, NF-κB, TNF-α, TOLLIP, PPARγ, COX-1, COX-2, and CDX-2

We did not find any statistically significant differences in genetic expression between gastric segments, gender or age and for that reason these factors were not considered in the per lesion analysis. The most expressed genes in normal mucosa were TOLLIP (TOLLIP/GAPDH = 3.62 ± 0.44) and PPARγ (PPARγ/GAPDH = 3.27 ± 0.45). Considering the different TLRs expression in normal mucosa, TLR5 was the most expressed with positivity in 90% of the samples and TLR5/GAPDH = 2.2 ± 0.6 compared with 70% sample positivity for TLR2 (TLR2/GAPDH = 1.12 ± 0.36) and TLR4 (TLR4/GAPDH = 0.85 ± 0.23). When comparing the expression of the several genes in the different lesions, independently of *H. pylori* status (Fig. 1), TLR2 and TLR4 were both overexpressed in intestinal metaplasia (TLR2 = 1.66 ± 0.46 AU, $p < .05$; TLR4 = 1.18 ± 0.21 AU, NS) and in dysplasia/cancer (TLR2 = 1.48 ± 0.20 AU; TLR4 = 1.34 ± 0.21 AU, $p < .05$) when comparing with normal mucosa (both genes = 1.0 ± 0.11 AU). This was associated with a statistically significant trend for decrease in TOLLIP ($p < .001$) and PPARγ ($p < .05$) from normal mucosa to metaplasia and finally to dysplasia/carcinoma, with these final lesions of gastric carcinogenesis presenting almost half the expression of both genes (TOLLIP = 0.52 ± 0.05 AU; PPARγ = 0.63 ± 0.10, $p < .05$). When considering *H. pylori* infection (Fig. 2), in normal mucosa *H. pylori* significantly increased TLR2 (2.23 ± 0.36 AU, $p < .05$) and TLR4 (1.92 ± 0.40, $p < .05$) and in intestinal metaplasia *H. pylori* significantly increased TLR4 (1.89 ± 0.45 vs 1.16 ± 0.21 in intestinal metaplasia without *H. pylori*, $p < .05$). *H. pylori* gastritis was also associated with lower TOLLIP (0.66 ± 0.15, $p < .05$) and lower PPARγ (0.73 ± 0.11, $p < .05$). *H. pylori* significantly augmented TNF-α expression both at the normal mucosa (2.14 ± 0.50, $p < .05$) and in intestinal metaplasia (1.84 ± 0.57 vs 0.89 ± 0.31 in intestinal metaplasia without *H. pylori*, $p < .05$). Moreover, TNF-α was also overexpressed in dysplasia/cancer, independently of *H. pylori* status (1.50 ± 0.26 AU, $p < .05$). Indeed, *H. pylori* did not alter significantly any gene expression on lesions with dysplasia or carcinoma. Interestingly, *H. pylori* also increased CDX-2 expression (Fig. 3) in intestinal metaplasia. In fact, there was a statistically significant trend for increase in CDX-2 ($p < .01$) from intestinal metaplasia to intestinal metaplasia with *H. pylori* and finally to dysplasia and carcinoma (Fig. 3).

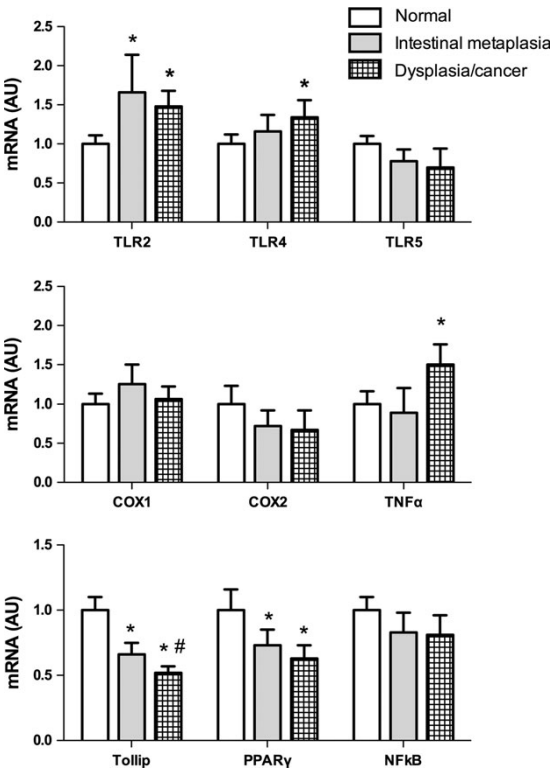

Figure 1 Expression levels (mRNA) of the several genes in the different lesions, independently of *Helicobacter pylori* status. Higher TLR2, TLR4, and lower TLRs antagonists levels characterized more advanced lesions. *$p < .05$ versus normal mucosa; #$p < .05$ versus intestinal metaplasia. TLR, Toll-like receptors.

Immunohistochemical Evaluation of TLR2, TLR4, TLR5

In Table 2 and Fig. 4, TLRs expression in the different gastric lesions is shown. Normal mucosa expressed all TLRs in a polarized manner, particularly at the basolateral membrane but also in the apical membrane in

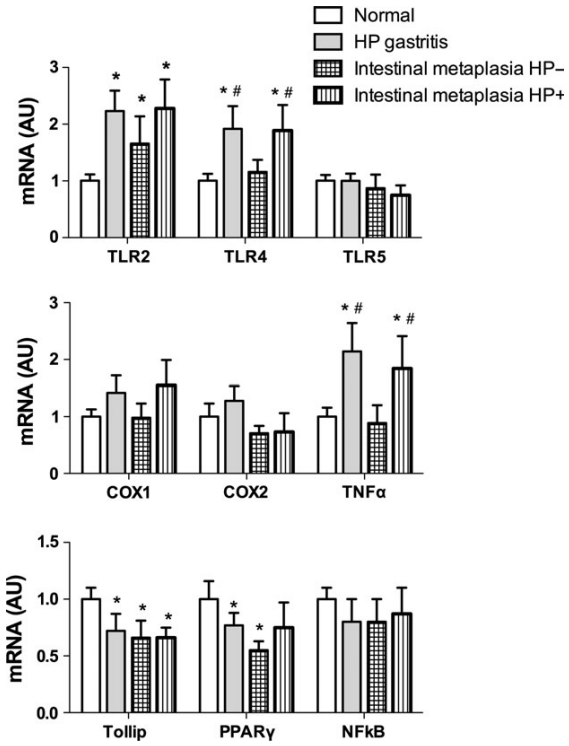

Figure 2 Effect of *H. pylori* on normal mucosa and intestinal metaplasia. *H. pylori* significantly changed genetic profile (mRNA) on normal mucosa with higher TLR2, TLR4, TNF-α and lower Toll-interacting protein (TOLLIP) and PPARγ. In intestinal metaplasia, the effect was less pronounced, nevertheless, it induced higher TLR4 and TNF-α. *$p < .05$ versus normal mucosa; #$p < .05$ versus intestinal metaplasia *H. pylori*-negative. TLR, Toll-like receptors

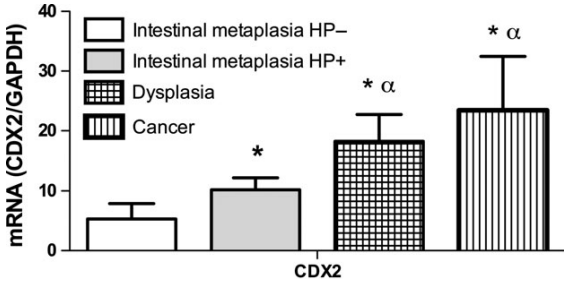

Figure 3 Expression levels (mRNA) of CDX-2 from intestinal metaplasia without *H. pylori* to adenocarcinoma. There was a progressive increase in CDX-2 from intestinal metaplasia without *H. pylori* to intestinal metaplasia with *H. pylori* and finally to dysplasia and carcinoma.

H. pylori gastritis. In contrast, metaplasia, dysplastic, and neoplastic epithelial cells expressed all TLRs diffusely and homogeneously throughout the cytoplasm with no apparent polarization. In normal mucosa, when positive, a mean of 10–50% of the cells were positive, however, with a weak to moderate intensity of expression. When *H. pylori* was present these values were significantly higher (50–75% of cells positive, $p < .05$), still, with a moderate intensity of expression. In metaplasia, dysplasia, and adenocarcinoma, almost all epithelial cells were strongly TLRs positive ($p < .05$ vs normal or *H. pylori* gastritis). Neither the presence of *H. pylori* nor the fact of complete or incomplete intestinal metaplasia changed these results. In dysplasia, TLRs expression was maximum in all areas (>90% gastric epithelial cells were positive with strong intensity of expression). In intestinal-type adenocarcinoma, the majority of the included tumors had a high level of TLRs expression in almost all the cells with a strong intensity. Two of them, however, showed some areas of weak expression for one or all TLRs, particularly at the profundity of the lesion in undifferentiated areas. There was a statistically significant trend for increase in TLRs expression from normal mucosa to dysplasia/carcinoma ($p < .05$).

Discussion

In the present study, we described for the first time in all the lesions of human gastric carcinogenesis the molecular expression of TLRs and of several other molecules that are involved or that modulate their signaling pathways. We have found that *H. pylori* early induce a lower expression of TLRs inhibitors associated with higher TLRs protein levels in normal mucosa and that these changes persist throughout all the spectrum of lesions of gastric carcinogenesis. Our results suggest that increasing activation of these receptors, initially by *H. pylori* but at later stages potentially by several other PAMPs or DAMPs, may have an important role in gastric carcinogenesis and tumor progression.

The intestinal type of gastric cancer (the most common form of gastric cancer) develops through a cascade of well-defined and recognizable precursors known as the Correa cascade for gastric carcinogenesis: inflammation; atrophy; metaplasia; dysplasia; carcinoma [8,9]. It is now undisputable that *H. pylori* plays a pivotal role in this cascade of lesions and for that reason it was classified as a type 1 carcinogen in 1994 by the WHO [48]. It is believed that the combination of a virulent bacterium in a genetically susceptible host is associated with more severe chronic inflammation and it is this inflammation that at long term may lead to cancer [49–51]. Indeed, several studies associate IL-1B, IL-1 receptor antagonist, IL-8, IL-10 and TNF-α polymorphisms to the risk of gastric cancer, confirming the important role

Table 2 Immunohistochemical evaluation of TLR2, TLR4, and TLR5

	Normal (n = 20)	*Helicobacter pylori* gastritis (n = 14)	Metaplasia (n = 15)	Dysplasia (n = 14)	Carcinoma (n = 7)
TLR2					
Grade	0.53 (0.11)	1.49 (0.13)*	2.20 (0.14)*#	2.86 (0.10)*#§	2.57 (0.20)*#§
Intensity	0.97 (0.10)	1.64 (0.13)*	2.20 (0.11)*#	2.79 (0.11)*#§	2.64 (0.18)*#§
TLR4					
Grade	0.53 (0.11)	1.42 (0.10)*	2.20 (0.14)*#	2.86 (0.10)*#§	2.57 (0.20)*#§
Intensity	0.97 (0.10)	1.64 (0.13)*	2.20 (0.11)*#	2.79 (0.11)*#§	2.71 (0.15)*#§
TLR5					
Grade	0.58 (0.08)	1.35 (0.12)*	2.13 (0.17)*#	2.86 (0.10)*#§	2.57 (0.20)*#§
Intensity	1.00 (0.07)	1.64 (0.13)*	2.27 (0.12)*#	2.79 (0.11)*#§	2.64 (0.18)*#§

TLR, Toll-like receptors. Values are presented as mean (SEM);
*$p < .05$ versus normal mucosa;
#$p < .05$ versus *H. pylori* gastritis;
§$p < .05$ versus intestinal metaplasia.

of inflammation as the link between *H. pylori* and cancer [52–57]. However, as *H. pylori* is not an invasive bacteria, it appears that the reaction of the host mucosa to the bacteria plays an essential role in inflammation. In that way, as TLRs are essential for *H. pylori* recognition and subsequent innate and adaptive immunity, they may be the crucial players in perpetuating this chronic inflammation [7].

After the first contact with the gastric mucosa, *H. pylori* interacts with several TLRs, with TLR2 being the receptor responsible for most of the immunologic reactions occurring as the result of infection [58,59]. This process of recognition and immunologic reaction to *H. pylori* depends also in a minor extent of TLR4 that acts in synergy with TLR2 [14,15,60–63]. On the other way, *H. pylori* flagellin appears to evade TLR5 recognition, although some initial studies suggested interaction between *H. pylori* flagellin and this receptor [58,64–67]. When activated these receptors initiate intracellular signaling pathways that promote NF-κB activation and production of different pro-inflammatory mediators such as TNF-α, IL-1, and several others inflammatory molecules [16–20]. Because TLRs are intrinsically related not only to inflammation but also to cell survival signaling and in that way to epithelial regeneration and cell proliferation, chronic activation of these receptors has been associated with tumorigenesis [21]. Indeed, our group and others previously showed that gastric dysplasia is associated with a more intense and diffuse cellular distribution of TLRs [13,40]. Moreover, recent studies associate TLR2 and TLR4 polymorphisms with the severity of gastric lesions associated with *H. pylori* infection and also with gastric cancer [36–38,68]. However, even though this data suggest an important role of TLRs in the multistep pathway to gastric cancer, scarce human data elucidates the sequence of events through which TLRs and *H. pylori* interact and promote progression of gastric lesions.

In that line of thoughts, to our knowledge, our study is the first one in humans that directly evaluated TLRs gene and protein expression throughout the entire Correa cascade of gastric carcinogenesis. Although we did not consider gastric atrophy, in this condition, the epithelial cells appear phenotypically the same as normal mucosa and we observed on immunohistochemistry that atrophic glands (without metaplasia) had a similar profile to normal mucosa. Our results allow us to create a potential sequence of events from *H. pylori* gastritis to cancer involving progressive activation of TLRs (Fig. 5). When interacting with normal mucosa *H. pylori* doubled the expression of TLR2 and TLR4 but not TLR5, indirect evidence that in fact *H. pylori* flagellin does not interact with this receptor. Nevertheless, we did not find any difference of expression between the three TLRs on immunohistochemistry. This may be because *H. pylori* also induced 25% decrease in the expression of PPARγ and TOLLIP. Indeed, besides antagonizing several intracellular kinases that are activated by TLRs, TOLLIP also blocks TLRs complexes and promote traffic of synthesized proteins into endosomes leading to TLR early degradation [69–74]. So, as we have shown in colon mucosa, this decrease in TOLLIP may be a crucial step leading to a more intense and apical protein expression of all TLRs, independently of the degree of mRNA expression [45]. We believe that the progressive activation of the overexpressed TLRs will eventually lead to aberrant transcription of CDX-2 and phenotypic change to intestinal metaplasia. This hypothesis is

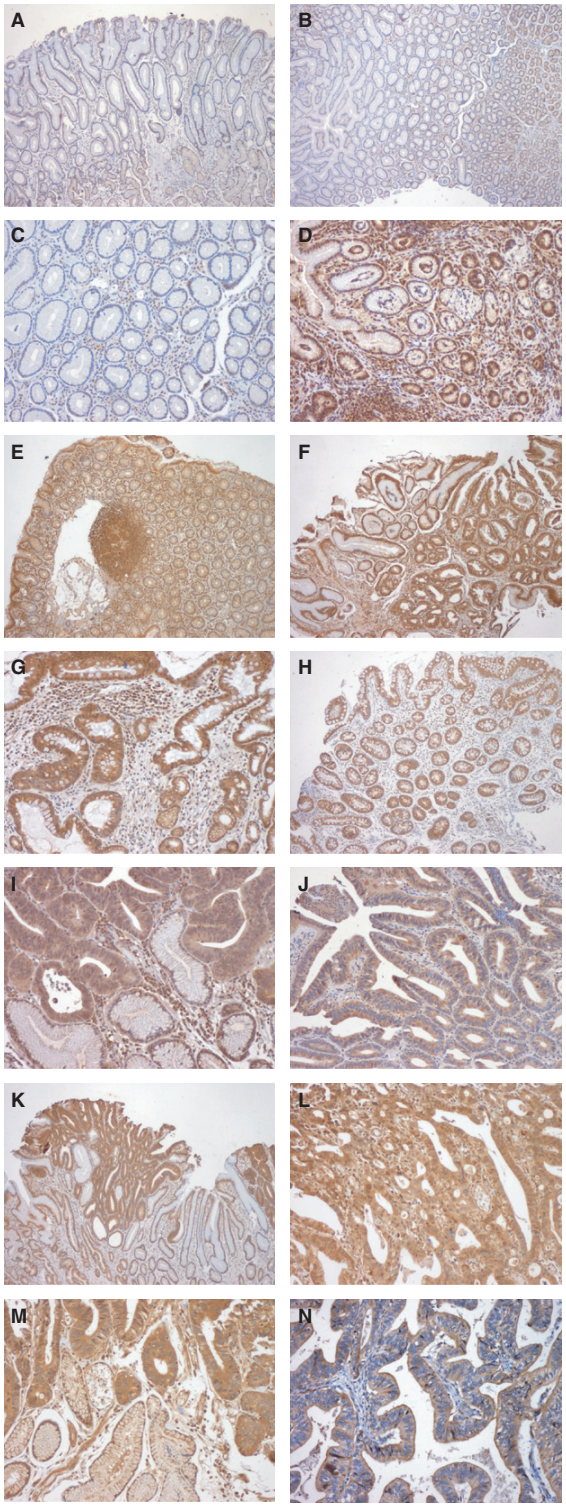

Figure 4 Toll-like receptors (TLRs) immunohistochemistry images of the different lesions. In brackets the letter of the image and the respective TLR being stained and amplification. Normal mucosa (*A* – TLR2, 40×; *B* – TLR4, 40×; *C* – TLR5, 100×) – Normal glands with a polarized basolateral staining and low intensity of expression; *H. pylori* gastritis (*D* – TLR2, 100×; *E* – TLR4, 40×; *F*, left – TLR5, 40×) – epithelial cells presented a more intense but still polarized TLR expression when compared to normal mucosa; Intestinal metaplasia (*G* – TLR2, 100×; *H* – TLR4, 40×; *F*, right – TLR5, 40×) – Diffuse and intense TLR expression throughout the cytoplasm with no apparent polarization. In (*F*) we see a transition of *H. pylori* gastritis to intestinal metaplasia and even in areas of gastritis the intensity of TLRs expression in intestinal metaplasia (right) was in clear contrast with normal mucosa (left); Dysplasia (*I*, up – TLR2, 100×; *J* – TLR4, 100×; *K*, up – TLR5, 40×) – Diffuse, homogenous and very strong intensity of TLR expression. In (*K*) we see a transition of normal mucosa to intestinal metaplasia and dysplasia with a growing intensity of TLR expression being evident from normal mucosa (right and below) to intestinal metaplasia (left and below) and then to dysplasia (up). In higher amplification (image *I*), we see a transition of normal mucosa to dysplasia with the polarized and low expression in normal mucosa (down) in clear contrast with the diffuse and intense TLR expression of dysplasia (up); Intestinal adenocarcinoma (*L* – TLR2, 100×; *M*, up – TLR4, 100×; *N* – TLR5, 100×) – The majority of adenocarcinomas in this study were well-differentiated and presented a high and strong intensity of TLRs expression. In (*M*) we can see that TLRs expression in carcinoma (up) was in clear contrast with normal mucosa (down).

supported by the study of Ikeda et al. [75] when they show that, at least in biliary epithelium, TLRs and NF-κB activation by PAMPs is essential to CDX-2 transcription and phenotypic change to intestinal metaplasia. In our study, intestinal metaplasia presented a different genetic profile when compared to normal mucosa with almost half of the expression of TOLLIP and PPARγ and 1.2–1.5 times more TLR2 and TLR4 expression dispersed through the entire cell, independently of *H. pylori* status. These results suggest that intestinal metaplasia is clearly more reactive to several PAMPs and might explain why this lesion is a preneoplastic condition. Nevertheless, our results suggest that at this stage the mucosa is still reactive to *H. pylori* because the presence of this bacterium not only doubled TNF-α levels in the mucosa but also increased TLR4 and CDX-2 expression. This is important because CDX-2 has been associated with progression of gastric lesions and cancer [76,77]. In this way, our results are in agreement with the current guidelines concerning gastric preneoplastic lesions that recommend *H. pylori* eradication at this stage because even though it is not likely that eradication will reverse intestinal metaplasia to normal mucosa it can prevent progression of the lesions [42,44]. Progressive activation of TLRs at this stage will eventually lead to dysplasia, characterized in our study by half of TOLLIP expression and

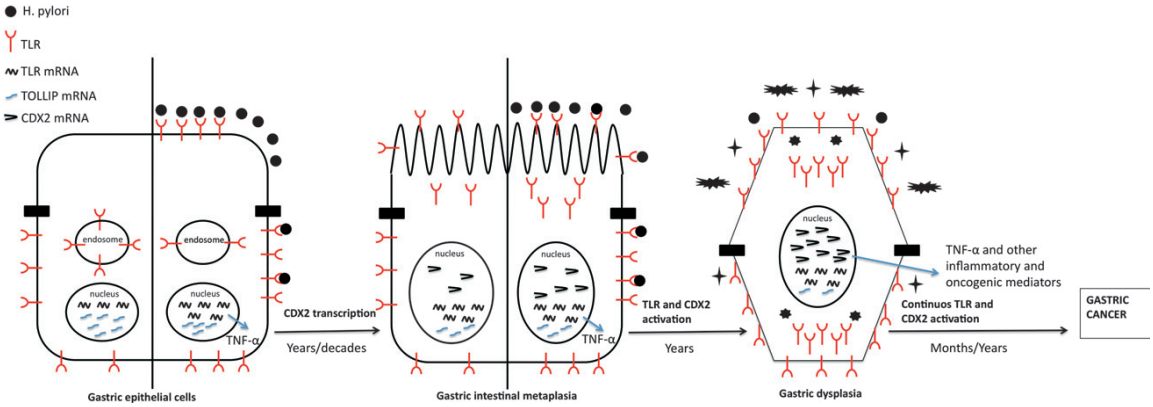

Figure 5 Proposed sequence for gastric carcinogenesis, involving progressive Toll-like receptors (TLRs) activation. Left: In normal mucosa, high Toll-interacting protein (TOLLIP) expression directs TLRs proteins to basolateral membrane or to degradation on endossomes, preventing in this way inadequate apical TLR activation to different antigens. *H. pylori* early induces higher TLRs expression and lower TOLLIP, allowing redistribution of TLRs to apical membrane where they can interact with *H. pylori*. Trying to combat the infection, TLRs interaction with *H. pylori* leads to activation of several proinflammatory pathways and cytokine production. Middle – The perpetuation of these intracellular pathways during years eventually leads to CDX-2 transcription and phenotypic change to intestinal metaplasia. These new state is characterized by even lower TOLLIP expression and consequently diffuse TLR protein throughout the cell, becoming the cell more reactive to *H. pylori* and eventually to other antigens. With TLRs chronically stimulated, CDX-2 levels progressively increase, and pro-oncogenic intracellular pathways eventually activate leading to cell dysplasia. Right: This state is associated with even lower levels of TOLLIP, high levels of CDX-2 and TLRs, that are disperse throughout the cell, and so, at this point, many different antigens may perpetuate the production of different inflammatory and oncogenic mediators eventually leading to cancer.

consequently more and diffuse TLR expression throughout the entire cell. At this point, *H. pylori* did not alter the genetic profile of the cell even though we found increased TNF-α levels in dysplasia and cancer. Probably, this intense and diffuse expression of TLRs make the cell more reactive to several PAMPs and even DAMPs and, so the effect of *H. pylori* becomes negligible at this stage. Interestingly, the progression of gastric lesions was associated with increasing levels of CDX-2. Although there is some controversy concerning the role of CDX-2 in gastric carcinogenesis, our results are in agreement with the study of Kang et al. [77] and clearly suggest an important role of CDX-2 in the progression of intestinal metaplasia, dysplasia, and even cancer.

In conclusion, our results suggest that TLRs signaling pathways may play an important role in gastric carcinogenesis and that they might be the link between *H. pylori* and cancer. A strategy of modulation of TLRs, either by blocking TLRs or by increasing TOLLIP levels, may be effective for the prevention of progression of gastric lesions. Considering the high TLRs expression in gastric cancer, blocking TLRs activation may also have an important role in cancer treatment. As modulation of TLRs activation may be accomplished by interventional measures, future studies should evaluate the clinical value of these novel findings.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1 Primers.

Data S2 Images from controls.

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CHAPTER V – TOLL-LIKE RECEPTORS AND COLON DISEASE

"Our bowels are outside of us – just a tucked-in portion"

William Osler (1849-1919)

A) DECREASED TOLL-INTERACTING PROTEIN AND PEROXISOME PROLIFERATED-ACTIVATED RECEPTOR γ ARE ASSOCIATED WITH INCREASED EXPRESSION OF TOLL-LIKE RECEPTORS IN COLON CARCINOGENESIS

Original article

Decreased Toll-interacting protein and peroxisome proliferator-activated receptor γ are associated with increased expression of Toll-like receptors in colon carcinogenesis

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ABSTRACT

Background Animal data suggest that Toll-like receptors (TLR) may play an important role in colon carcinogenesis. Studies in humans to support that hypothesis are scarce.

Aim To evaluate the expression of TLR2, TLR4 and TLR5, and the expression of several other related molecules, in different human colonic lesions.

Methods Colon biopsy samples from normal mucosa, normal mucosa adjacent to lesion, adenoma or sporadic carcinoma were obtained from 35 consecutive patients undergoing colonoscopy. Quantification of TLR2, TLR4, TLR5, Toll-interacting protein (TOLLIP), peroxisome proliferator-activated receptor γ (PPAR-γ), nuclear factor κB, tumour necrosis factor (TNF) α, cyclooxygenase (COX) 1 and 2 mRNA was performed by real-time reverse transcription PCR. TLR2, TLR4 and TLR5 protein expression was quantified by immunohistochemistry.

Results When compared with normal mucosa (1.0 arbitrary unit (AU)), adjacent normal mucosa presented higher expression of COX-2 (1.86±0.3 AU, p=0.01) and TNFα (1.44±0.18 AU, p=0.04) and lower TOLLIP expression (0.75±0.05 AU, p=0.004). Adenomas and carcinomas presented higher expression of COX-2 (1.63±0.27 and 1.38±0.14 AU, p=0.03 and p=0.05, respectively) and lower expression of TOLLIP (0.44±0.04 AU, p<0.001), with diffuse and higher TLR protein expression (p<0.001). Carcinomas also expressed higher TLR2 (2.31±0.32 AU, p=0.006) and lower PPAR-γ (0.56±0.12 AU, p=0.003). There was a trend towards decreased TOLLIP (p<0.001) and PPAR-γ (p=0.05) from normal mucosa to adenoma/carcinoma.

Conclusions Persistently positive TLR expression and lower expression of TLR inhibitors was associated with higher TLR protein levels throughout the spectrum of lesions of colon carcinogenesis. Increasing activation of these receptors by bacteria may play a crucial role in colon carcinogenesis and tumour progression.

Colorectal cancer (CRC) is one of the most common cancers in the world. Its incidence appears to be growing, particularly in developed countries.[1–3] Three distinct molecular pathways for colorectal tumorigenesis have been described, namely chromosomal instability, microsatellite instability and epigenetic pathways.[4 5] Conversely, other factors such as cyclooxygenase (COX) 2 and peroxisome proliferator-activated receptor γ (PPAR-γ), by promoting genomic instability and controlling cell growth, may be important for the progression of these extensively described mutagenic pathways.[6–8]

Toll-like receptors (TLR) are the most important family of innate immunity receptors, with 10 different TLR being ubiquitously expressed in humans.[9 10] In general, the most important for bacterial recognition are TLR2, which recognise pathogen-associated molecular patterns (PAMP) from Gram-positive bacteria, TLR4, the receptor for Gram-negative bacteria lipopolysacharide and TLR5, which recognise bacteria flagellin.[11–13] Activation of these receptors initiates intracellular signalling pathways that promote the production of different pro-inflammatory mediators such as tumour necrosis factor α (TNFα) and COX-2 through activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases.[9 14–17] In order to prevent an inflammatory response to the million different commensal PAMP, a strict regulation of TLR activation appears fundamental for gastrointestinal homeostasis. This may be done in a number of ways such as the downregulation of surface expression and compartmentalisation of TLR, proteolytic degradation of TLR or its signalling molecules and high expression of several TLR antagonists, such as Toll-interacting protein (TOLLIP) and PPAR-γ (figure 1).[18–31]

By promoting chronic inflammation and consequently predisposing to genetic instability, inflammatory bowel disease (IBD) is an established risk factor for CRC.[32 33] TLR may be the link between IBD and CRC because in induced colitis murine models, bacterial-induced inflammation through TLR/MyD88 signalling appears essential for colon carcinogenesis.[14 34 35] In contrast, data linking TLR to sporadic CRC are scarce.[23] Nevertheless, studies showing that rats given carcinogens under sterile conditions are protected from CRC,[36] that cancer and adenoma present higher bacteria levels than normal mucosa,[37] and that TLR activation (particularly TLR2 and TLR4) of colon cells induce the enhanced expression of several oncogenic factors[14 35 38 39] suggest that TLR response to bacterial PAMP may play a role in colon carcinogenesis.[23]

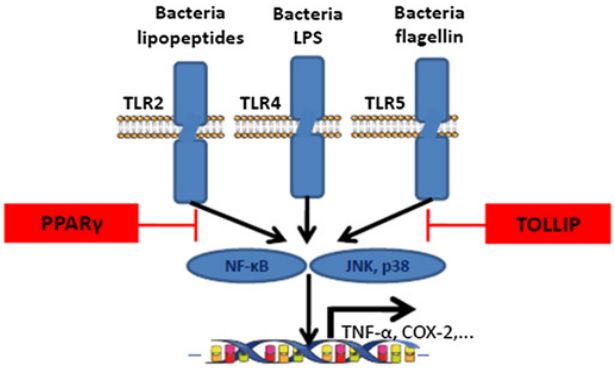

Figure 1 Interaction of Toll-like receptors (TLR) with bacterial pathogen-associated molecular patterns leads to nuclear factor κB (NF-κB) activation and transcription of several pro-inflammatory molecules such as tumour necrosis factor α (TNFα) and cyclooxygenase (COX) 2. In the colon, under normal physiological conditions, high levels of Toll-interacting protein (TOLLIP) and peroxisome proliferator-activated receptor γ (PPAR-γ) block TLR signalling pathways being fundamental for colon homeostasis. JNK, c-jun N-terminal kinase; LPS, lipopolysacharide.

We hypothesised that common CRC risk factors, such as diet and obesity, may change normal microbiota equilibrium with modification of the physiological expression of TLR and/or their antagonists, inducing a subclinical pro-inflammatory state that may facilitate carcinogenesis. Therefore, in the present study, we evaluated the expression of TLR2, TLR4 and TLR5 in different human colon lesions, from normal mucosa to CRC. The expression of NF-κB, TNFα, COX-1, COX-2 (factors induced by TLR activation) as well as TOLLIP and PPAR-γ (TLR antagonists) was also evaluated in order to characterise their association with TLR expression.

MATERIALS AND METHODS

Patients and biological samples

This cross-sectional study included patients from Portuguese Oncology Institute of Porto. The study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Portuguese Oncology Institute of Porto. Informed consent was obtained from each patient.

Patients referred for colonoscopy aged 50 years or more were recruited consecutively during 2010. Hereditary syndromes (confirmed or suspicion of), IBD, diarrhoea as an indication for the procedure, oral anticoagulation or haematological diseases, other active oncological disease, previous or current pelvic radiotherapy, endoscopic or histological inflammatory changes and deficient bowel preparation were exclusion criteria. Three groups of patients were defined: control (patients undergoing colonoscopy for screening, with a normal total colonoscopy); adenoma group (patients with history of adenoma or with at least one adenoma identified in the procedure); cancer group (patients with a history of CRC or CRC identified in the procedure). Four groups of tissue samples obtained by endoscopic biopsy were considered and analysed: normal mucosa (mucosa with normal endoscopic and histological appearance with at least 5 cm distance from an adenoma or cancer suspicious lesion); normal mucosa adjacent to a lesion (mucosa with normal endoscopic and histological appearance within 3 cm of a histological confirmed adenoma or cancer); adenoma (endoscopic lesion compatible with an adenoma of more than 5 mm, confirmed by histology in the resected specimen); cancer (endoscopic lesion compatible with a CRC, confirmed by histology in the biopsy sample).

In every patient at least two biopsy samples (one for molecular analysis and the other for histological and immunohistochemical evaluation) were obtained for at least one normal segment of mucosa, either from the caecum/ascendent colon, transverse colon, left colon or rectum. Whenever possible a pair of samples was obtained from each of these anatomical regions. When a lesion (adenoma of more than 5 mm or CRC) was identified an additional pair of samples was obtained from normal mucosa within 3 cm of the lesion (adjacent normal mucosa).

One of the biological samples was immediately placed in RNAlater (Qiagen, Valencia, Santa Clarita; California, USA) and stored at −80°C for messenger RNA isolation and quantification; the other was fixed in 10% buffered formalin and embedded in paraffin for histological and immunohistochemical evaluation.

mRNA isolation and quantification of TLR2, TLR4, TLR5, NF-κB, TNFα, TOLLIP, PPAR-γ, COX-1 and COX-2

These methods were described elsewhere.[40] Briefly, total mRNA was extracted from tissue samples using the TriPure isolation reagent (Roche, Grenzach, Germany). Two-step real-time reverse transcription (RT)—PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and the PCR threshold cycle of graded dilutions from a randomly selected sample from the control group. For relative quantification of specific mRNA levels, 100 ng of total mRNA from each sample underwent two-step real-time RT—PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all experimental groups, which enabled the use of this gene as an internal control. Specific PCR primer pairs for the studied genes (GAPDH, TLR2, TLR4, TLR5, NF-κB, TNFα, TOLLIP, PPAR-γ, COX-1 and COX-2) are presented as a supplement (available online only). Results of mRNA quantification were expressed as an arbitrary unit (AU) set as the average value of the control group, after normalisation for GAPDH.

Immunohistochemical evaluation of TLR2, TLR4 and TLR5

Our immunohistochemical protocol was described previously.[41] Briefly, tissue specimens were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. Deparaffinised tissue slides were submitted to antigen retrieval using a high temperature antigen unmasking technique. Immunostaining was performed using an immunoperoxidase method according to the manufacturer's instructions. The slides were incubated with normal horse serum (Vector Laboratories, Burlingame, California, USA) 1/50 in phosphate-buffered saline—bovine serum albumin 1% at room temperature for 20 min in a humid chamber. Sections were then incubated with primary antibody at 4°C overnight. The following primary antibodies were used: rabbit polyclonal antibody anti-TLR2 (H-175, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit polyclonal anti-TLR4 (H-80, 1:100 dilution; Santa Cruz Biotechnology) and rabbit polyclonal anti-TLR5 (H-80, 1:100 dilution; Santa Cruz Biotechnology). Bound antibody was detected by applying biotilynated secondary antibody (Vectastain Universal Elite ABC Kit, Peterborough,United Kingdom) for 30 min. Following counterstaining with haematoxylin, the slides were washed, dehydrated and mounted with Entellan (Merck

KGaA, Darmstadt, Germany). Samples without the primary antibodies were considered as negative controls. Normal gastric mucosa and lymph node tissue were additionally used as negative and positive controls, respectively. In order to quantify TLR expression in tissue samples two parameters were considered: (1) Grade of expression: A score of 0–3 was considered according to the number of epithelial cells marked (0, no cells; 1, <10% of epithelial cells; 2, 10–75% cells; 3, more than 75% cells); (2) Intensity of expression: A score of 0–3 was considered according to a subjective evaluation of the intensity of marked cells (0, no immunostaining; 1, weak positive staining; 2, moderate positive staining; 3, strong positive staining). Immunohistochemical evaluation was performed independently by two experienced pathologists.

Statistical analysis

Data analysis was performed using the computer software SPSS for Windows (V.17.0). Data are presented as mean±SEM, as median and range or as a proportion, according to the type of distribution. One-way analysis of variance and Student's t test for paired and unpaired data (or a corresponding non-parametric test) were used for comparison between groups and lesions. When justified the χ^2 test was used for comparison of proportions. Stratified analysis according to gender was done for all the genes and data presented separately when appropriate. In order to evaluate the tendency for increased or decreased expression, the t test for trend (analysis of variance with polynomial function analysis) was used. Correlation between TLR protein expression and the several genes mRNA expression was evaluated by univariate analysis. Statistical significance was set at $p<0.05$.

RESULTS

Baseline characteristics of patients

The baseline characteristics of patients are shown in table 1. A total of 35 patients was included in the study. All included patients were Caucasian. The age was similar between the groups, with an age range of 51–89 years. The proportion of men was higher in the adenoma and cancer group when compared with the control group ($p<0.01$). Screening was the indication for the procedure in 29%. A substantial proportion of patients (31%) underwent the colonoscopy because of cancer, the majority of whom for the exclusion of synchronous lesions. A median of three pairs of biopsy samples (range 1–10) for analysis was obtained per patient. At the end a total of 25 pairs of fragments of normal mucosa, 28 adjacent to lesion, 21 of adenoma and 14 of carcinoma were collected for molecular analysis.

Table 1 Baseline characteristics of patients (n=35)

	Control (n=6)	Adenoma (n=15)	Cancer (n=14)	Total (n=35)
Age, years (mean±SEM)	62 (±4.8)	62 (±2.5)	65 (±2.8)	64 (±2.1)
Sex (male)	2 (33%)	11 (73%)	10 (71%)	23 (66%)
Indication for endoscopy				
Screening	6	3	1	10
Adenoma	0	12	2	14
Cancer	0	0	11	11
Endoscopy findings*				
Normal	6	5	2	13
Adenoma	0	15	6	21
Cancer	0	0	14	14

*Values are presented as number of lesions identified per group.

mRNA expression of TLR2, TLR4, TLR5, NF-κB, TNFα, TOLLIP, PPAR-γ, COX-1 and COX-2

Considering normal mucosa, we did not find any statistically significant difference in genetic expression between segments, gender or age. However, we did find a tendency for higher expression of TOLLIP (1.1±0.08 vs 0.8±0.07 AU, p=0.07), COX-1 (1.22±0.16 vs 0.75±0.13 AU, p=0.07) and TLR5 (1.17±0.1 vs 0.78±0.09 AU, p=0.06) and for lower COX-2 (0.88±0.1 vs 1.14±0.1 AU, p=0.1) in the women with normal mucosa when compared with men. When considering all fragments of normal mucosa (normal and adjacent to lesion normal mucosa), the expression of TOLLIP was significantly higher (1.04+0.07 vs 0.79+0.05 AU, p=0.004) and COX-2 significantly lower (1.1 +0.15 vs 1.7+0.26 AU, p=0.04) in women. When comparing normal mucosa between groups (controls vs patients with adenoma and carcinoma), controls expressed more COX-1 (1.4±0.17 vs 0.74±0.1 AU, p=0.006) but also more TOLLIP (1.16±0.07 vs 0.88±0.07 AU, p=0.02) and a tendency for more PPAR-γ (1.15±0.09 vs 0.89±0.09 AU, p=0.08), with no differences in the other studied genes. Quantification of all genes in the different lesions is shown in figure 2. The genetic profile of adjacent normal mucosa was different from that of normal mucosa with higher expression of COX-2 (1.86±0.3 vs 1±0.08 AU, p=0.01) and TNFα (1.44±0.18 vs 1±0.09 AU, p=0.04) and lower TOLLIP expression (0.75±0.05 vs 1±0.06 AU, p=0.004). Adenoma and carcinoma also presented higher expression of COX-2 (1.63±0.27 and 1.38±0.14 vs 1±0.08 AU, p=0.03 and p=0.05, respectively) and lower expression of TOLLIP (0.44±0.04 and 0.45±0.06 vs 1±0.08 AU, p<0.001) when compared with normal mucosa. Carcinoma expressed more TLR2 when compared with adenoma or normal mucosa (2.31±0.32 vs 1±0.14 AU, p=0.006) and less PPAR-γ when compared with normal mucosa (0.56±0.12 vs 1±0.07 AU, p=0.003). Ninety per cent of the patients presented higher COX-2 expression in the lesions when compared with normal mucosa, but 60% of these expressed more COX-2 in the adjacent mucosa than in the lesion itself. There was a statistically significant trend for a decrease of TOLLIP (p<0.001) and PPAR-γ (p=0.05) from normal mucosa to adenoma/carcinoma. In an individual analysis, 90% of patients with lesions presented this tendency for lower TOLLIP expression and 60% for lower PPAR-γ expression.

Immunohistochemical evaluation of TLR2, TLR4 and TLR5

In table 2 and figure 3, TLR expression in the different colonic lesions is shown. Only 15% of the samples of normal mucosa and 10% of adjacent normal mucosa were considered negative for TLR expression. When positive, a mean of 50% of the cells were positive; however, with a weak to moderate intensity of expression. All the samples of adenoma and adenocarcinoma were considered positive with more than 90% of the cells immunoreactive, and the grade and intensity of expression was significantly higher when compared with normal or adjacent mucosa (p<0.001). Normal mucosa expressed all TLR in a polarised manner, particularly at the basolateral membrane (figure 3A). On the other hand, adenoma and carcinoma expressed all TLR diffusely and homogeneously throughout the cytoplasm and also apical membrane with no apparent polarisation (figure 3B–F). Concerning individual analysis, 90% of the patients presented increased TLR2 expression and 70% of the patients increased TLR4 and TLR5 expression from normal mucosa to adenoma or adenocarcinoma. We did not find any significant statistical correlation between TLR protein levels and mRNA levels of the several genes.

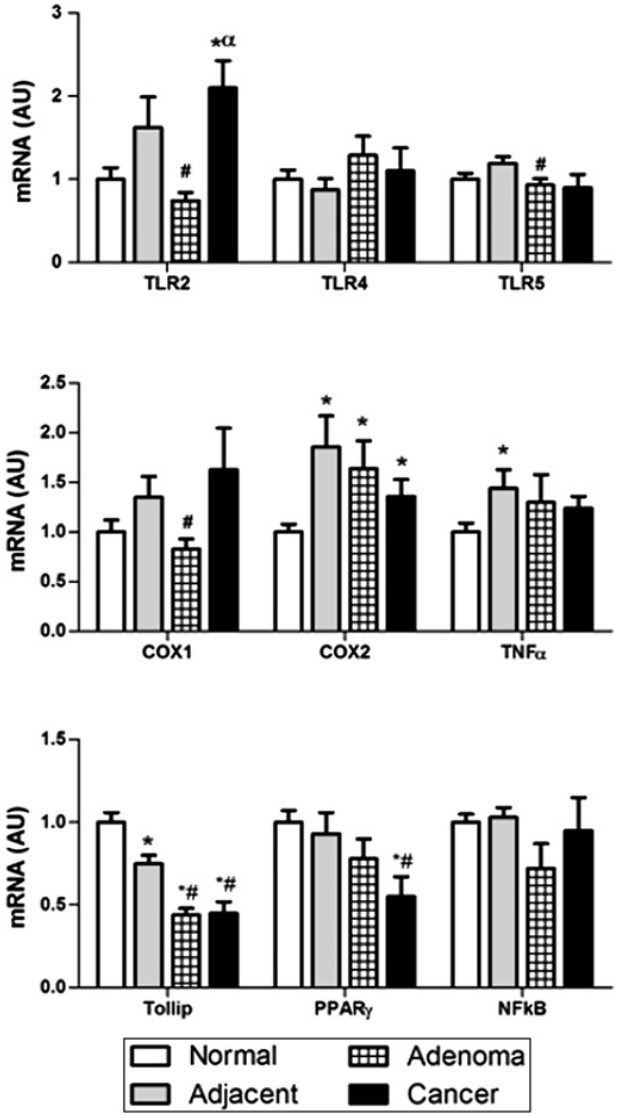

Figure 2 mRNA quantification according to the studied lesions. Adenoma and carcinoma presented lower Toll-interacting protein (Tollip) (−56%) and higher cyclooxygenase (COX) 2 (+63% and +38%) when compared with the normal mucosa. Carcinoma also presented a 46% decrease in peroxisome proliferator-activated receptor γ (PPAR-γ). *p<0.05 versus normal mucosa; #p<0.05 versus adjacent normal mucosa; αp<0.05 versus adenoma. AU, arbitrary unit; NF-κB, nuclear factor κB; TLR, Toll-like receptor; TNFα, tumour necrosis factor α.

DISCUSSION

In the present study we described for the first time in human colonic lesions the molecular expression of TLR and of several other co-factors that modulate their signalling pathways. We have found a persistently positive TLR expression and lower expression of TLR inhibitors associated with higher TLR protein levels throughout all the spectrum of lesions of colon carcinogenesis. Our results suggest that increasing activation of these receptors by bacteria may play a crucial role in colon carcinogenesis and tumour progression.

It is current knowledge that three distinct molecular mutagenic pathways are involved in colon carcinogenesis:

Table 2 Immunohistochemical evaluation of TLR2, TLR4 and TLR5

	Normal (n=21)	Adjacent (n=25)	Adenoma (n=21)	Adenocarcinoma (n=14)
TLR2				
Grade	1.7 (±0.11)	1.95 (±0.14)	2.79 (±0.11)*	2.62 (±0.13)*
Intensity	1.25 (±0.1)	1.4 (±0.1)	2.57 (±0.17)*	2.47 (±0.13)*
TLR4				
Grade	1.75 (±0.1)	2.0 (±0.14)	2.79 (±0.11)*	2.57 (±0.13)*
Intensity	1.25 (±0.1)	1.4 (±0.1)	2.64 (±0.13)*	2.57 (±0.13)*
TLR5				
Grade	1.75 (±0.1)	1.9 (±0.15)	2.79 (±0.11)*	2.57 (±0.13)*
Intensity	1.25 (±0.1)	1.4 (±0.1)	2.28 (±0.16)*	2.47 (±0.13)*

Values are presented as mean (±SEM).
*p<0.001 versus normal or adjacent mucosa.
TLR, Toll-like receptor.

Chromosomal instability (inactivating mutations of adenomatous polyposis coli (APC) gene and sequential activation of oncogenes and inactivation of tumour suppressor genes); microsatellite instability (mutations in DNA mismatch repair genes predisposing to mutations in genes with repetitive sequences); epigenetic pathway (hypermethylation and gene silencing).[4] All these pathways of the adenoma–carcinoma sequence are now extensively described.[4 5] Nevertheless, not considering the hereditary syndromes, it is still not clear which factors initiate and promote tumour progression. Indeed, a number of environmental risk factors have been described; yet it is unknown how environment and diet influence genetic pathways and predispose to cancer. For example, age, male gender, race, obesity, diabetes and diet, among many others, are considered risk factors for non-hereditary CRC. Even so, the molecular mechanisms through which these factors increase the risk of cancer and influence colon carcinogenesis are still not known.[2 42 43] In contrast, IBD is a risk factor for CRC by promoting chronic inflammation and consequently predisposing to mutations.[32 33] So, inflammation (even subclinical inflammation) may be an important factor in the initiation and progression of the mutagenic pathways described. In fact, COX-2 upregulation has long been associated with the adenoma–carcinoma sequence and inhibition of COX-2 reduces the risk of developing new adenomas and may also reduce the growth of colon cancer cells.[6 8 44] Loss-of-function mutations in PPAR-γ, a factor that controls inflammation by reducing NF-κB activation, were also associated with colon cancer.[7]

The colon is colonised by a number of microorganisms and their associated PAMP without inducing an inflammatory response. A stringent regulation of TLR activation appears fundamental for that to happen, maintaining colon homeostasis in this way.[23] Our results, in agreement with other studies,[24–29] show that normal colon mucosa constitutively express TLR; however, this is associated with high expression of TOLLIP and PPAR-γ, which appears to circumscribe protein expression to basolateral membrane where they are not continuously exposed to PAMP. This is important because TLR in that location are central in controlling auto-immunity processes and for epithelial regeneration, in that way maintaining normal physiology in the gastrointestinal tract.[45–49] On the other hand, by activating cell survival signalling pathways (eg, NF-κB activation and augmented COX-2 expression) abnormal TLR activation may theoretically promote colon carcinogenesis in a different number of ways.[47 50] As TLR are the first line of recognition for bacterial antigens, studies showing that germ-free rats given carcinogens are protected from colonic cancer,[36] and that cancer and adenomas present higher bacteria levels than normal mucosa,[37]

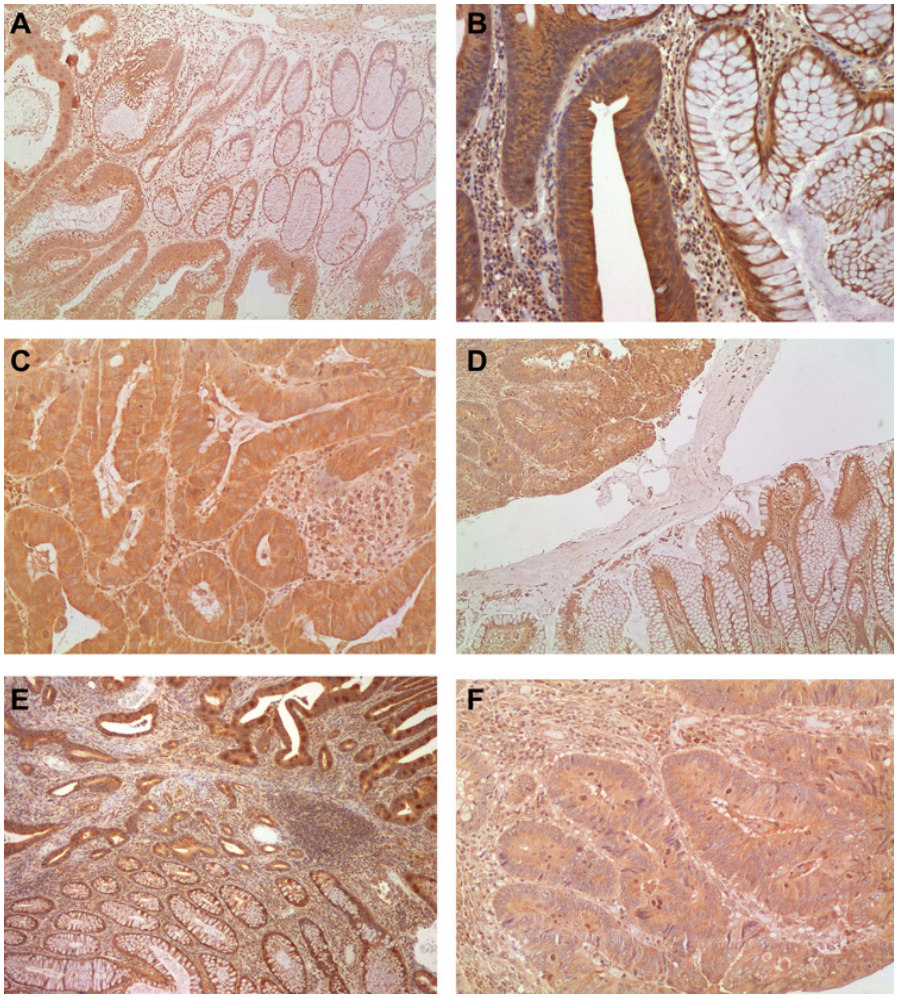

Figure 3 Immunohistochemistry images of the different lesions. (A) Normal mucosa/adenoma (low power field)—transition of normal mucosa (top and right), with weak and polarised expression, to low grade tubular adenoma (down and left) with very strong, diffuse, expression in almost all epithelial cells in this case for Toll-like receptor (TLR) 4 (similar to the other TLR). (B) Normal mucosa/adenoma (high power field)—on the right, normal mucosa with polarised, basolateral, and moderate TLR5 expression, with the transition in the left for adenoma with diffusely and strong TLR expression. (C) Adenoma (high power field)—high grade tubulovillous adenoma with strong and diffuse expression of TLR2. (D) and (E) Normal/adenocarcinoma (low power field)—TLR2 (D) and TLR5 (E) strong immunoreactivity in adenocarcinoma cells (up and left) in contrast to weak to moderate reactivity in normal mucosa (down and right). (F) Adenocarcinoma (high power field)—the majority of the tumours presented very strong TLR expression, in this case for TLR2.

provide indirect evidence that TLR activation may be essential for the development and progression of colon cancer. Indeed, Fukata *et al*[35] showed that, at least in mice, TLR4 activation is critical for colon cancer development, because TLR4$^{-/-}$ mice were protected against colitis-induced cancer. Other authors showed that silencing of TLR4 in mice decreased CRC tumour burden and metastasis,[51 52] and that TLR2 activation also induced the production of oncogenic factors in cultured human colon cancer cells.[39] Still, data in humans that associate TLR with colon cancer are scarce. Recently, Nihon-Yanagi *et al*,[53] using human colon cancer tissue, suggested that TLR2 activation might be involved in sporadic colon carcinogenesis. Also using human tissue other studies suggested that TLR4 expression may have prognostic value in CRC.[54 55] More importantly, a study found an association of polymorphisms of the TLR2 gene and Asp299Gly polymorphism of the TLR4 gene with sporadic CCR.[56]

In that line of thought, to our knowledge, our study is the first in humans that directly evaluated TLR gene and protein expression as well as other related factors throughout the whole adenoma–carcinoma sequence. First of all, we have found that controls expressed 24% more TOLLIP and a tendency for more PPAR-γ when compared with persons with a history of adenoma or carcinoma. These results, however, must be interpreted with caution because our primary aim was not to detect differences between the groups, there was a higher proportion of women in the controls and, finally, all the studied segments of normal mucosa from the controls were provided only from six different individuals. Nevertheless, when considering all the fragments of normal mucosa (adjacent or not), women, a protective factor for CRC, also presented 24% higher expression of TOLLIP and 35% lower expression of COX-2. Associating these facts with the 56% decrease in TOLLIP expression in the tumour lesions, which came predominantly from men, we may hypothesise that higher mucosa levels of TOLLIP may at least partly explain why some people do not develop tumours and why women have a lower incidence of CRC.

The major findings of our study were the decreasing levels of TOLLIP from normal mucosa to the adenoma–carcinoma lesions associated with high TLR protein levels. Importantly, this applied globally but also in an individual analysis, with 90% of the patients presenting this tendency. TOLLIP is an ubiquitin-binding protein that regulates inflammatory signalling by interacting with several TLR signalling cascade components and, therefore, is fundamental for controlling abnormal TLR activation.[57–61] Besides interacting with several intracellular kinases that are activated by TLR, TOLLIP also appears to interact and block TLR complexes.[57 61] Indeed, TOLLIP appears

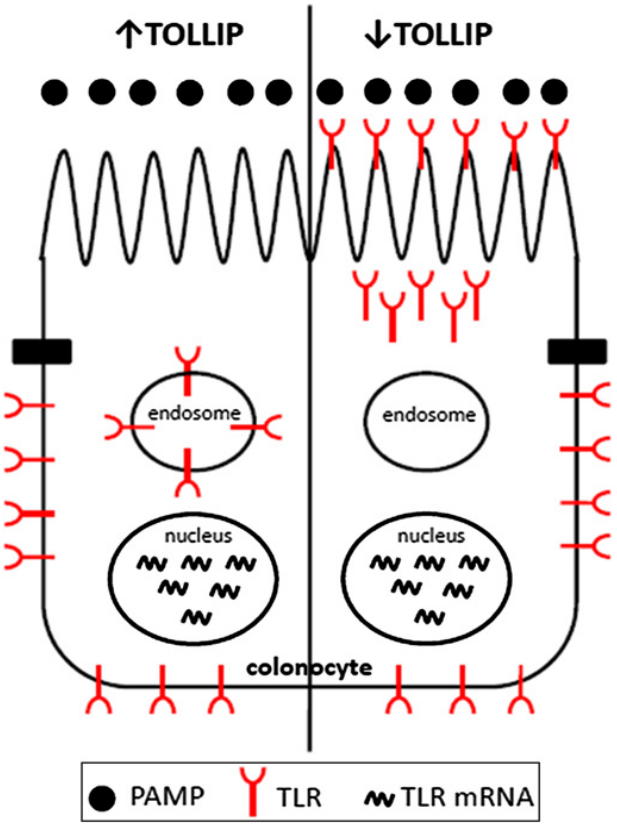

Figure 4 We hypothesised that high levels of Toll-interacting protein (TOLLIP), as in normal mucosa, interact with Toll-like receptor (TLR) molecules leading to early protein degradation and directing TLR to basolateral membrane. With low levels of TOLLIP, as in adenoma and adenocarcinoma, TLR are diffusely dispersed through the cell. PAMP, pathogen-associated molecular pattern.

to promote the traffic of synthesised proteins into endosomes and may lead to protein early degradation.[59 62–64] This might explain why in our study despite similar mRNA TLR expression we found a substantial increase in protein levels and also receptor dispersion through all the cytoplasm and apical membrane (figure 4). PPAR-γ is also associated with the attenuation of colon inflammation through modulation of TLR signalling and NF-κB inhibition, and its expression was also 46% lower in carcinoma cells. Combining the decreased levels of TOLLIP and PPAR-γ with the 38–63% increase in COX-2 and the high immunohistochemistry positivity of TLR receptors in the adenoma/carcinoma lesions we believe that continuous TLR activation by colonic bacteria is an important factor for tumour progression.

In a similar way, we have previously described an increased expression of TLR in gastric cancer, suggesting that progression of gastrointestinal malignancies may depend, at least partly, on bacteria–TLR interaction.[41] This may be even more important in colon carcinogenesis because all carcinomas included in our study were extremely positive for TLR, even the two tumours with poor to moderate differentiation, in contrast with the 25% of gastric adenocarcinomas that were TLR negative.[41] Moreover, we did not find any important differences in TLR expression between adenomas and adenocarcinomas. This suggest that TLR may become highly active after neoplastic transformation (adenoma) and continue extremely actively in all the process of colon carcinogenesis (from malignant transformation to tumour progression).

Another interesting aspect of our study, which to our knowledge was not previously described elsewhere, was that the adjacent normal mucosa presented a distinct genetic profile when compared with normal mucosa. A 25% statistically significant decrease in TOLLIP was associated with high levels of mucosal COX-2 and TNFα. The reason for this is not linear as it may represent a consequence of altered microbiota that colonises adenoma/carcinoma lesions or it may be a marker for an increased risk of neoplastic transformation. It is possible that these two aspects may be simultaneously true. It has been shown that commensal non-pathogenic bacteria may regulate TLR activation by increasing mucosal levels of TOLLIP and PPAR-γ.[18–22] With this in mind, it can be hypothesised that some risk factors for CRC that have been shown to alter commensal microbiota, such as diet and obesity,[65–67] may increase cancer risk by changing the mucosal genetic profile with lower TOLLIP expression. As a consequence, it might lead to higher TLR activation, promoting in that way higher COX-2 expression and subclinical inflammation. By promoting a pro-inflammatory and pro-mutagenic environment this might be the initial event for neoplastic transformation of the mucosa.

In conclusion, our results suggest that TLR signalling pathways play a crucial role in colon carcinogenesis and that they might be the missing link between diet, bacteria and cancer. For adenoma prevention, a strategy of modulation of TLR, by acting earlier in the cascade of events, may be cost effective when compared with COX-2 inhibitors and their side effects. Considering the high TLR expression in colon cancer cells, blocking TLR activation may also play an important role in cancer treatment. Future studies should evaluate the clinical value of these novel findings as the modulation of TLR activation may be accomplished by dietetic or pharmacological measures.

Take-home messages

Increasing activation of TLR may play a crucial role in colon carcinogenesis and tumour progression. TLR and innate immunity modulation may help to reduce the risk of colon cancer.

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Competing interests None to declare.

Patient consent Obtained.

Ethics approval The study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Portuguese Oncology Institute of Porto.

Provenance and peer review Not commissioned; externally peer reviewed.

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B) FUNCTIONAL POLYMORPHISMS OF TOLL-LIKE RECEPTORS 2 AND 4 ALTER THE RISK FOR COLORECTAL CARCINOMA IN EUROPEANS

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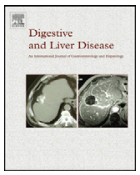

Oncology

Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans

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ABSTRACT

Background: Colon carcinogenesis is associated with increased expression levels of Toll-like receptor 2 and Toll-like receptor 4.
Aim: To determine in a Caucasian population the role of Toll-like receptor 2 and Toll-like receptor 4 polymorphisms in colorectal cancer development.
Methods: Hospital based multicentre case control study involving 193 colorectal cancer patients and 278 healthy individuals. DNA samples were extracted from blood cells and genotyping of *TLR2+597T>C*, *TLR2−4760T>C*, *TLR4−3745A>G*, *TLR2Arg753Gln*, *TLR4Asp299Gly* was performed. Functionality of risk polymorphisms was evaluated through production of TNF-α in cell culture and Toll-like receptors levels quantified by real-time RT-PCR.
Results: TLR2+597CC homozygous had 5-fold decreased risk (odds ratio (OR) = 0.21, 95% CI: 0.09–0.50, $p < 0.001$) and *TLR4 299Gly* homozygous 3-fold increased risk of colorectal cancer (OR = 3.30, 95% CI: 1.18–9.28, $p = 0.015$). In stratified analysis, *TLR2+597CC* genotype protective effect was even higher in overweight individuals (OR = 0.17, 95% CI: 0.06–0.53, $p < 0.001$) and in never smokers (OR = 0.11, 95% CI: 0.02–0.51, $p = 0.001$). Also, the increased risk effect for *TLR4 299Gly* homozygous genotype was higher in overweight individuals (OR = 8.67, 95% CI: 1.11–87.85, $p = 0.011$). *TLR2+597T>C* polymorphism conferred 41% less ($p = 0.03$) and *TLR4Asp299Gly* 65% more TNF-α production ($p = 0.02$) with no differences in Toll-like receptors levels.
Conclusion: Functional Toll-like receptor 2 and Toll-like receptor 4 polymorphisms significantly alter the risk to have colorectal cancer. Obesity and smoking may influence the risk for colorectal cancer in individuals presenting these genetic profiles.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, being the third most common in males and the second one in females. Its incidence rates are rapidly increasing in several areas in the world, probably related to a combination of factors like diet, obesity and smoking [1–3]. It is clear that there are at least three distinct molecular pathways for CRC development [4,5]. Nevertheless, modifier genes and inflammatory molecules, by promoting genomic instability and controlling cell growing, may also be important for the progression of these CRC carcinogenic pathways [6–8]. Indeed, COX-2 polymorphisms have been associated to CRC risk, suggesting that other factors, namely pro-inflammatory ones, significantly influence the adenoma–carcinoma sequence [9–11].

Toll-like receptors (TLR) are key players in immune system, with ten different TLRs being expressed in humans [12,13]. TLR2

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recognizes a number of pathogen-associated molecular patterns (PAMP) from Gram positive bacteria and TLR4 is the receptor of the Gram negative bacteria lipopolysaccharide (LPS) [14–16]. Activation of these receptors initiates intracellular signalling pathways that promote cell survival and production of different pro-inflammatory mediators such as COX-2 [12,17–20]. Because they are not only intrinsically related to inflammation but also to cell survival signalling, epithelial regeneration and cell proliferation, recent reports associate these receptors function to tumourigenesis [21,22]. Concerning gastrointestinal system, current evidence suggests that TLR innate immune responses to PAMPs from luminal microbiota may be essential for the development of tumours [21–24]. In fact, our own group and other authors have shown that human colon carcinogenesis is associated with increasing expression levels of TLR2 and TLR4 [25–28].

Playing an important role in the interface between host and the environment, dysregulation of the TLR2 and TLR4 signalling pathways due to functional single nucleotide polymorphisms (SNPs) can disrupt the normal cellular immune response and consequently conditioning cytokines cellular levels, contributing for inflammation and cancer. Genetic variants in TLRs encoding genes may contribute to different response phenotypes, including susceptibility to cancer development.

A potential functional genetic polymorphism in TLR4 gene has been described responsible for an A-to-G transition in exon 3, causing an aspartic acid/glycine substitution Asp299Gly (rs4986790). This transition affects the extracellular domain of TLR4 receptor, in a ligand-recognition area [29]. *TLR4 Asp299Gly* polymorphism has been subject of investigation in several studies involving different types of cancer [30–35]. Despite some studies observed lack association of *TLR4 Asp299Gly* polymorphism and the risk of CRC development [36,37], one study associated this SNP to CRC [38] and others address its role in tumour prognosis [39,40]. Several TLR2 SNPs have also been associated to cancer [38,40,41], namely, it has been reported, that TLR2+597T>C (rs3804099) polymorphism can alter the risk of colon cancer development [40].

We hypothesized genetic SNPs, with potential influence on TLR2 and TLR4 receptor expression and/or function, may have impact in CRC development. Our purpose was to address the role of potential functional TLR SNPs on CRC risk in a European Caucasian population.

2. Materials and methods

2.1. Study population and data collection

The study population has been described previously [11]. This hospital-based case–control study included 471 participants: 193 histologically confirmed CRC patients and 278 cancer-free controls from the northern and central region of Portugal recruited at the Portuguese Institute of Oncology, Porto (IPOP) and Coimbra (IPOC). Eligible cases included patients aged 50–75 years with a newly diagnosed of CRC between January 2002 and September 2007 and CRC patients submitted to chemotherapy between January 2004 and March 2008 that were under follow-up between February and March 2008 at IPOP and IPOC. Controls were healthy individuals aged 50 years or more without clinical evidence of cancer (blood donors) recruited at IPOP between July 2005 and October 2007. The characteristics of the study population are summarized in Table 1. Cases were significantly older than controls' with a median age of 62 years (50–75) [vs 56 years in controls (50–65), $p < 0.001$]. There were no significant differences in the distribution of gender, BMI and smoking habits between both groups. Written informed consent was obtained from all participants before their inclusion in the study, according to the Declaration of Helsinki.

Table 1
Description of participants (cases and controls): age, gender, body mass index, smoking habits, and summarized clinical characteristics of cases (patients with cancer).

	Cases $n = 193$	Controls $n = 278$	p
Demographics			
Age (years)			
Mean (SD)	62 (7)	56 (4)	
Median [min–máx]	62 [50–75]	55 [50–65]	<0.001
Gender, *n* (%)			
Male	123 (64)	176 (63)	0.926
Female	70 (36)	102 (37)	
Lifestyle behaviours[b]			
BMI category[a], *n* (%)			
<25 kg/m^2	49 (26)	41 (23)	0.598
≥25 kg/m^2	143 (74)	136 (77)	
Smoking status, *n* (%)			
Never smokers	142 (74)	110 (66)	0.095
Ever smokers	51 (26)	58 (34)	
Tumour characteristics			
Tumour location, *n* (%)			
Rectum	82 (42)	–	
Colon	111 (58)	–	
Stage, *n* (%)			
I or II	76 (40)	–	
III or IV	116 (60)	–	

BMI, body mass index.
[a] Categorization based on the cut-off defined by WHO for overweight people.
[b] The numbers may not add-up since we were unable to gathered this information for all subjects, namely in controls' group.

Furthermore, the Ethics Committee of the IPOP and IPOC approved this research.

2.2. Sample DNA extraction and TLR2/TLR4 polymorphisms genotyping

Genomic DNA was extracted from peripheral blood leukocytes, using the QIAamp® DNA Blood Mini Kit (Qiagen, Madrid, Spain) following manufacturer's instructions.

The selection of studied TLR2 and TLR4 polymorphisms was based on expected functional repercussion (FastSNP) and/or previous associations with cancer development of SNPs retrieved from literature and public database search (dbSNP). The following polymorphisms were selected: TLR2 Arg753Gln (rs5743708), TLR2−4789T>C (rs4696483), TLR2+597T>C (rs3804099); TLR4 Asp299Gly (rs4986790) and TLR4−3869A>G (rs2737191). TLR2 Arg753Gln and TLR4 Asp299Gly variants were analysed through PCR-RFLP method. Briefly, DNA was amplified in a 50-μL reaction mixture containing TLR4 Asp299Gly primers (forward, 5′-AGC ATA CTT AGA CTA CTA CCT CCA TG-3′; reverse, 5′-GAG AGA TTT GAG TTT CAA TGT GGG-3′), and TLR2 Arg753Gln primers (forward, 5′-CAT TCC CCA GCG CTT CTG CAA GCT CC-3′; reverse, 5′-GGA ACC TAG GAC TTT ATC GCA GCT C-3′) (Metabion Martinsried, Germany), respectively, 1× PCR buffer, 1 unit Taq polymerase, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, and 20 ng DNA. TLR2 PCR products (129 bp) were incubated with MspI restriction endonuclease at 37 °C, in the presence of allele G the fragment is cleaved by the enzyme giving arise two fragments (104 and 25 bp), whereas the A allele is not cleaved by the enzyme. TLR4 PCR products (188 bp) were incubated overnight with NcoI restriction endonuclease at 37 °C, the polymorphism was defined by the presence (G) or absence (A) of a restriction site. TLR2+597T>C, TLR2−4789T>C and TLR4−3869A>G polymorphisms were analysed by allelic discrimination using 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR were carried out using a 6-mL reaction mixture, containing 1× Master Mix (Applied Biosystems), with 1× probes (TaqMan assay, C_22274563_10, C_27313261_10, C_1844485_10, respectively

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Table 2
TLR-2+597T>C and *TLR-4 Asp299Gly* polymorphisms-related odds ratios for colorectal cancer and genotype frequencies in patients and controls.

	Controls *n* (%)	Cases *n* (%)	OR	95% CI	*p*
TLR-2+597T>C					
TT/TC	235 (86)	184 (97)			
CC	37 (14)	6 (3)	**0.21**	**0.086–0.501**	**<0.001**
TLR-4 Asp299Gly					
AA/AG	186 (97)	169 (92)			
GG	5 (3)	15 (8)	**3.30**	**1.175–9.279**	**0.015**

OR, odds ratio; 95% CI, 95% confidence interval. Bold values represent statistical significant results and the significance of that values (*p*) is in the right column.

Applied Biosystems) and 20 ng of the DNA sample. Quality control procedures implemented for genotyping included double sampling in about 10% of the samples to assess reliability and the use of negative controls to step-away false-positives. In PCR-RFLP method, two authors obtained the results independently, and the ambiguous were reanalysed.

2.3. Functional evaluation of TLR's genotypes – culture and activation of peripheral blood monocytes (PBM)

Blood samples were obtained from 14 healthy blood donors according to the different genotype of *TLR2+597T>C* and *TLR4 Asp299Gly* polymorphisms. Our culture cell protocol was described elsewhere [42]. Briefly, PBM were isolated from whole blood by density-gradient centrifugation with Ficoll-Paque (GE Healthcare Lifesciences, UK) followed by positive selection isolation with anti-CD11b Microbeads (MACS, Miltenyi Biotec, Germany). Afterwards, PBM primary culture was performed. The monocytes samples were adjusted to 1×10^5 cells per well and cultured in quadruplicate in RPMI-1640 medium (GE Healthcare Lifesciences, UK), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine and 12% foetal bovine serum (GE Healthcare Lifesciences, UK) at 37 °C and 5% of CO_2. After 3 h incubation, nonadherent cells and supernatants were removed and fresh medium was added (time 0 h). PBMs from the different genotypes were separately incubated in four different wells with zymosan (Zym) [2 μg/mL] for TLR2/TLR6 stimulation, with Lipopeptide (Lp) Pam3Cys-SK4 [1 μg/mL] for TLR2/TLR1 activation, with LPS [1 μg/mL] for TLR4 stimulation, and 0.9% NaCl as internal control. The supernatants were collected after 24 h stimulation. After collection, supernatants were frozen at −80 °C until analysis of TNF-α levels (R&D Systems, USA; sensitivity 1.6 pg/mL).

2.4. Isolation of mRNA from PBM and quantification of TLR2 of TLR4 expression

These methods were described elsewhere [42]. Briefly, after separation and isolation of PBM, 1×10^5 cells were collected and the final cell pellet was used for mRNA isolation with TriPure Isolation Reagent (Roche, Germany). Two-step real-time RT-PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle of graded dilutions from a randomly selected sample. For relative quantification of specific mRNA levels, 100 ng of total mRNA from each sample underwent two-step real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all genotypes, which enabled the use of this gene as internal control. Specific PCR primers pairs for the studied genes were: **GAPDH** – fw (P1) 5′-TTG GCC AGG GGT GCT AAG-3′ and rev (P2) 5′-AGC CAA AAG GGT CAT CAT CTC-3′; **TLR2** – fw 5′-GAT CCC AAC TAG ACA AAG ACT-3′ and rev 5′-CTG CGG AAG ATA ATG AAC ACC-3′; **TLR4** – fw 5′-CTA AAC CAG CCA GAC CTT GAA-3′ and rev 5′-ACC TGT CCC TGA ACC CTA TGA-3′. Results of mRNA quantification were expressed as the ratio gene/GAPDH.

2.5. Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences – SPSS for Windows (version 17.0). The Hardy–Weinberg equilibrium was tested by a Pearson goodness-of-fit test to compare the observed vs the expected genotype frequencies. Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Statistical differences between mean values were evaluated applying the Mann–Whitney test. Multivariate logistic regression analysis was used to estimate odds ratio (OR) and its 95% confidence interval (CI) as a measure of the association between variant allele carriers and the risk for the development of CRC. The potential confounding variables: age, gender, BMI and smoking habits were addressed through data stratification. For each OR estimation dominant and recessive models of analysis were followed and results presented according to the tendency observed. The Kaplan–Meier method and log-rank test were used to compare genotype influence in the age at CRC diagnose. One-way ANOVA and Student's *t* test for paired and unpaired data (or correspondent non-parametric test) were used for group comparison of TNF-α production in cell culture and for mRNA levels. Statistical significance was set at $p < 0.05$.

3. Results

3.1. SNP analysis and risk evaluation

We did not find any differences between cases and controls concerning *TLR2−4760T>C*, *TLR2Arg753Gln* and *TLR4−3745A>G* polymorphisms. *TLR2+597T>C* and TLR4 *Asp299Gly* polymorphisms genotypes' distribution in cases and controls and genetic profile-associated risk of CRC are presented in Table 2. According to *TLR2+597T>C* polymorphism, the CC genotype was under-represented in CRC group (3% vs 14% in controls', $p < 0.001$). The present results show lower risk for developing CRC in CC genotypes carriers than in those individuals' carriers of TT/TC genotypes (OR = 0.21, 95% CI: 0.09–0.50, $p < 0.001$). In *TLR4 Asp299Gly* genotype distribution, we observed that GG genotype was more frequent in CRC group than in control group (8% vs 3%, $p = 0.015$). Furthermore, we observed that GG genotype carriers had higher risk for developing CRC than AA/AG genotype carriers (OR = 3.30, 95% CI: 1.18–9.28, $p = 0.015$). We observed an interaction between *TLR2+597T>C* polymorphism and BMI and smoking status but not with gender (Table 3). Both, female and male CC genotype carriers had lower risk to CRC development (OR = 0.10, 95% CI: 0.01–0.76, $p = 0.005$ and OR = 0.27, 95% CI: 0.10–0.73, $p = 0.004$, respectively). We observed that CC genotype is associated with lower risk to CRC development in individuals with BMI ≥ 25 (OR = 0.17, 95% CI: 0.06–0.53, $p < 0.001$) and in individuals never smokers (OR = 0.11, 95% CI: 0.24–0.51, $p = 0.001$). Concerning the *TLR4 Asp299Gly*

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Table 3
Potential interaction between gender, body mass index, smoking status and *TLR-2+597T>C and TLR-4 Asp299Gly* polymorphisms in the development of colorectal cancer.

	Controls *n* (%)	Cases *n* (%)	OR	95% CI	*p*
TLR-2+597T>C					
Stratification					
Gender					
Female					
TT/TC	84 (87)	67 (98)			
CC	13 (13)	1 (2)	**0.10**	**0.012–0756**	**0.005**
Male					
TT/TC	151 (86)	117 (96)			
CC	24 (14)	5 (4)	**0.27**	**0.100–0.726**	**0.004**
BMI					
<25					
TT/TC	37 (97)	46 (96)			
CC	1 (3)	2 (4)	1.61	0.140–18.441	0.588
≥25					
TT/TC	114 (86)	138 (97)			
CC	19 (14)	4 (3)	**0.17**	**0.058–0.526**	**0.001**
Smoking status					
Never smokers					
TT/TC	92 (88)	138 (99)			
CC	12 (12)	2 (1)	**0.11**	**0.024–0.508**	**0.001**
Ever smokers					
TT/TC	50 (86)	46 (8)			
CC	8 (14)	4 (8)	0.54	0.153–1.026	0.260
TLR-4 Asp299Gly					
Stratification					
Gender					
Female					
AA/AG	57 (98)	61 (91)			
GG	1 (2)	6 (9)	5.61	0.655–48.015	0.083
Male					
AA/AG	129 (97)	87 (92)			
GG	4 (3)	9 (8)	2.77	0.805–8.969	0.084
BMI					
<25					
AA/AG	27 (96)	42 (93)			
GG	1 (4)	3 (7)	1.93	0.191–19.512	0.502
≥25					
AA/AG	91 (99)	126 (91)			
GG	1 (1)	12 (9)	**8.67**	**1.107–87.845**	**0.011**
Smoking status					
Never smokers					
AA/AG	74 (100)	123 (91)			
GG	1 (1)	12 (9)	**7.22**	**1.08–56.660**	**0.004**
Ever smokers					
AA/AG	36 (95)	46 (94)			
GG	2 (5)	3 (6)	1.174	0.186–7.403	0.620

OR, odds ratio; 95% CI, 95% confidence interval. Bold values represent statistical significant results and the significance of that values (*p*) is in the right column.

polymorphism, we observed that individual' carriers of GG genotype and BMI ≥ 25 had a higher risk to CRC development (OR = 8.67, 95% CI: 1.11–87.85, $p = 0.011$). Furthermore, the GG genotype was also associated with risk to CRC development in never smokers' individuals (OR = 7.22, 95% CI: 1.08–56.67, $p = 0.004$). No difference was found considering different cancer locations, namely comparing rectal or colon cancer.

3.2. Influence of TLR2+597T>C and TLR4 Asp299Gly polymorphisms on the time-to-diagnosis of CRC

When we evaluated the influence of *TLR2+597T>C* polymorphism in the age at CRC diagnose (Fig. 1), we observed that the TT/TC genotype carriers tend to be younger than CC genotype carriers at diagnose (66 vs 69 years, $p = 0.073$, respectively). Concerning *TLR4 Asp299Gly* polymorphism we observed a lack of association of the polymorphism and the age at CRC, despite GG carriers being younger at the age of diagnosis (GG vs AA/AG, 63 vs 65 years, $p = 0.4$ respectively). No other statistical important association or tendency between the studied polymorphisms and any other clinical parameter (e.g. survival, answer to therapy) was found.

3.3. Functional characterization of TLR2+597T>C and TLR4 Asp299Gly polymorphisms

The genotypes of the 14 participants involved in the functional study were: *TLR2+597T>C*, 5 CC, 6 TC and 3 TT; *TLR4Asp299Gly*, 5 GG, 2 AG and 7 AA. Statistical differences were found when comparing *TLR2+597T>C* CC homozygous with T carriers after Lp stimulation (TNF-α production of 127.0 ± 18.7 vs 214.3 ± 23.2 pg/mL, $p = 0.03$) and after LPS stimulation when comparing *TLR4 299Gly* carriers vs AA homozygous (TNF-α production of 259.5 ± 27.7 vs 157.9 ± 22.2 pg/mL, $p = 0.02$) (Fig. 2). The TLR2 mRNA levels for the different TLR2 genotypes were 0.58 ± 0.11 (CC), 0.41 ± 0.08 (CT) and 0.47 ± 0.17 (TT) and the TLR4 mRNA levels for the different TLR4 genotypes were 1.5 ± 0.59 (AA), 0.52 ± 0.35 (AG) and 0.87 ± 0.26 (GG), without any statistical difference between the groups. T carriers for *TLR2+597T>C* had TLR2 levels of 0.42 ± 0.07 ($p = 0.2$ vs CC

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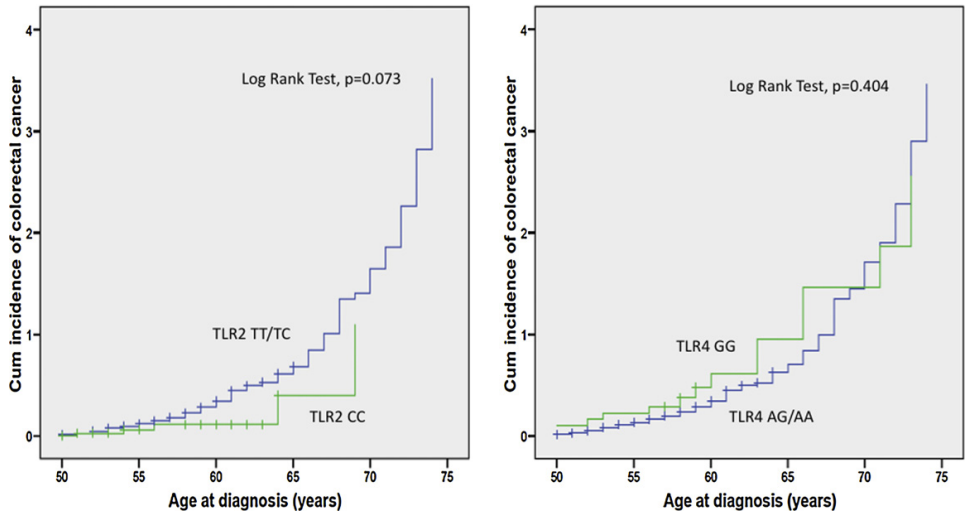

Fig. 1. TLR2 (*TLR-2+597T>C*) and TLR4 (*TLR-4 Asp299Gly*) genotype influence in the age of CRC diagnosis (Kaplan–Meier curves and log-rank test). The effect of the *TLR-2+597T>C* CC genotype in the age of diagnosis was stronger than the *TLR4 299Gly* homozygous genotype.

homozygous) and G carriers of *TLR4Asp299Gly* had TLR4 levels of 0.82 ± 0.25 ($p = 0.2$ vs AA homozygous).

4. Discussion

In the present study we describe that functional TLR2 and TLR4 SNPs significantly influence the risk of CRC. Our results suggest that small changes in the normal function of these receptors due to functional SNPs may contribute to an unbalanced cytokine and pro-oncogenic cellular microenvironment and thus to a higher risk for cancer development.

Why should TLRs SNPs influence the risk of CRC development? It is current knowledge that a strict regulation of TLRs activation is fundamental for maintaining colon homeostasis [24]. Normal colon mucosa constitutively express TLRs, however, it also presents a high expression of TLRs inhibitors, like TOLLIP and PPARγ, which circumscribe TLRs protein expression to basolateral membrane where they are not continuously exposed to PAMPs preventing in this way inadequate inflammation to commensal bacteria [43–48]. Basal TLRs expression in that particular cellular location appears important for maintaining gastrointestinal homeostasis, preventing at the same time auto-immunity processes, controlling bacterial infections and allowing epithelial regeneration [21,49–52]. Since TLRs also activate cell survival signalling pathways, abnormal TLR activation could promote colon carcinogenesis [21,22]. Indeed, several groups including our own have shown in human studies that colon carcinogenesis is associated with decrease expression of TLRs inhibitors and conversely with higher protein expression of TLR2 and TLR4 [26,28]. It was previously shown that TLR4 expression in tumours may have prognostic value [25,27] and several animal studies suggest that TLR2 and TLR4 activation may be essential for CRC development [23,53–55]. So, it appears that dysregulation of these receptors activation may influence the risk of cancer.

In this line of thoughts, we found that *TLR2+597T>C* and *TLR4 Asp299Gly* SNPs significantly influence the risk of CRC development, suggesting that these TLRs SNPs may be genetic susceptibility markers for CRC. The CC genotype of the *TLR2+597T>C* SNP was associated with 5-fold decreased risk of CRC development (OR = 0.21), which is a remarkable result for a SNP. In our study, the CC genotype frequency in controls was similar to that observed in healthy European Caucasian [56] and Korean individuals [57] and higher than that observed in Thailand [58]. The *TLR2+597T>C* polymorphism in exon 3 does not appear to induce any amino acid change, remaining its functional impact and molecular mechanism poor understood. According to in silico analysis, this SNP can introduce alterations in splicing regulation, possibly leading to an alteration in TLR2 molecule. On the other hand, it may be in linkage disequilibrium with another functional SNP in TLR2 and thereby influencing promoter activity or the stability of the transcript [59]. Previous reports have shown associations of this polymorphism in TLR2 gene with melanoma susceptibility [41], sepsis [60] and reverse reaction in leprosy [59]. To the best of our knowledge we showed for the first time that TLR2+597T>C SNP may confer hypofunctionality to the receptor. Indeed, monocytes with the CC genotype produced 41% less TNF-α in cell culture. Moreover, we did not find any differences in TLR2 levels between the different

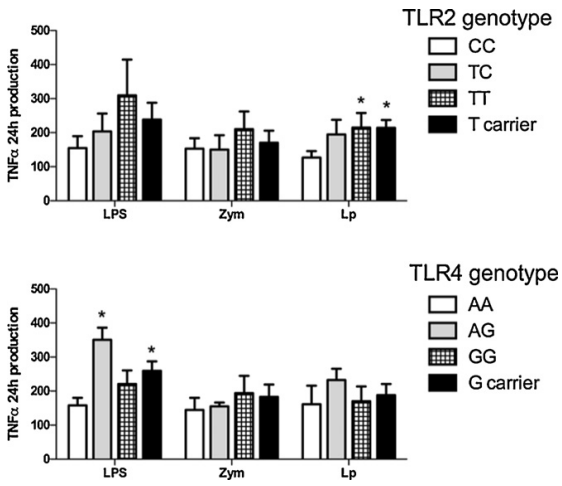

Fig. 2. TNF-α 24 h production (pg/mL) after stimulation with LPS (lipopolysaccharide), Zym (zymosan), and Lp (lipopeptide) in culture cell of monocytes with the different TLR2 (*TLR-2+597T>C*) and TLR4 (*TLR-4 Asp299Gly*) genotypes. *$p < 0.05$ vs CC (TLR2) or vs AA (TLR4).

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genotypes suggesting that the potential hypofunctionality conferred by this polymorphism is not dependent of TLR2 levels. So, for any given stimulus, individuals with this SNP may have less production of inflammatory cytokines and less cell survival signalling and this might help to explain the increase risk of melanoma and sepsis and the decrease risk of CRC with an early age of diagnosis.

The *TLR4 Asp299Gly* polymorphism leads to missense replacement of a conserved aspartic acid residue with a glycine amino acid that alters the structure of the extracellular domain of this receptor. *TLR4 Asp299Gly* has been subject of investigation in several studies involving different type of cancer with controversial results [31,33,34,39,61–64]. Due to evolutionary pressure and human migration, this TLR4 polymorphism has a distinct distribution in different populations, and may or not be cosegregated with the *TLR4 Thr399Ile* polymorphism, which may change the functionality of the receptor and may help to explain the discrepancies between the studies [65]. We observed that in our control population the frequency of *TLR4 Asp299Gly* polymorphism tend to be similar to those observed in healthy European Caucasian [31,33,34] and American [61,62] populations. The significance of this SNP led to contradictory conclusions about its functional role [66]. Studies performed by Arbour et al. reported that this SNP was associated with a blunted response o inhaled LPS [29]. However, Lundberg et al. suggested that not only genetic variant in TLR4 should be considered in functional studies but also the origin of LPS [67]. Furthermore, Ferwerda et al. showed that cells from individuals' carriers of *299Gly* variant significantly produce higher amounts of pro-inflammatory cytokines than homozygous wild-type [65]. Recently, a study performed by Eyking et al. demonstrated that Caco-2 cells which expressed *TLR4 Asp29Gly* polymorphism had a significant increase in expression levels of genes associated with inflammation and/or tumourigenesis compared with cells that expressed other forms of TLR4 [68]. Our results, although they are not definitive concerning the functionality of this SNP, are in agreement with the results of Eyking et al. showing that monocytes from carriers of G allele produce 64% more TNF-α when stimulated with LPS. Clinical studies also confirm the oncogenic potential of this SNPs since this variant allele has been associated with a more quickly relapse in patients submitted to radiotherapy and chemotherapy [30]. Other study showed that this TLR4 SNP might alter prognosis on patients that receive oxaliplatin [37]. So, this gain-of-function genetic variant implies the *TLR4 Asp299Gly* in malignant progression of human colon cancer [68]. Future studies should study the role of these SNPs also for prognosis and answer to therapy.

The other aspect that is interesting in our study is that both the protective effect of the *TLR2+597T>C* SNP and the risk effect of the *TLR4 Asp299Gly* SNP appear to be stronger in overweight and never smokers' individuals. Obesity is a well known risk factor for CRC and in last decade, increase evidence has suggested the relevance of a chronic inflammatory state in obesity [69]. Long-term smoking also causes systemic inflammation with an increase of inflammatory mediators concentration (C-reactive protein, IL-6, IL-8, TNFα) [70]. Indeed, and in agreement with our results, recently it was shown that *TLR2+597T>C* polymorphism can interact with nonsteroidal anti-inflammatory drug use and cigarette smoking to alter risk of colon cancer [40]. Individuals never-smokers and CC genotype carriers have even lower risk for CRC development probably due to their genetic background, with attenuated TLR2 function, and due to lower exposure to environmental factors. In that line of thoughts it is easily understood why overweight individuals *TLR4 Asp299Gly* homozygous have greater risk of cancer, however, why never smokers have greater risk than smokers is not so comprehensive. We may speculate that the genetic influence of the TLR4 Asp299Gly SNP may be blunted in the face of the deleterious effect of smoking and, so, this SNP may strongly interact with the inflammatory process of obesity but not with the distinctive inflammation process of smoking. Indeed, it is well known that TLR4 may have an important influence on adiposity and metabolic syndrome [71].

Two main drawbacks could be noticeable in our study. First, even if a match for ages were attempted by including only controls aged 50 or more, a difference of ages between cases and controls existed and secondly, the level of certainty of absence of CRC among controls. The first was addressed in the statistical analysis and for the second point, we should consider that controls were recruited in 2005–2007, and, up to now, 85% of them (235/278) were asymptomatic and still blood donors, so no clinical evidence of CRC is present 5 years after the recruitment. Moreover, of the 43 controls that were not blood donors in 2012, 31 quit because of age criteria and there were no record of CRC in any of the 278 participants 5–7 years after recruitment. Thus, taking altogether, we may well consider that our control population represents individuals without CRC and that the difference of ages at the time of recruitment was not an issue to our results and conclusions.

In conclusion, functional TLRs SNPs modulate in a significant way the individual susceptibility for CRC development with the *TLR2+597CC* genotype decreasing 5-fold, whereas *TLR4 299Gly* homozygous genotype increasing 3-fold the CRC risk. Factors like obesity and smoking habits may influence the risk of CRC in individuals presenting these genetic profiles. In future, the identification of these genetic profiles may help to define more efficacious strategies for screening of CRC through an individual fitted schedule.

Conflict of interest statement
None to declare.

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CHAPTER VI - CONCLUSION

"Research is to see what everybody else has seen, and to think what nobody else has thought."

Albert Szent-Györgi (1893-1986)

In this dissertation we have analyzed the role of TLRs, mainly TLR2, TLR4 and TLR5, in several gastrointestinal pathologies, particularly in ones related with malignant or premalignant lesions. Although it was not possible to study TLRs in all gastrointestinal diseases, we have described TLRs expression in gastrointestinal normal epithelia as well as in preneoplastic and neoplastic lesions. Expression of several other TLRs related molecules was also analyzed and interesting results were provided. We have shown that gastrointestinal carcinogenesis was associated with increased expression of TLRs and/or decreased expression of their antagonist molecules. Moreover, we have provided data that suggest that single nucleotide polymorphisms of these receptors may significantly impact the risk of an individual to develop gastrointestinal cancer. Furthermore, emphasizing the multifaceted role of TLRs, we have shown that attenuation of these receptors function may on the other hand contribute to cirrhotic infection risk. These previously not described data suggest that in the future TLRs modulation may be an interesting therapeutic option not only to prevent infectious complications but more important to prevent cancer development. In the following sections we will discuss the role of TLRs in the different gastrointestinal organs studied in this thesis individually and then an integrated conclusion will be provided.

TLRs and Liver

In this dissertation we have seen evidence that link TLR to chronic liver disease infection risk and to the inflammation-fibrosis-carcinoma sequence. Although an attenuation of TLR2 and TLR4 activation was associated with infection, increasing levels of these receptors were involved in the progression of liver lesions to cirrhosis and hepatocellular carcinoma.

Previously to our work, some other authors had suggested that TLRs might be involved in the cirrhotic infection risk. However, the results were somewhat contradictory and not conclusive. Lin *et al* and Testro *et al* suggested that advanced cirrhotic patients presented an attenuated TLR4 response to LPS and that this blunted response to LPS might be dependent of decreased TLR4 levels with the possibility of antibiotic restoring the immunologic response to LPS (1, 2). Riordan *et al* did not find attenuation of TLR4 function but a blunted TLR2 activation in immunological cells was found also with decreased levels of this receptor and with symbiotic therapy also able to reverse TLR2 towards normal (although with decreased TLR2 function) (3). Other studies suggested that TLR function might in fact be increased or unaltered and that TLRs levels might be increased despite low function (4-6).

In that line of thoughts we believe that our original and review studies provided some lights about the subject. In our study, TLR2 and TLR4 innate immune responses as well as several endotoxaemia markers were analyzed in a group of patients with stable alcoholic cirrhosis (7). Our patients were selected from an outpatient hepatology clinic and presented no recent history of infection, gastrointestinal bleeding, hospital admission

or evidence of recent alcohol ingestion. Moreover, we also studied a group of decompensated cirrhotic patients and obtained clearly distinct results, further supporting the stability of our group of patients. In our study patients with stable alcoholic chronic liver disease showed attenuated TLR2-mediated innate immune response with an impaired TLR4 response only found in the unstable patients. Moreover, we did not find any difference in genetic or protein expression of TLR2 and TLR4 levels to explain this attenuated function of these receptors.

Taken altogether our results and also our review (8) suggest that TLR2 function at an early stage and that TLR4 function in advanced stages of disease are compromised and that this may constitute an important mechanism of acquired immunodeficiency in cirrhotic patients. Moreover, by analyzing all the data and in agreement with our results we might conclude that this deficiency is independent, at least in part, of TLRs levels and it is probably related to dysfunction in intracellular signalling pathways. Finally, this process may in part be reversible with antibiotics and/or probiotics (2, 6). Future studies should consider if modulation of TLRs function in cirrhotic patients would be beneficial.

Concerning the role of TLRs in the progression of liver diseases, many data imply activation of these receptors, particularly TLR4, in the progression of the inflammation-fibrosis-carcinoma sequence. Several studies show that liver disease is associated with endotoxaemia and bacteremia, TLRs main agonists (9-13). Other studies using different models of fibrosis and cirrhosis and different mechanisms to block TLR4 signalling

confirmed that LPS-TLR4 activation is essential for hepatic fibrogenesis (14, 15). Even though TLR4 activation in Kupffer cells promotes the production of several pro-inflammatory and also pro-fibrogenic mediators (16-19), TLR4 activation in hepatic stellate cells appears to be the vital step for collagen production and consequently fibrosis and cirrhosis (15, 20, 21). Recent studies confirmed that variants of TLR4 gene modulate risk of liver fibrosis (22-24). The data linking other TLRs to hepatic fibrogenesis are not so strong. However, some studies associate not only TLR4 but also TLR2 activation to fibrosis and cirrhosis, independently of the cause of liver disease (alcohol, metabolic, virus) (25-28). Although the important role of TLRs, mainly TLR4, in liver inflammation and fibrosis is consensual, regarding the progression to hepatocellular carcinoma the results are not so strong. Nevertheless, a recent study has revealed TLRs, in particular TLR4, as major factors linking hepatic chronic inflammation and carcinoma (29). The problem with these data, although important, is that the suggested implication of TLR2 and TLR4 in the pathogenesis of hepatic inflammation-fibrosis-carcinoma sequence is mainly based on evidence obtained from animal studies or in vitro hepatocyte culture models. Studies using human liver tissue to confirm or refute the in vitro and animal findings are scarce and have evaluated TLR2 and TLR4 in each stage of this sequence separately.

In our study, we evaluated for the first time in humans the expression of TLR2 and TLR4 in liver samples from patients in each stage of virus-induced hepatic inflammation-fibrosis-carcinoma sequence (30). We found an increased TLR2 and TLR4 mRNA and protein expression in virus-induced

chronic hepatitis and cirrhosis and a maintained TLR2 and TLR4 levels in virus-induced carcinoma. Moreover, we found that hepatic TNF-α and COX-2 mRNA levels, factors induced by TLR activation and implicated in liver inflammation and carcinogenesis (31-33), were also increased in this sequence of liver disease. Thus, we showed for the first time in humans that upregulation of TLR2 and TLR4 is an early and persistent event in the hepatic inflammation-fibrosis-carcinoma sequence. Our results confirmed previous animal data and open the door to a new line of research concerning modulation of TLRs pathways in order to prevent progression of liver diseases.

TLRs and Stomach

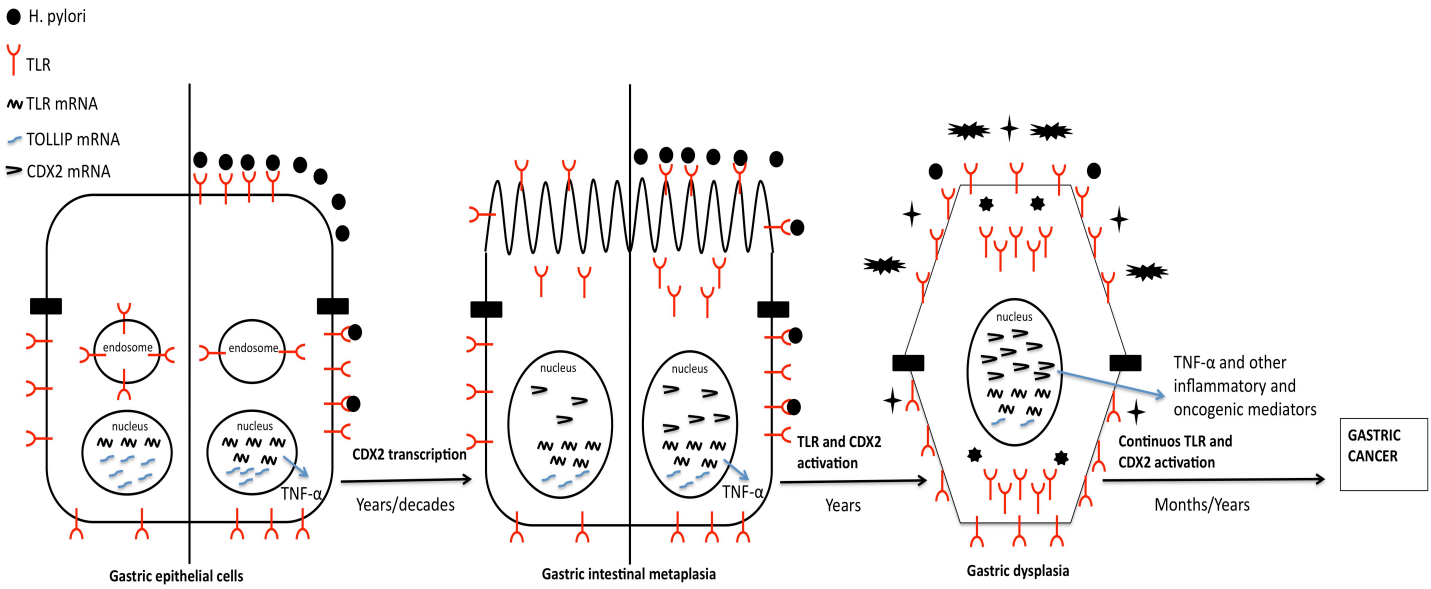
Current knowledge provides conclusive data that TLRs have an essential role in *H. pylori* recognition and subsequent innate and adaptive immunity against this bacterium (34). After the first contact with the gastric mucosa *H. pylori* interacts with several TLRs, with TLR2 being the receptor responsible for most of the immunologic reactions occurring as the result of infection (35, 36). In fact, although being a Gram-negative bacterium, TLR2 appears to be the receptor responsible for most of the inflammatory changes occurring as the result of *H. pylori* infection. Indeed, several studies showed that TLR2 was required for *H. pylori*-induced NF-kappa B activation and cytokine production by epithelial (35) and antigen presenting cells (37). Cytotoxin-associated gene A (Cag A), an important virulence factor of *H. pylori*, promotes a higher production of IL-8 by TLR2 and not by TLR4 signalling (36). In spite of that, other studies suggest that TLR4 also play an important role in *H. pylori* infection by recognizing several other *H. pylori* antigens (38-40). More conclusive studies demonstrate that either in epithelial or dendritic cells, TLR2 is in fact the principal receptor for recognition and immunologic response to *H. pylori*, but this process depends also in a minor extent of TLR4 that acts in synergy with TLR2 (34, 41-45). TLR9 recognizes *H. pylori* DNA and appears to have a complementary and synergistic interaction with the other two receptors (42, 46). On the other hand, the role of TLR5 is very controversial, despite some initial studies had showed interaction between *H. pylori* flagellin and this receptor (35, 47), other studies suggested that TLR5 is unresponsive to *H. pylori* flagellin (48-50).

Although it is certain that TLRs are essential for *H. pylori* recognition and subsequent immunologic response to this bacterium, their role in the progression of gastric lesions was not established. Nevertheless, there is some data suggesting that they might have an important role. Indeed, some studies showed that chronic *H. pylori* infection increased TLR4 expression as well as promoted chronic activation of NF-kB (39, 40). Additionally, other studies showed an association between TLR4 polymorphisms and the severity of gastric lesions associated to *H. pylori* infection (51-53). The role of TLR2 in the progression of lesions was even more blurred. However, a single nucleotide polymorphism of TLR2 was associated with the severity of intestinal metaplasia and mucosal atrophy (54). Bringing it all together and remembering the essential role of TLR2 in the recognition of *H. pylori*, it looks like TLR2 can have an important role in the progression of gastric lesions.

So, although there was some rationale to say that TLRs might be important in gastric carcinogenesis, scarce human data elucidated the sequence of events through which TLRs and *H. pylori* interact and promote progression of gastric lesions with no single study simultaneously studying TLRs throughout the entire cascade of gastric carcinogenesis. Therefore, we evaluated for the first time in humans the expression of TLR2, TLR4 and TLR5 as well as their interacting molecules expression in all the lesions of gastric carcinogenesis, from normal mucosa to adenocarcinoma. We have shown for the first time in humans that *H. pylori* early induce a lower expression of TLRs inhibitors associated with higher TLRs protein levels in normal mucosa and that these changes persist throughout all the spectrum

of lesions of gastric carcinogenesis. Moreover, we have created an immunohistochemistry score of TLRs' expression that could be applied to the diagnosis of gastric precancerous conditions. Our results suggest that increasing activation of these receptors, initially by *H. pylori* but at later stages potentially by several other PAMPs or DAMPs, may have an important role in gastric carcinogenesis and tumour progression. Indeed we were able to create a potential sequence of events from *H. pylori* gastritis to cancer involving progressive activation of TLRs (Figure VI.1). We believe that understanding of this proposed sequence of events can open the door to a new line of research in gastric cancer prevention and treatment.

FIGURE VI.1 – Proposed sequence for gastric carcinogenesis, involving progressive TLRs activation.



Left - *H pylori* early induces higher TLRs expression and lower TOLLIP, allowing redistribution of TLRs to apical membrane where they can interact with *H pylori*. Trying to combat the infection, TLRs interaction with *H pylori* leads to activation of several pro-inflammatory pathways and cytokine production. **Middle** - The perpetuation of these intracellular pathways during years eventually leads to CDX2 transcription and phenotypic change to intestinal metaplasia. These new state is characterized by even lower TOLLIP expression and consequently diffuse TLR protein throughout the cell, becoming the cell more reactive to *H pylori* and eventually to other antigens. With TLRs chronically stimulated, CDX2 levels progressively increase, and pro-oncogenic intracellular pathways eventually activate leading to cell dysplasia. **Right** - This state is associated with even lower levels of TOLLIP, high levels of CDX2 and TLRs, that are disperse throughout the cell, and so, at this point, many different antigens may perpetuate the production of different inflammatory and oncogenic mediators eventually leading to cancer.

TLRs and Colon

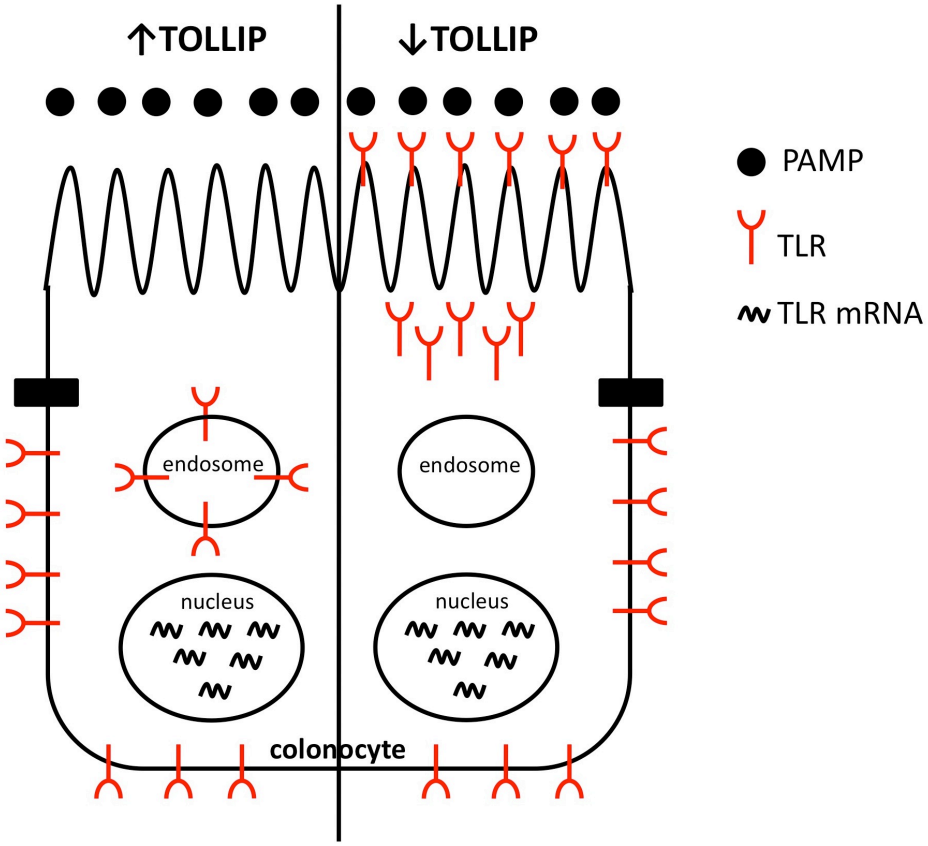
Probably, before our studies relating TLRs to sporadic colorectal cancer, this was the weakest scientific research area of all three carcinogenesis pathways considered in this thesis. Nevertheless, there was some indirect scientific evidence that allowed us to speculate that TLRs might also have a role in colorectal cancer, in spite of not having a clear infectious agent involved in its pathogenesis, contrarily to gastric cancer (*H. pylori*) and hepatocellular carcinoma (hepatitis virus).

Several facts made us to assume a potential link between innate immunity receptors and colorectal cancer. First, inflammatory bowel disease is an established risk factor for colon cancer by promoting chronic inflammation of the mucosa (55). Second, germ-free rats given carcinogens are protected from colonic cancer (56). Third, cancer and adenomas appear to present higher bacteria levels than normal mucosa (57). More conclusive data came from animal and cell lines studies. In fact, in induced-colitis murine models, bacterial-induced inflammation through TLR/MyD88 signalling appears essential for progression of adenoma to carcinoma (58). Fukata *et al* showed with TLR4 knockout mice that TLR4 signalling was critical for colon carcinogenesis following chronic colitis (59, 60). Although the data from humans was scarce, it was shown that human cancer cells overexpress TLR4 when compared to normal mucosa and that colon cancer cell lines when stimulated with LPS promoted intracellular signalling pathways involved in tumour growth and progression (61). The data relating other TLRs to colorectal cancer was even weaker than for TLR4. However, in cultured human colon cancer cells TLR2 activation also induced production

of oncogenic factors (62) and a small study found association of polymorphisms of TLR2 and TLR4 with sporadic colorectal cancer (63).

Having this in mind, we hypothesized that common colorectal cancer risk factors, like diet and obesity, could change normal microbiota equilibrium with modification of the physiologic expression of TLRs and/or their antagonists, inducing a subclinical pro-inflammatory state that might facilitate carcinogenesis. Therefore, we decided to evaluate for the first time in humans the expression of TLRs and related molecules in different human colon lesions, from normal mucosa to cancer. We found a persistently positive TLRs expression and lower expression of TLRs inhibitors, particularly TOLLIP, associated with higher TLRs protein levels throughout all the spectrum of lesions of colon carcinogenesis. Moreover, we proposed for the first time that TOLLIP levels were essential for intestinal homeostasis by controlling not only TLRs activation but also its protein levels (Figure VI.2). Another interesting and novel result was that the adjacent to lesion normal mucosa presented a distinct genetic profile when compared to the normal mucosa, with lower expression of TOLLIP. Normal mucosa from control population also presented higher expression of TOLLIP. This is in agreement with our hypothesis that some risk factors for colorectal cancer that have been shown to alter commensal microbiota, like diet and obesity (64-66), may increase cancer risk by changing the mucosa genetic profile with lower TOLLIP expression and as a consequence with higher TLR activation. This can promote higher COX-2 expression and sub-clinical inflammation facilitating a pro-mutagenic environment that can be the initial event for neoplastic transformation of the mucosa.

FIGURE VI.2 - Regulation of epithelial colon cell TLR protein levels and localization by TOLLIP



Left - High levels of TOLLIP, like in normal mucosa, interact with TLRs molecules leading to early protein degradation and directing TLRs to basolateral membrane. **Right** - With low levels of TOLLIP, like in adenoma and adenocarcinoma, TLRs are diffusely dispersed trough the cell.

Our results have suggested that increasing activation of TLRs by bacteria could have an important role in colon carcinogenesis and tumour progression. For that reason we decided to study the role of genetic polymorphisms with potential influence on TLR2 and TLR4 receptor expression and/or function in the risk of colorectal cancer development. In

a Hospital based multicentre case control study we showed that functional TLR2 and TLR4 single nucleotide polymorphisms significantly altered the risk of colorectal cancer. Indeed, we showed that a single nucleotide change could significantly alter the function of the receptors and that the hipofunctional CC genotype of the TLR2+597T>C polymorphism was associated with 5-fold decreased risk of CRC development (OR=0.21). On the other hand, the hiperfunctional TLR4 299Gly homozygous conferred a 3-fold increased risk of cancer (OR=3.30). Furthermore, both obesity and smoking influenced the risk for cancer in individuals presenting these genetic profiles.

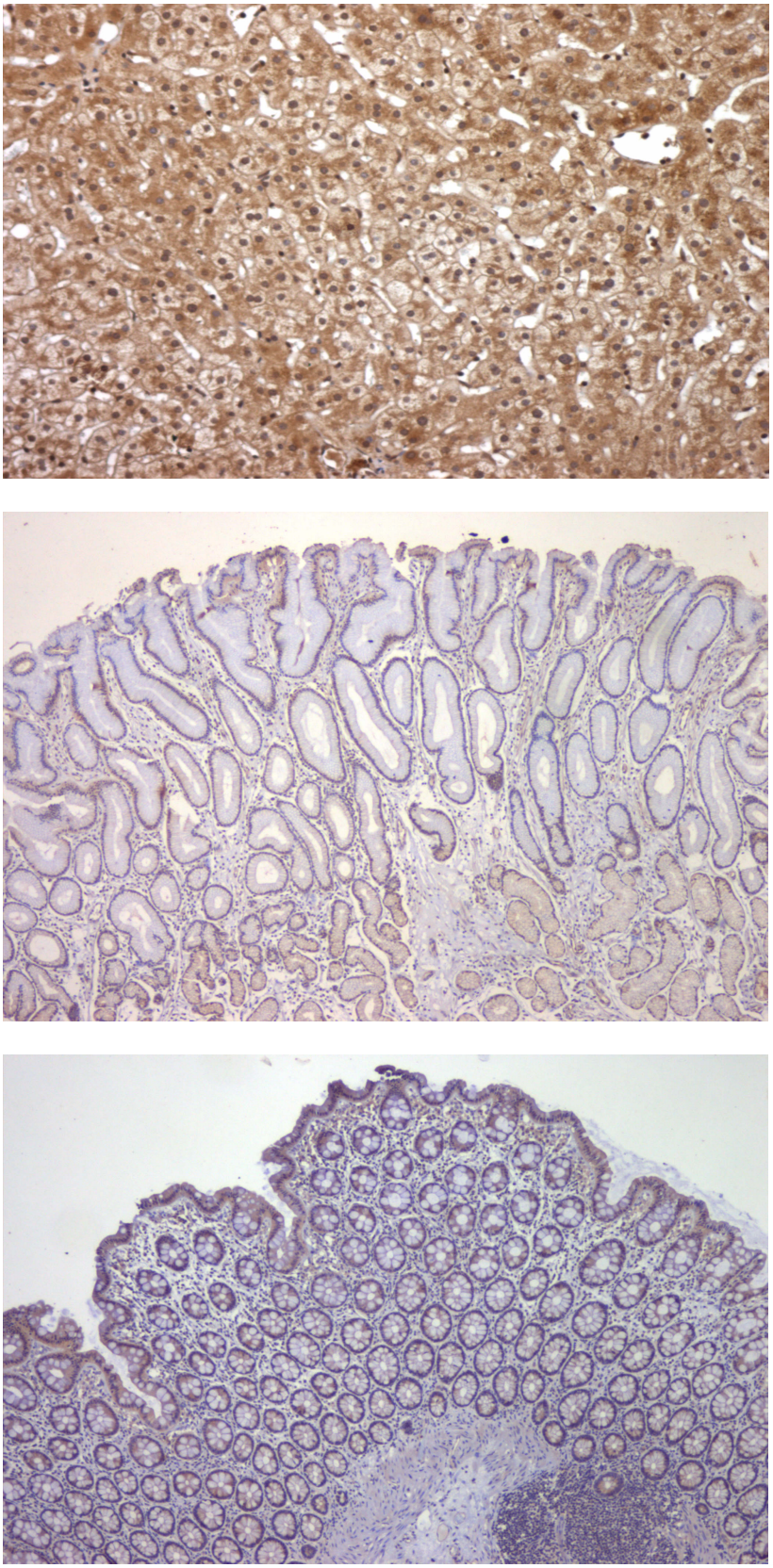
In our opinion these results suggest that TLR2 and TLR4 progressive activation to colon bacteria has an important role in colon carcinogenesis. Small changes in the normal function of these receptors due to functional polymorphisms may contribute to an unbalanced cytokine and pro-oncogenic cellular microenvironment and thus to a higher risk for cancer development, particularly when associated with other pro-inflammatory conditions like obesity. If confirmed by other studies, in the future, identification of these genetic profiles may help to define more efficacious strategies for screening of colorectal cancer through an individual fitted schedule.

Integrated conclusion

Although we were able to extract some interesting conclusions regarding the role of TLRs in each of the three pathways of carcinogenesis considered individually, when we analyze all the data together some interesting conclusions can be drawn. Indeed, the pattern of TLRs expression between the three organs shares some similarities but also some important differences. We believe that the different exposure to several number and type of microorganisms in the distinct parts of the gastrointestinal tract can help us to explain the obtained results.

If we look to normal cells of stomach, liver and colon, TLRs expression was positive in almost all epithelial cells. Indeed, although some authors suggested that low TLR mRNA expression could be a mechanism for maintaining gastrointestinal homeostasis, particularly in the colon that is exposed to million different microorganisms, our data contradicts this theory. In fact, at the mRNA level the expression of TLRs was similar to other constitutive cell genes suggesting that TLRs may also have an important regulative function. Moreover, although not directly comparable, we did not find any apparent difference in TLR mRNA expression between the three organs although the exposure to microorganisms is clearly lower in the liver than in the stomach or colon. However, as we can see in figure VI.3, at the protein level there are some differences with the liver presenting a higher and more diffuse expression when comparing to the low intensity and polarized TLR protein expression of gastric and colonic epithelial cells.

FIGURE VI.3 – TLR protein expression in normal epithelial cells



Although with similar mRNA levels, the intensity of TLR protein expression in the liver **(up)** was in clear contrast with stomach **(middle)** or colon **(down).**

We believe that the reason why liver has more TLR protein expression than gastric or colon epithelia is because this organ has to provide a rapid and effective immune response to any bacteria that come in contact with liver parenchyma. On the other hand gastrointestinal epithelia has to gain tolerance to the commensal bacteria in order to prevent an inadequate inflammatory response to non-pathogenic microorganisms, which could have deleterious consequences to the organism. So, although maintaining a constitutive genetic expression of TLRs, the gastrointestinal tract had to create mechanisms to diminish TLRs protein levels and to direct these receptors mainly to basolateral membrane where they could answer to any pathogenic microorganism that overcomes the first line of epithelial defence but not to the luminal commensal bacteria.

Our initial hypothesis was that was made through a lower TLRs mRNA expression, however, as we have seen, that was not the case. Indeed, our work had a setback when we clearly saw that colon adenomas and adenocarcinomas had a much higher protein expression when comparing with normal mucosa, but with almost similar levels of mRNA. Fortunately, literature review allowed us to find TOLLIP. Of all the TLRs antagonist molecules, besides interacting with several intracellular kinases, TOLLIP also appeared to interact and to block TLRs complexes (67, 68). Indeed, although not described with TLR molecules, TOLLIP promoted traffic of some synthesized proteins like IL-1 receptors into endosomes, leading to protein early degradation (69-72).

So, we hypothesized that lower levels of TOLLIP could allow higher TLRs protein expression with similar mRNA. It was thrilling to see that both

at gastric and colon mucosa decreasing levels of TOLLIP were associated with increasing protein levels of TLRs throughout the carcinogenesis sequence. In fact, although the carcinogenesis sequence of all the three organs was associated with increasing TLRs protein levels, in the liver that was associated with a direct elevation of TLRs mRNA while in the colon that was mainly associated with a decrease of TOLLIP with almost similar levels of mRNA. The stomach had an intermediate pattern between these two organs with lower TOLLIP but also with some increase in TLRs mRNA. So, it appears that in organs with lower exposure to microorganisms (liver), TLR protein levels must be constitutively high and TLRs inhibitors might not have an essential role since these organs have to rapidly answer to microorganisms in order to promote systemic sterile environment. In contrast, organs that are constantly exposed to microorganisms (colon) have low TLRs protein levels but high expression of TLR inhibitors and, when necessary, this levels decrease in order to allow a more robust TLR activation.

Although interesting and allowing evidence-based scientific theories, our results should, nevertheless, be interpreted with some caution. In fact, although increased protein levels of TLRs and decreased TLRs inhibitors (TOLLIP, PPAR-γ) suggest that there is more TLRs activation by PAMPs in the sequence of lesions of gastrointestinal carcinogenesis, the lack of functional studies does not allow us to be definitive about that. Nevertheless, we believe that this is the case mainly because we showed that this sequence of events was also associated with increased inflammatory markers.

In conclusion, our results suggest that TLRs signalling pathways may play an important role in gastrointestinal carcinogenesis and that they might be the link between diet, infectious agents and cancer. If confirmed by more definitive functional studies, a strategy of modulation of TLRs, either by blocking TLRs or by increasing TOLLIP levels, may be effective for the prevention of progression of pre-neoplastic lesions. Also, considering the high TLRs expression in all the gastrointestinal cancers studied in this thesis, blocking TLRs activation may also have an important role in cancer treatment. Since modulation of TLRs activation may be accomplished by interventional measures, future studies should evaluate the clinical value of these novel findings.

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CHAPTER VII – FUTURE RESEARCH

"Science may set limits to knowledge, but should not set limits to imagination"

Bertrand Russell (1872-1970)

This thesis gave many answers but created even more questions. In this chapter a brief summary of future studies planned by our group of investigation will be presented taking in account the results previously presented. This does not intend to be a plan of future projects nor it intends to be a list of all possible lines of research that can follow the presented studies.

Functional studies.

Although we now know the pattern of TLR expression in normal and diseased gastrointestinal tissue, the functionality of these receptors in these lesions is still not fully established. In our opinion, tissue culture studies where biopsy samples of the different lesions are studied under laboratory conditions with TLRs agonists and antagonists will provide some answers regarding not only functionality but also interaction of all TLR signalling pathways. Cell lines could be used to complement information. The limitation of this line of research is that until now only few groups of investigation were able to study human gastrointestinal living tissue samples under in vitro conditions.

Polymorphisms studies

We obtained very interesting results regarding the role of some TLRs polymorphisms in the risk of colorectal cancer development. Taking in account that both gastric and liver cancer also presented similar pattern of TLR expression when compared to colorectal cancer, it is predictable

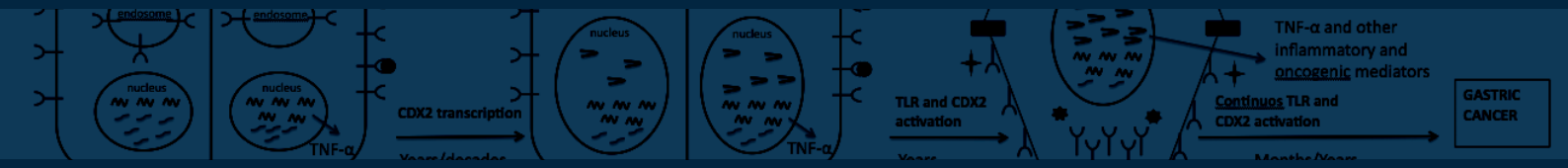
that TLRs polymorphisms may also alter the risk of these cancers. Moreover, we may speculate that TLRs polymorphisms can alter not only the risk of cancer but also the risk of developing pre-neoplastic conditions like cirrhosis in the liver or intestinal metaplasia in the stomach. This should be worth to study. However, a huge limitation of these studies will be a relative great number of participants that would be necessary to include.

Prevention and interventional studies

The main aim of scientific biomedical research is, in the end, to prevent or to treat diseases. The better way to prevent is to know exactly who is at risk. In fact, the main limitation of prevention of gastrointestinal cancers is that in order to prevent one case we have to screen and follow far more cases. So, although we know some risk factors we still have to know which individuals exposed to that risks factors would at the end develop cancer. It should be interesting to study if a specific pattern of TLR/TOLLIP (or other related molecules) expression could predict which patients with pre-neoplastic conditions would develop cancer. Furthermore, since it is possible to modulate TLRs function with interventional measures such as antibiotics, probiotics or even specific TLRs agonists or antagonists, the effect of these interventions in persons at risk would be interesting – could modulation of TLRs with these interventions change the molecular profile of the mucosa? In the end could it prevent the progression of lesions? This hypothesis should be tested first

with animal studies and only afterwards in humans. The follow-up of these patients and ethical concerns will be issues to these studies.

Someone said that science never solves a problem without creating ten more. Fortunately, trough the path of Science our patients and our spirit live longer.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