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Combination of genetic polymorphisms in TLR influence cytokine profile in HCV patients treated with DAAs in the State of Amazonas

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ABSTRACT

Hepatitis C is a public health problem and affects approximately 3% of the world's population. HCV infections have a wide spectrum of clinical manifestations, and several single nucleotide polymorphisms (SNPs) in the genes of the toll-like receptors are cited to influence the clinical outcomes. A cross-sectional study was conducted in the Amazonas State, Brazil in which SNPs in *TLR4* and *TLR9* genes were genotyped by PCR-RFLP in 151 HCV chronic liver disease patients and 206 healthy donors. The circulating cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL-17A were measured by cytometric bead array (CBA) which revealed that the combined genotypes of *TLR9 -1237T/T* and *-1486C/T* seem to influence the cytokine profile under lipopolysaccharide (LPS) stimulation of the Th17 profile, especially among patients with advanced chronic liver disease when treated with DAAs.

1. Introduction

Chronic hepatitis C (CHC) is a major global health problem worldwide and in 2018 more than 71 million people suffered chronic hepatitis C infections [1]. The liver damage which occurs during chronic infection is commonly attributed to mechanisms mediated by the host immune response associated with a marked release of inflammatory mediators, which favors changes in the patterns of adhesion molecule expression and recruitment of cells to the hepatic inflammatory tissue microenvironment [2]. Immunological events linked to chronic hepatitis C can lead to dysregulated architecture and function of the liver and increases intestinal permeability through the release of cytokines that alter the portal circulation and intestinal epithelial junctions, which allows the LPS derived from the intestinal microbiota to enter the portal circulation [3–5].

Previous studies showed that TLR-4 and TLR-9 pathways can activate hepatic stellated cells (HSCs) in patients with advanced liver disease through an increase of LPS levels in systemic/portal vein blood and DNA from dying hepatocytes, respectively [6–9]. Several studies have already investigated the association between TLR single nucleotide polymorphisms (SNPs) and different outcomes in HCV infection [10], and direct-acting antivirals (DAAs) are known to inhibit the tumor suppressor p53 in patients with successful clearance of HCV, though the inflammatory process continues to persist and places patients at risks of developing HCC [11].

Clearance of HCV in patients treated with interferon-free therapy and the genetic background of the host may contribute to the progression of the disease via the persistence of the inflammatory process after virologic immune response [12–17].

In the present study, we analyzed whether the polymorphisms in *TLR4* rs4986790 *(A299G* A/G), *TLR4* rs4986791 *(T399I* C/T), *TLR9* rs5743836 *(-1237C/T)* and *TLR9* rs187084 *(-1486C/T)* are associated with susceptibility to HCV infection and cytokine profile changes after treatment with DAAs in the sample group of the population from the Amazonas state.

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2. Material and methods

2.1. Ethics approval

This study was approved by the Ethics Committee of the Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) (1.405.965/2015 and 00240112000-10/2010). Participants read and signed the written informed consent form prior to the enrollment in the study, in accordance with the Declaration of Helsinki and Resolution 466/12 of the Brazilian National Health Council regarding research involving human subjects. All patients were treated according to the recommendations of the Brazilian Ministry of Health [18].

2.2. Samples and clinical data

The study was carried out at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD) in Manaus, the capital city of the Amazonas State, during 2016–2017. The study population was a non-probability convenience sample that consisted of 206 healthy blood donors (HD) who were randomly included during donations at HEMOAM and 151 HCV infected patients treated at the FMT-HVD. All the 357 participants were submitted to a serological screening at HEMOAM, which is recommended by Brazilian Blood Donor Bank Authorities in order to monitor blood borne pathogens and includes serological analysis for the Hepatitis B and C virus, HIV, DENV, HTLV, Syphilis and Chagas Disease. Among the HCV infected patients, 84 and 67 had ≤F2 and > F2, respectively.

2.3. Genomic DNA extraction

10 mL of blood were collected by venipuncture from each participant in two tubes, one containing EDTA (BD Vacutainer® EDTA K2) and one tube containing Gel separator (Gel BD SST® II Advance®) for carrying out the genotyping of polymorphisms and assay of circulating cytokines, respectively. Sera samples were kept at −80 °C until cytokines were assayed. Genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions. DNA samples were quantified with a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) to evaluate the concentration and purity of nucleic acids.

2.4. Genotyping of TLR

The following polymorphisms, *TLR4* rs4986790, *TLR4* rs4986791, *TLR9* rs5743836 and *TLR9* rs187084 were investigated. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used for allelic discrimination as described previously [19–21]. The PCR reaction for each SNP consisted of 1 μL genomic DNA (~20 ng) added to 24 μL amplification mix containing 0.2 μL (2U) Platinum™Taq polymerase (Thermo Fisher Scientific), 2.5μL10x buffer (100 mmol/L Tris-HCl (pH 8.3) and 500 mmol/L KCl), 1 μL MgCl2 (1.5 mmol/L), 1μLdNTPs (40 mmol/L), 0.5 μL each of forward and reverse primer (0.25 pmol/L) and 18.3 μL ultrapure dH_2O. A total of 10 μL of PCR product was digested with 5 U of respective restriction endonuclease NcoI, HinfI, BstNI, and AfIII (New England Biolabs, Ipswich, MA, USA) in enzyme buffer, according to the manufacturer's instructions. The primers, PCR protocols conditions, and restriction endonucleases are shown in Supplementary Table 1. The fragments generated by PCR-RFLP were separated by electrophoresis in either a 2% or 4% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized with the UV light Gel Doc™ XR + System (Bio-Rad Corporation, Hercules, CA, USA) with a photo documentation system.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2020.155052.

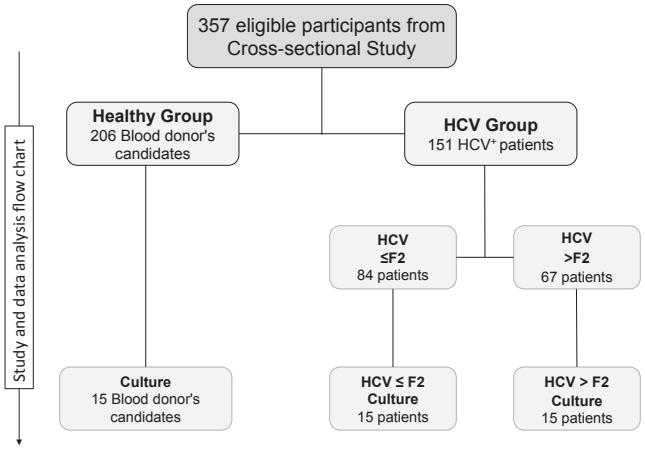

Fig. 1. Study and data analysis flow chart. We included 357 eligible participants:206 Healthy blood donors and 151 that were infected with HCV and were attended by FMT-HVD. Of these, thirty biological samples were collected for cell culture after being obtained from patients with chronic hepatic disease who were treated with direct-acting antivirals (DAAs) and sustained virological response (SVR).

2.5. Isolation of peripheral blood mononuclear cells (PBMCs) and cell culture

PBMCs were collected from 45 individuals for culture assay as described previously [22]. 15 samples were from healthy donors (HD) and 30 from HCV infected patients with chronic liver disease (15 ≤ F2 and 15 > F2) who were treated with DAAs and sustained virological response (SVR) confirmed by the absence of viral RNA by quantitative molecular tests (Fig. 1). PBMCs from each participant were isolated from 20 mL of venous venipuncture in four tubes containing Heparin (BD Vacutainer® PST™), using the Ficoll-Hypaque protocol. After centrifugation, PBMCs were collected from the interface and washed with RPMI 1640 medium. After washing twice, the supernatant was discarded and the PBMCs were resuspended at a concentration of 2×10^6 cells/ml in 4 mL of RPMI supplemented with 1% streptomycin and 10% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 12 h. LPS stimulus was performed in duplicate using 100 μL of exogenous endotoxin (LPS/1 μg / mL).

2.6. Serum cytokine assay

The cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL-17A in human blood serum and supernatant of PBMC culture were measured by Cytometric Bead Array (CBA), Kit BD™ Human Th1/Th2/Th17 Cytokine (Cat: N° 560484, Lot: 29132, BD® Biosciences, San Diego, CA, USA) according to the manufacturer's technical guidelines and protocols. A FACSCanto II flow cytometer (BD® Biosciences) at the HEMOAM was used for sample acquisition. The FCAP-Array software v3 (Soft Flow inc, USA) was used to calculate the cytokine levels.

2.7. Genotype association test and statistical analyses

Comparison between groups was performed with the chi-squared (χ^2) or Fisher's exact test with 95% confidence interval (CI). The Hardy-Weinberg equilibrium (HWE) was determined by comparing the frequency of the observed and expected number of genotypes. Tests for Hardy-Weinberg equilibrium were performed using an online application (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Graphpad Prism v.5 software (San Diego, CA, USA) was used for comparative analyses of cytokines among the genotype combination of the SNPs studied. The continuous variables presented a non-normal distribution and data were analyzed with a non-parametric Mann-Whitney test to compare

Table 1
Clinical and demographics characteristics of the study population.

Variables	Healthy Donor's	HCV patients		
		All	≤F2	> F2
	(n = 206)	(n = 151)	(n = 84)	(n = 67)
Age (mean ± SD)	32 ± 11	58 ± 11	55 ± 11	61 ± 9
Gender (male/female)	134/72	83/68	45/39	38/29
ALT (IU/L)	–	69.2 ± 56.8	59.8 ± 44.6	80.9 ± 67.4
AST (IU/L)	–	63.2 ± 43.3	44.1 ± 25.8	86.9 ± 48.7

pairs. A Spearman correlation test was performed to assess the association between the levels of each cytokine tested. The positive and negative correlations are considered significant when $p < 0.05$. The correlation index (r) was used to categorize the correlation strength as weak ($r \leq 0.35$), moderate ($r \geq 0.36$ to $r \leq 0.67$), or strong ($r \geq 0.68$), as previously described [23]. Networks were assembled to assess the associations among the circulating cytokines. Significant correlations were compiled using the open access software Cytoscape v3.3 (Cytoscape Consortium, San Diego, CA), as previously reported [24,25]. Statistical significance was considered $p < 0.05$ in all cases.

3. Results

3.1. Baseline demographic, laboratorial and clinical characteristics of the study population

The median age of the healthy blood donors and HCV patients were 32 and 57 years, respectively ($p < 0.0001$). In both groups, male subjects were predominant (65% and 55%). Of note, the healthy blood donors were randomly selected and are younger than the HCV patients since the development of the disease may take 15–20 years to occur. At the time of blood collection, the HCV group were ≤F2 (55.6%) and > F2 (44.4%), and presented statistical differences in follow-up markers (AST $p < 0.0317$ and ALT $p < 0.0001$), as shown in Table 1.

3.2. Polymorphisms in TLR4 and TLR9 are not associated in patients with chronic liver disease

In order to systematically examine the association of TLR4 and TLR9 SNPs with HCV infection, allelic and genotypic frequencies of two TLR4 SNPs (rs4986790 and rs4986791) and two TLR9 SNPs (rs5743836 and rs187084) were analyzed and the results are shown in Table 2. Homozygosity for TLR4 A299G G/G and TLR4 T399I T/T was absent in the studied population. The genotype distributions for TLR4 A299G G/G, TLR4 T399I T/T, TLR9 -1237C/T and TLR9 -1486C/T were not significantly different between the groups. Carriers of the TLR9 -1237 T/T and TLR9 -1486C/T variants represent 40% of the studied population. All the SNPs studied were in HWE, only TLR9

Table 2
Genotypes and alleles association of *TLRs* polymorphisms in HCV patients.

Polymorphism, Genotype or Allele	Healthy Donor's	HCV patients	OR (CI 95%)	(p) value	HCV ≤F2	HCV > F2	OR (CI 95%)	(p) value	
	(n = 206)	(n = 151)			(n = 84)	(N = 67)			
***TLR4 A299G* (rs4986790)**									
A/A	196 (95%)	147 (97%)	0.656 (0.219–1.96)	0.447	81 (96%)	66 (99%)	0.656	0.429	A/A vs A/G
A/G	10 (5%)	4 (3%)			3 (4%)	1 (1%)	(0.042–4.025)		
G/G	–	–			–	–			
A	402 (97%)	298 (99%)	0.662	0.452	165 (98%)	133 (99%)	0.414	0.660	A vs G
G	10 (3%)	4 (1%)	(0.224–1.958)		3 (2%)	1 (1%)	(0.043–4.021)		
***TLR4 T399I* (rs4986791)**									
C/C	199 (96%)	146 (96%)	0.952	0.934	80 (95%)	66 (99)	0.303	0.264	C/C vs C/T
C/T	7 (4%)	5 (4%)	(0.296–3.066)		4 (5%)	1 (1%)	(0.033–2.777)		
T/T	–	–			–	–			
C	405(98%)	297 (98%)	0.953	0.935	164 (98%)	133 (99%)	0.308	0.399	C vs T
T	7(2%)	5 (2%)	(0.299–3.035)		4 (2%)	1 (1%)	(0.034–2.791)		
***TLR9 -1237C/T* (rs5743836)**									
T/T	137 (66%)	99 (65%)	0.286	0.115	56 (67%)	43 (64%)	1.204	0.551	C/C vs
			(0.055–1.496)				(0.195–7.420)		C/T + TT
C/T	67 (33%)	47(31%)	0.281	0.117	25 (30%)	22 (33%)	1.320	0.771	C/C vs C/T
			(0.052–1.508)				(0.202–8.639)		
C/C	2(1%)	5 (4%)	0.289	0.120	3 (3%)	2 (3%)	*0.868*	*0.01*	C/C vs T/T
			(0.055–1.520)				*(0.139–5.428)*		
T	341 (83%)	245(81%)	0.895	0.572	137 (82%)	108 (80%)	1.064	0.833	C vs T
C	71(17%)	57(19%)	(0.609–1.316)		31 (18%)	26 (20%)	(0.596–1.898)		
***TLR9 -1486C/T* (rs187084)**									
C/C	26 (13%)	17 (11%)	1.065	0.851	8 (10%)	9 (13%)	0.678	0.400	C/C vs
			(0.548–2.069)				(0.247–1.866)		C/T + TT
C/T	114(55%)	93 (62%)	1.190	0.617	54 (64%)	39 (60%)	0.642	0.400	C/C vs C/T
			(0.600–2.360)				(0.227–1.812)		
T/T	66 (32%)	41 (27%)	0.856	0.682	22 (26%)	19 (27%)	0.768	0.647	C/C vs T/T
			(0.407–1.801)				(0.247–2.384)		
C	166 (40%)	127 (42%)	0.893	0.479	70 (42%)	57 (43%)	0.965	0.878	C vs T
T	246 (60%)	175 (58%)	(0.652–1.223)		98 (58%)	77 (57%)	(0.609–1.529)		

Table 3
Association of genotypic combinations of deferent polymorphisms with HCV patient's susceptibility.

Polymorphism, genotypic combinations	Healthy Group	HCV patients	OR (CI 95%)	(p) value	HCV ≤F2	HCV > F2	OR (CI 95%)	(p) value
	(n = 206)	(n = 151)			(n = 84)	(n = 67)		
***TLR9 -1237C/T* (rs5743836) / *TLR9 -1486C/T* (rs187084)**								
TT/TT	41(20%)	23(15%)	1.38 (0.78–2.42)	0.267	14(17%)	9(13%)	1.28 (0.52–3.19)	0.652
TT/CC	18 (9%)	12(8%)	1.10 (0.51–2.37)	0.848	4(5%)	8(12%)	0.36 (0.10–1.28)	0.134
CT/CT	35 (17%)	29(19%)	0.86 (0.49–1.48)	0.675	16(19%)	13(19.5%)	0.97 (0.43–2.20)	1.000
TT/CT	78(38%)	64(42%)	0.82 (0.53–1.27)	0.443	38(45%)	26(39%)	1.30 (0.67–2.50)	0.507
CT/CC	8 (4%)	5 (3%)	1.18 (0.37–3.68)	1.000	4(5%)	1(1.5%)	3.30 (0.35–30.26)	0.382
CC/TT	1 (0.5%)	4(3%)	0.17 (0.01–1.62)	0.166	3(3%)	1(1.5%)	2.44 (0.24–24.07)	0.629
CC/CC	–	–	–	–	–	–	–	–
CC/CT	1(0.5%)	1(1%)	0.73 (0.04–11.80)	1.000	–	1(1.5%)	0.26 (0.01–6.54)	0.443
CT/TT	24 (11%)	13 (9%)	1.40 (0.68–2.84)	0.384	5(6%)	8 (12%)	0.46 (0.14–1.50)	0.246

-1486C/T SNP deviated slightly, in both HCV patients (p = 0.002) and HD (p = 0.04).

3.3. Lack of association of genotypic combinations of polymorphisms in TLR9 -1237T/T and -1486C/T with chronic liver disease

None of the polymorphisms of TLR4 and TLR9 analyzed in this study exhibited any significant association with chronic liver disease. TLR4 A299G A/A + A/G and TLR4 T399I C/C + C/T variants were the most frequent in the study population – 97% + 3% and 96% + 4%, respectively. We therefore assessed the influence of the combination genotypes for TLR9 variants -1237T/T and -1486C/T on the cytokine profile in HCV patients treated with DAAs. Statistical analysis revealed that combination of TLR9 -1237T/T and TLR9 -1486C/T variants was higher in the HD and HCV groups when compared to other genotype combinations between the groups, as can be seen in Table 3.

3.4. Influence of genotypic combinations of polymorphisms in TLR9 -1237T/T and -1486C/T on the circulating profile of cytokines in HCV patients

Human antiviral response against HCV is characterized by induction of Th1 cytokines profile. Analysis of genotypic combinations in regard to the Th1, Th2, Th17 and Treg cytokines profiles among the studied groups indicated a different profile between the HCV patients in regard to liver damage. A significant increase of IL-6 (p = 0.005) and IL-4 (p = 0.0007) (Fig. 2A and F) was observed in the serum of these patients compared with HD. The cytokines TNF, IL-10, IL-2, IFN-γ and IL-17A did not show any difference between HCV patients and HD group (Fig. 2B–E and G). A significant increase of IL-10 was observed in HCV > F2 patients (p = 0.028) compared to ≤F2, whereas the cytokines IL-6, TNF, IL-2, IFN-γ, IL-4 and IL-17A were not significant when compared between HCV patients with different stages of liver disease (Fig. 3A).

3.5. Influence of genotypic combinations regarding cytokine profiles in the culture supernatant stimulated with Lipopolysaccharide (LPS)

No significant difference in IL-6, TNF, IL-10, IL-2, IFN-γ and IL-4, (Fig. 3B) was observed on stimulated PMBCs with LPS. Only, IL-17A showed a significant increase in the HCV > F2 patients (p = 0.043) when compared to the ≤F2 group (Fig. 3B).

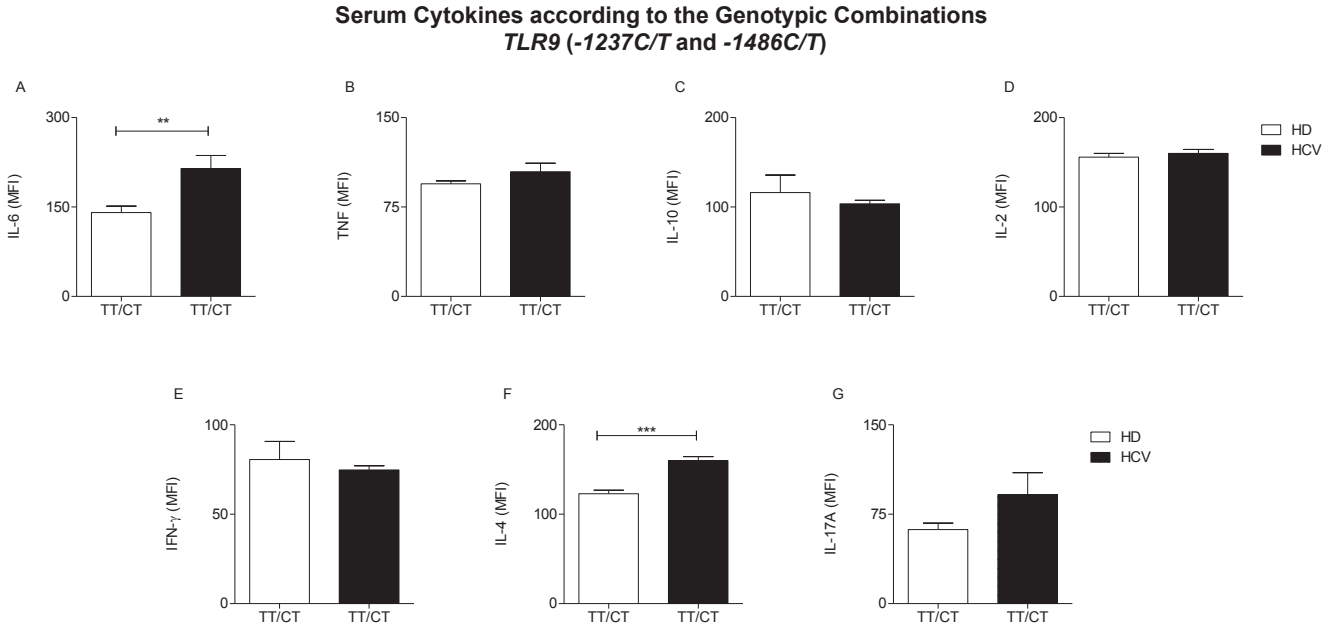

Fig. 2. Concentration in Mean Fluorescence Intensity (MFI) of serum cytokines according to the genotypic combinations (rs5743836/ rs187084) between the control group (HD) and HCV patient group (HCV). Results are expressed as median and standard deviation. Statistical analyses were performed using the non-parametric Mann Whitney test. Significant statistical difference was considered when *p < 0.05, **p < 0.01 and *** < 0.0001.

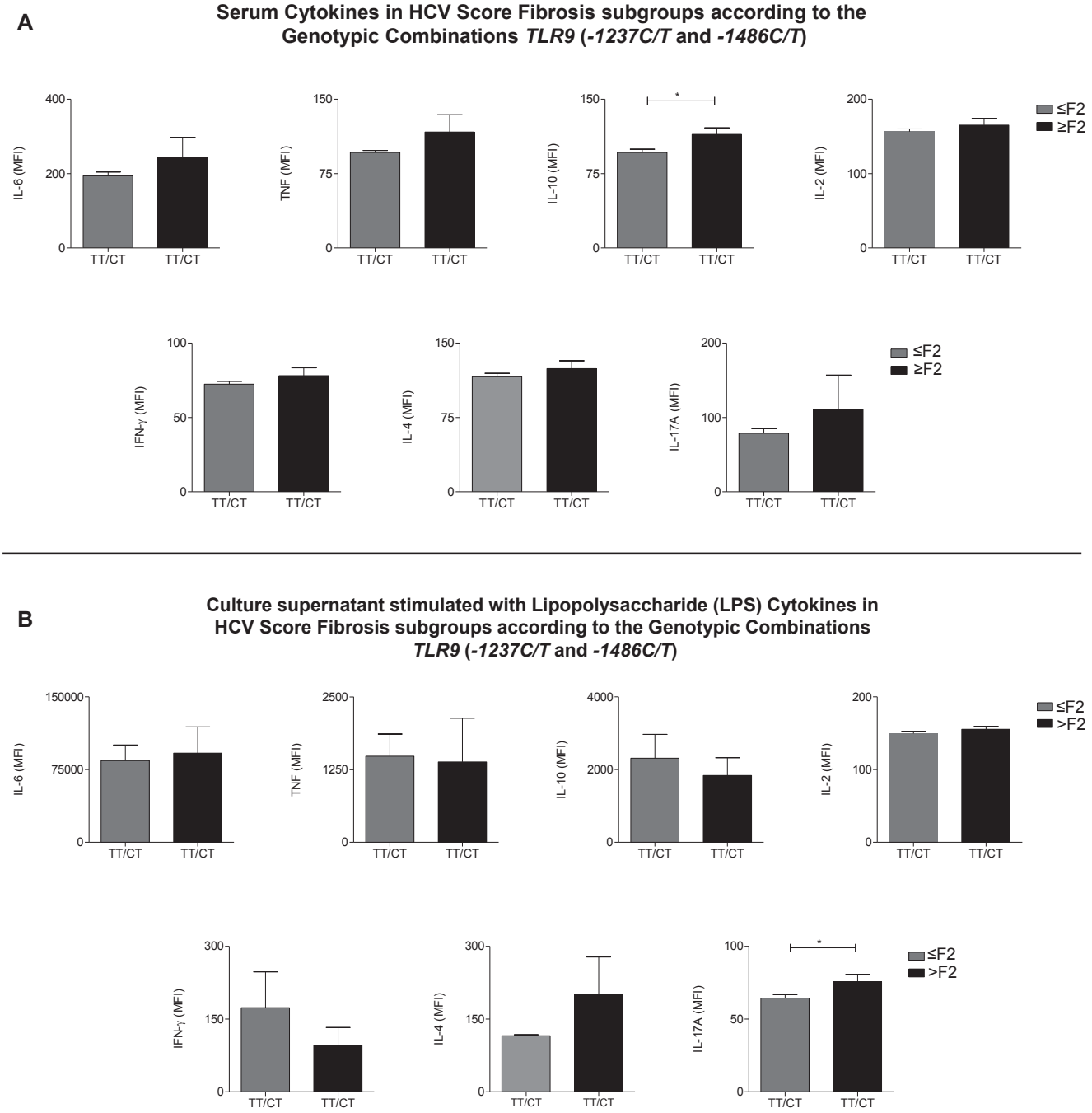

Fig. 3. (A) Concentration in Mean Fluorescence Intensity (MFI) of serum cytokines according to the genotypic combinations (rs5743836/ rs187084) between HCV patient group (≤F2 and > F2). (B) Concentration in Mean Fluorescence Intensity (MFI) in the culture supernatant stimulated with Lipopolysaccharide (LPS) (1 μg/ml), according to the genotypic combinations (rs5743836/ rs187084) in HCV patient group (≤F2 and > F2). Results are expressed as median and standard deviation. Statistical analyses were performed using the non-parametric Mann Whitney test. Significant statistical difference was considered when *p < 0.05, **p < 0.01 and *** < 0.0001.

3.6. Lipopolysaccharide (LPS) changes the interaction between of cytokines in HCV patients

To test the relationship between altered levels of cytokines after LPS stimulus, a series of correlation analyses was performed (Fig. 4). The numbers of interactions between the cytokines analyzed were also different according to the hepatic score in HCV patients. No interactions were observed in the HD group carrying the same genotype combinations as the HCV group, which exhibited a strong positive relation between IL-6, IL- 10, IL-2 and IL-17A (Fig. 4A). A moderate relation between IL-6, TNF and IL-10 was also observed in the HCV group. HCV ≤F2 patients exhibited a strong positive relation between IL-6, IL-10 and TNF. In addition, in HCV > F2 patients, a strong positive correlation between IL-6, IL-10, IL-2 and IL-17A was preserved, while IL-6, IL-17A and IL-10 exhibited a strong negative correlation (Fig. 4B).

4. Discussion

The results of the present study revealed the low frequency of polymorphic alleles in *TLR4* (rs4986790 and rs4986791) in the sample group of the population from the Amazon state. Studies conducted using other Northern Brazilian samples have reported frequencies of

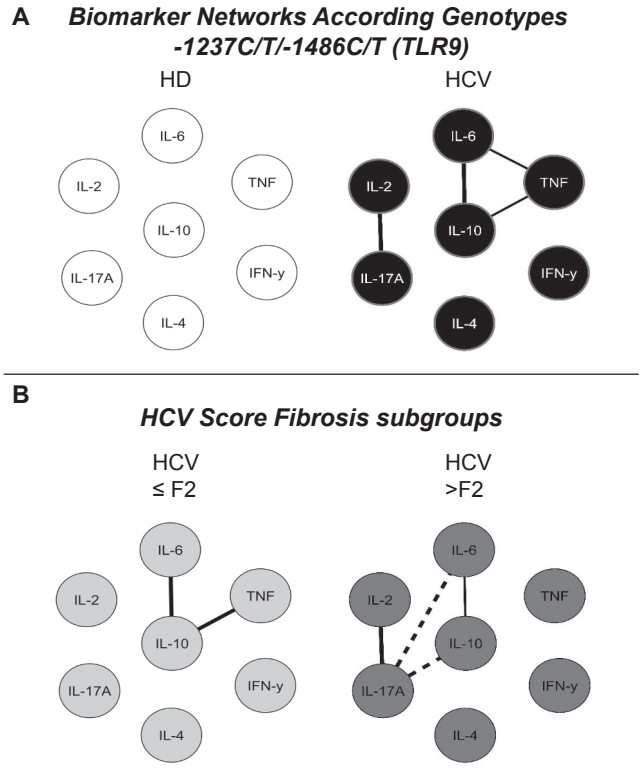

Fig. 4. "Cytokine Networks" in the HCV patients according to the fibrosis score. A. Concentration in Mean Fluorescence Intensity (MFI) in the culture supernatant stimulated with Lipopolysaccharide (LPS) (1 μg/ml), according to the genotypic combinations (rs5743836/ rs187084) between the control group (HD) and HCV patient group (HCV) (A) and patient group (≤F2 and > F2) (B). Customized biomarker network layouts were built to identify the relevant association between proinflammatory IL-6, TNF, IL-2 and IL-17A cytokines, modulatory IL-10 axis, using a clustered distribution of nodes. Significant Spearman's correlations at $P < 0.05$ were represented by connecting edges to highlight positive (strong ($r \geq 0.68$; thick continuous line) or moderate ($0.36 \geq r \leq 0.67$; thin continuous line) and negative (strong ($r \leq -0.68$; thick dashed line) or moderate ($-0.36 \geq r \leq -0.67$; thin dashed line) as proposed by Taylor (18). The overall statistical analysis of the network node neighborhood connections point to an almost linear-chain pattern in the HCV groups with a clear shift towards a more imbricate profile in HCV patients > F2. A persistent IL-6/IL-10 loop was observed in all HCV subgroups with differential neighborhood connections for the IL-17A node in HCV patients > F2.

heterozygotes similar to those found in our study [19–21,26,27]. Increasing evidence suggests that the rs4986790 allele of *TLR4* that leads to an amino acid change (Asp299Gly) might interfere with the interaction between the receptor-ligand and the protein's stability. This may cause deficiency in the recruitment of Myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), and contribute to the increase risk of bacterial infections and a decrease in the proinflammatory response in patients with cirrhosis [28–35]. Our data does not show any association between these SNPs and susceptibility to HCV infection and hepatic impairment.

Another important receptor that also participates in this process of hepatic disease evolution is TLR-9, which recognizes the CpG-rich DNA of bacteria. Watanabe et al., 2007 demonstrated that host-derived denatured DNA from apoptotic hepatocytes stimulates HSCs through TLR-9 in hepatic fibrosis [9]. Several studies showed possible associations between *TLR9* gene SNPs and a greater risk of cancer [10,36]. The T allele of the TLR9 rs5743836 is transcribed more effectively under basal conditions compared to the variant C allele [37,38].

IL-6 up-regulates TLR9 expression and leads to exacerbated cellular responses to CpG, including IL-6 production and B-cell proliferation in mononuclear cells carrying the TLR9 -1237C/T SNP [39]. Furthermore, the increased transcriptional activity of TLR9 in mononuclear cells from patients harboring TLR9 -1237C/T SNP supports a functional effect of this polymorphism on non-Hodgkin lymphoma susceptibility [40]. It has been suggested that TLR9 -1486C/T heterozygote may be a genetic risk factor for cervical cancer [41]. The C allele of TLR9 -1486C/T SNP showed lower TLR9 expression and correlated with the risk of systemic lupus erythematosus [42]. TLR9 expression by fibroblast-like cells has been shown to be significantly correlated with shortened overall survival in patients with hepatocellular carcinoma [43].

Successful treatment of hepatitis C is associated with SVR and declining liver inflammation through a decrease in cytokine and chemokine levels during the treatment of hepatitis C [44] and the DAAs dramatically increased the number of patients that will be able to clear a hepatitis C virus infection with the use of this new medication. However, risk of developing hepatocellular carcinoma (HCC) after DAAs is still a great concern among patients with advanced cirrhosis. It is known that the ER-stress response and p53 are normalized after HCV clearance when induced by interferon-alpha-based antiviral therapies, whereas HCV clearance by DAAs does not restore p53. The p53 is a tumor suppressor and coordinates diverse cellular responses to stress, damage, and in the suppression of autoimmune and inflammatory diseases [11,45].

TLRs may play an important role in a wide range of human diseases by triggering early events in the immunological response. The polymorphisms studied have been associated with greater risks of cancer and the most frequently found genotype combination in our population is TLR4 299A/A, TLR4 299C/C, TLR9 -1237T/T and TLR9 -1486C/T.

The data presented in this study suggest the influence of combination of *TLR9 (-1237T/T* and *-1486C/T*) on the production of circulating cytokines IL-6 and IL-4 in HCV patients when compared to healthy donors. Furthermore, these combined polymorphisms also affect the production of circulating IL-10 in patients with moderate fibrosis. These cytokines are important in chronic liver disease caused by chronic HCV infection. Nieto et al., (2014) observed a spontaneous increase in L-6 and IL-10 production in patients with cirrhosis which carried the wild-type variants of *TLR4 A299G* and/or *T399I* [46]. Yet, the production of IL-6, TNF-α and IL-10 after TLR stimulation was similar between the patients. This different pattern of cytokine production could be a factor in the development of complications in cirrhosis [46].

An increase of IL-17A in PBMCs in patients who had a greater severity of hepatic disease and were treated with DAAs was observed in this study. LPS can up-regulate the expression of several TLRs and nuclear factor kappa B (NF-kB), extracellular-signal-regulated kinase (ERK) and p38 kinase signal pathways. This phenomenon might explain the synergy between bacterial DNA and LPS in activating macrophages against invading bacteria [47,48].

LPS also downregulated the expression of p53 protein in mouse Kupffer cell and RAW 264.7 macrophage cell line [49]. This observation may contribute to the hypothesis that the p53 inhibition perpetuates through TLR4 activation by LPS in patients with moderated fibrosis. In fact, the p53 tumor suppressor protein is related to carcinogenesis and is suggested to cause prolonged NF-κB activation at a late stage after LPS stimulation and possibly sustain prolonged proinflammatory responses [49]. Several inflammatory factors encoded by NF-κB target genes and IL-6 are important activators of transcription 3 (STAT3). NF-κB and STAT3 regulate the expression of numerous oncogenic and inflammatory mediators, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatic growth factor (HGF), IL-10, IL-17 and IL-6, which favor the emergence of tumors [50]. In this study, the interactions between the cytokines analyzed seem to change after LPS stimulation. A strong positive correlation between IL-6, IL-10, IL-2 and IL-17A was observed.

The present study reveals different interactions between cytokines in HCV patients according to polymorphism combinations and liver damage. During the disease, the changes in the cytokines' dynamic

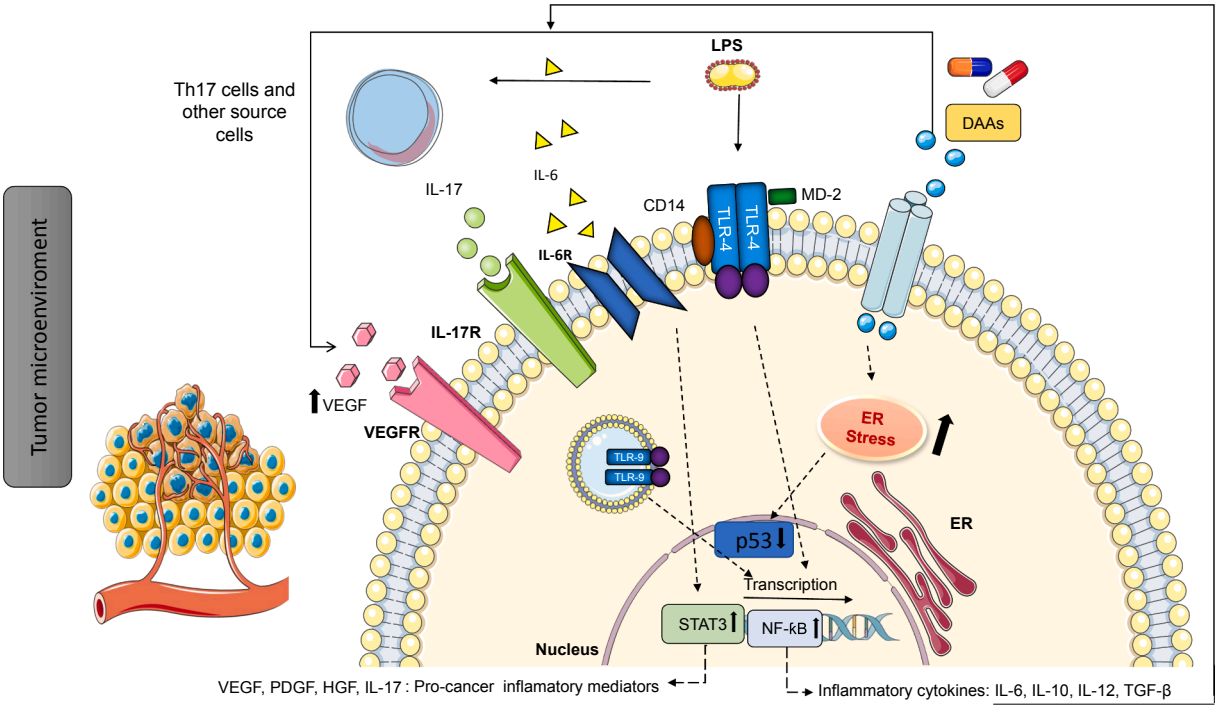

Fig. 5. Schematic diagram for the role of the interplay between the effects caused by DAAs and sever chronic hepatic disease.

connections during the disease associated with these genotypes may lead to a progressive oncogenic milieu. This would signal an increased risk of cancer in patients treated with DAAs who carry the combination of *TLR9* (*-1237T/T* and *-1486C/T*) even after successful viral clearance treatment (Fig. 5). In addition, understanding the influence of these polymorphisms may be useful in clinical practice for designing new therapeutic scenarios.

This study has some limitations. The recruitment of the group of healthy donors served randomly as a normal parameter for analysis purposes. Nevertheless, age discrepancy between patients and healthy donors is still a limitation. The study population size is small and may influence in the levels of associations with HCV infection and disease severity. The small sample size does not allow intra-comparison of the genotype combination when studied with the profile of cytokines. However, it did show that the combinations of these polymorphisms seem to influence chronic hepatic disease, although more studies are needed in order to confirm this preliminary finding.

5. Conclusion

Altogether, our findings demonstrated that *TLR9 -1237T/T* and *-1486C/T* variants may change the cytokine correlations between IL-6, IL-10, IL-2 and IL-17A, especially among patients with advanced chronic liver disease, and who are treated with DAAs. The possibility of these patients presenting an unresolved ER stress after HCV clearance by DAAs is linked to the genetic background, and the pathology already installed could increase the risk of HCC development. Our results suggest the relevance of these sets of biomarkers for further clinical investigations, with a view to being used as a potential tool for predicting adverse outcomes in patients with chronic hepatic disease caused by HCV infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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