Journal of Immunology Research

Toll-Like Receptors and Innate Immunity

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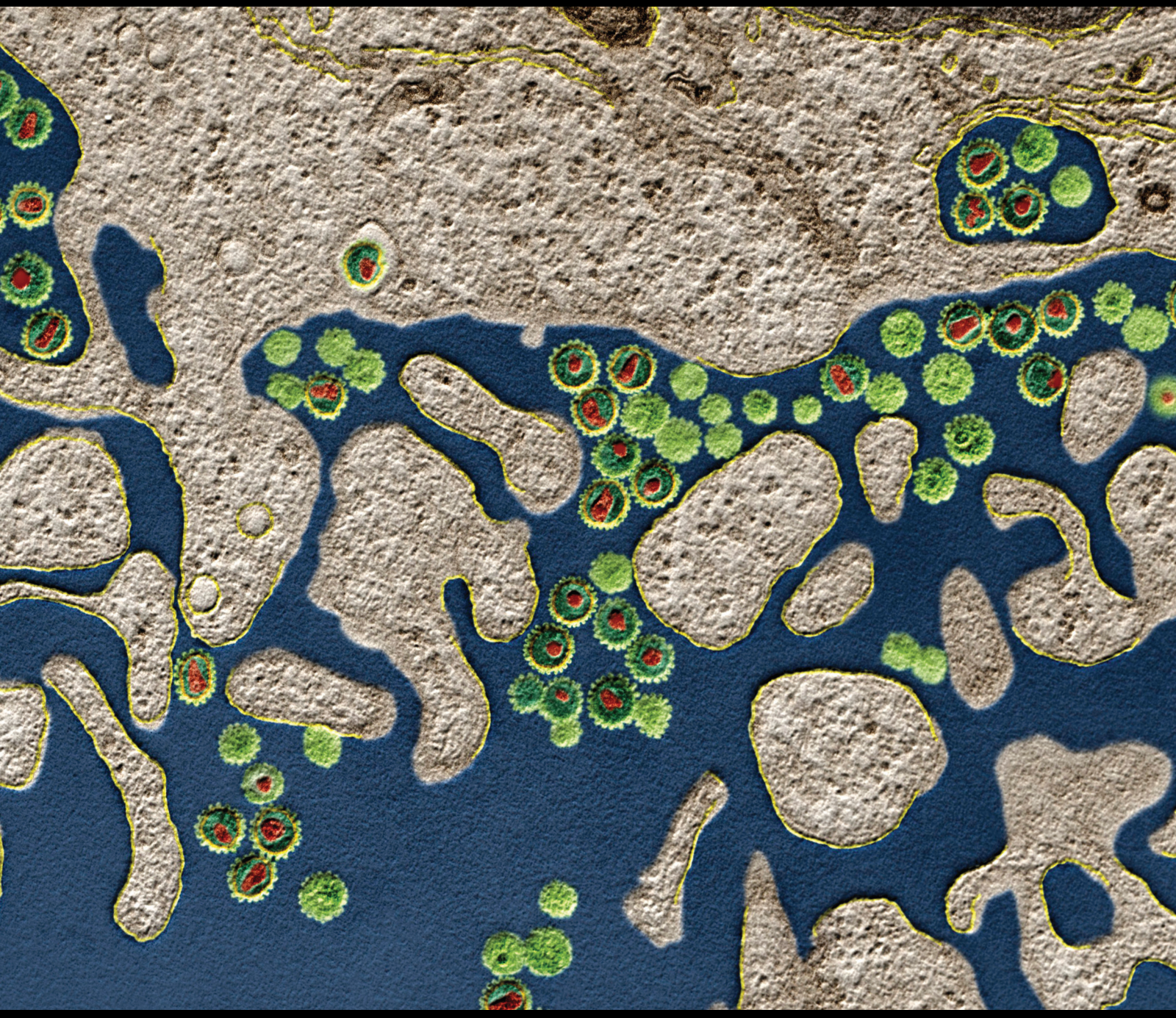
Guest Editors: Raouia Fakhfakh and Darius Widera



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Research Article

Single Nucleotide Polymorphisms in *TLR4* Affect Susceptibility to Tuberculosis in Mexican Population from the State of Veracruz

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Tuberculosis is still a global public health problem, with an estimated 10 million new cases and 1.6 million deaths in 2017. Of all humans infected with *M. tuberculosis*, only 10-15% will develop active tuberculosis disease during their lifetime, and data suggest that along with environmental factors, genetic factors influence susceptibility to develop active disease. Toll-like receptors (TLRs) are pattern recognition receptors that play a central role in the initiation and shaping of adaptive immune responses, and several TLRs have been shown to recognize mycobacterial components. In this work, we performed a case-control study to determine if common single nucleotide polymorphisms (SNPs) in genes encoding TLRs 1, 2, 4, 6, and 10 are associated with susceptibility to develop active tuberculosis in population from the state of Veracruz, Mexico. The study included 279 cases and 569 controls. The results show that the frequency of two SNPs in TLR4 was significantly higher in controls than in tuberculosis patients. The minor allele (G) of rs4986790 in *TLR4* (D299G) decreased the risk of active tuberculosis in the allelic (A vs. G, OR = 0.31, 95%CI = 0.09-0.81, $p = 0.01$) and in the dominant genetic model (AA vs. GG+AG, OR = 0.26, 95%CI = 0.09-0.77, $p = 0.02$). Similarly, the minor allele (T) of rs4986791 in *TLR4* (T399I) decreased the risk of active disease in the allelic model (C vs. T, OR = 0.29, 95%CI = 0.10-0.90, $p = 0.03$). We did not find an association of SNPs in *TLR1* (N248S), *TLR2* (R753Q), *TLR6* (S249P), and *TLR10* (A153S and V298I) with tuberculosis disease. These results suggest that in this population, genetic variants of *TLR4* affect the susceptibility for suffering active tuberculosis disease.

1. Introduction

Tuberculosis (TB), an infectious disease usually caused by *Mycobacterium tuberculosis* (Mtb), is a global public health problem, with an estimated 10 million new cases of TB and 1.6 million deaths in 2017 [1]. Despite significant advances in its detection and treatment, TB remains the leading cause of death by a single infectious agent worldwide [1].

In humans, infection with Mtb can result in the development of active TB disease or in an immune response that can maintain the infection in a latent state (>85% of infected individuals). Under conditions causing immunosuppression, latent TB can progress to active disease. Many factors affect the outcome of the initial infection and determine the progression from latent infection to active disease. In addition to factors such as age, gender, microbial infections, chronic diseases, and environmental aspects, there is strong evidence that genetic factors are important in determining the individual susceptibility to develop active TB [2].

Several studies in different human populations have identified many candidate genes in which polymorphisms can affect susceptibility or resistance to develop TB disease (reviewed in [2, 3]). Among them, genes encoding Toll-like receptors (TLRs) have received particular attention because of the central role of these receptors in the initiation and shaping of an adaptive immune response, and various TLRs have been shown to recognize mycobacterial components [4, 5]. TLRs are a group of pattern recognition receptors that

play a key role in the immune system by detecting pathogens and initiating a signaling cascade that results in the secretion of inflammatory cytokines, type I IFN, chemokines, and antimicrobial peptides. In turn, these mediators orchestrate an inflammatory reaction and recruit and activate macrophages and other effector cells of the innate immune response. TLRs also contribute to the activation and maturation of dendritic cells that are essential for the initiation and for shaping the adaptive T cell response [6].

Various TLRs have been shown to recognize mycobacteria and their extracellular products. Thus, TLR2-TLR1 and TLR2-TLR6 heterodimers, as well as TLR4, have been shown to recognize mycobacterial PAMPs and mediate activation of dendritic cells and macrophages [7–10]. TLR10, on its part, serves as a modulatory receptor with inhibitory properties on TLR2-derived immune responses. Single nucleotide polymorphisms (SNPs) in TLRs can alter ligand-receptor interactions or modulate receptor signaling and thus can influence susceptibility or resistance to diseases. It is therefore not surprising that several studies have looked for an association between genetic polymorphisms in TLRs and susceptibility to TB in several human populations. However, results are in many instances inconsistent and inconclusive, emphasizing the need for more studies analyzing larger numbers of samples from different human populations.

In this study, we investigated the association of polymorphisms in the genes for *TLR1*, *TLR2*, *TLR4*, *TLR6*, and *TLR10*, with susceptibility to TB in a population of the Mexican state of Veracruz, a state with a relatively high prevalence of TB (27.4 cases/100,000 people) [11]. Our results found that two common SNPs in *TLR4* that are in strong linkage disequilibrium confer a protective effect to develop active TB in this population.

2. Materials and Methods

2.1. Study Population. A total of 279 TB cases and 569 healthy controls were included in this case-control association study. All participants, as well as their parents and grandparents, were born in Mexico. We consider participants in this study as Mexican mestizos, i.e., having both European and Amerindian ancestry, as only indigenous groups living in very remote and isolated areas of Mexico can be considered to have pure indigenous ancestry. All participants in this study have a Spanish-derived last name and spoke Spanish, and a small proportion also spoke an indigenous language. All participants were older than 18 years and were living in three regions of the state of Veracruz, México: Coatzacoalcos (31 cases, 71 controls), Poza Rica (156 cases, 298 controls), and Veracruz (92 cases, 200 controls). TB patients were diagnosed with pulmonary TB by clinical symptoms and at least one of the following: sputum smear examinations for acid-fast bacilli, or a positive culture for *Mycobacterium tuberculosis*, or chest X-ray. All patients have been diagnosed as TB patients by the medical staff of the Health Services of the state of Veracruz, Mexico, and were receiving anti-TB medication. Patients coinfected with human immunodeficiency virus (HIV), or having diabetes mellitus or an autoimmune disease, were excluded from the study. Healthy controls were unrelated individuals with no history and no suggestive symptoms of TB, living in the same area and similar socioeconomic conditions as patients. Written informed consent was obtained from all patients and controls. This study was reviewed and approved by the Committee for Research in Humans of the Instituto de Investigaciones Biomédicas, UNAM, and by the Committee for Research, Bioethics, and Biosafety of the Health Services of the state of Veracruz, México.

2.2. Blood Samples and DNA Isolation. Blood samples were collected from May 2011 through June 2013. From each participant, samples of 4 ml of venous blood were collected directly into EDTA-containing tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). Genomic DNA was extracted from blood samples following standard protocols [12]. DNA from each sample was quantified in a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and was kept frozen at -20°C until used.

2.3. Gene Polymorphism Genotyping. Seven nonsynonymous single nucleotide polymorphisms (SNPs) in five genes were investigated in all participants: *TLR1* (N248S; rs4833095), *TLR2* (R753Q; rs5743708), *TLR4* (D299G; rs4986790 and T399I; rs4986791), *TLR6* (S249P; rs5743810), and *TLR10* (A163S; rs11466649 and V298I; rs11466651). These SNPs were selected based on previous reports of their association with TB and because these polymorphisms have been reported to have functional consequences.

SNP genotyping was performed using validated predesigned TaqMan™ SNP Genotyping Assays (Life Technologies/Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All assays were carried out on an ABI 7500 HT Real-time PCR System. The TaqMan call rates for genotyping were over 99%.

2.4. Statistical Analysis. Wilcoxon 2-sample rank-sum test was used to compare median ages of cases and controls. The first step of the statistical analysis was to compare the distribution of demographic characteristics and allelic and genotypic frequencies between cases and controls using the χ^2 test, Fisher's exact test, and univariate unconditional logistic regression. In this crude analysis, TLR4 polymorphisms, age, sex, speaking an indigenous language, and educational level were associated with TB infection risk (Table 1). Odds ratios (OR) and 95% confidence intervals (CI) for genetic associations were assessed by using univariate and multivariate unconditional logistic regression (LR), with adjustment for age, sex, rural dwelling, speaking an indigenous language, and educational level (Table 2). All analyses were performed using Stata 12 (StataCorp, College Station, TX, USA). Hardy-Weinberg equilibrium (HWE) was evaluated in controls using goodness-of-fit χ^2 tests within each SNP. The D' values of LD plots were produced using the Haploview 4.2 program. Since this was an exploratory study, we did not introduce a correction for multiple comparisons [13]. Statistical significance was defined as $p < 0.05$.

Table 1: Demographic characteristics of cases and controls.

	TB cases $n = 279$	Controls $n = 659$	OR (95% CI)	p value
Median age (years) (range)∗	37 (18-97)	38 (18-97)	—	0.13∗
Gender				
Female (%)	107 (38.4)	410 (72.1)	1	Reference
Male (%)	172 (61.6)	159 (27.9)	4.15 (3.03-5.68)	<0.001
Smoking (%)				
No	254 (91.1)	505 (88.8)	1	Reference
Yes	25 (8.9)	64 (11.2)	0.75 (0.46-1.29)	0.24
Alcohol use (%)				
No	232 (83.3)	455 (80.0)	1	Reference
Yes	47 (16.7)	114 (20.0)	0.80 (0.54-1.19)	0.20
Direct contact with patients				
No	21 (7.5)	46 (8.1)		Reference
Yes	257 (92.1)	516 (90.7)	1.09 (0.62 - 1.97)	0.89
Unknown	1 (0.4)	7 (1.2)		
Living in a rural area (%)				
No	205 (74.2)	447 (78.6)	1	Reference
Yes	74 (26.3)	122 (21.4)	1.32 (0.93-1.86)	0.10
Speak an indigenous language (%)				
No	229 (82.1)	514 (90.3)	1	Reference
Yes	50 (17.9)	55 (9.7)	2.04 (1.33-3.15)	<0.001
Educational level (%)				
Higher	15 (5.4)	133 (23.4)		
Complete high school	41 (14.7)	109 (19.2)		
Complete secondary school	71 (25.4)	127 (22.3)	1	Reference
Complete elementary school	100 (35.8)	145 (25.5)		
None	52 (18.6)	55 (9.7)	2.22 (1.66-2.97)	<0.0001

Abbreviations: TB: tuberculosis; OR: odds ratio; CI: confidence interval. ∗p value for age was calculated by Wilcoxon 2-sample rank-sum test. OR and 95% CI and p values for all other characteristics were assessed by using univariate logistic regression.

3. Results

We used a case-control population study design to evaluate whether SNPs in genes for various TLRs were associated with an increased risk to develop active TB disease in Mexican adults from the state of Veracruz. All individuals participating in the study were descendants of Mexican parents and grandparents. Demographic characteristics of TB cases and controls are shown in Table 1. The distribution of age, personal habits such as smoking and alcohol consumption, and conditions such as having direct contact with patient(s) and residence in a rural area showed no statistically significant differences between cases and controls ($p > 0.05$). There was a significant difference between the proportion of females and males among groups ($p < 0.001$). The higher proportion of males in the patient group is expected as the male : female ratio among adult TB patients is approximately 2 : 1 [1]. The control group had a higher proportion of females, although this result is very probably biased because of the sampling, as persons accompanying the patient to the clinic that were recruited to participate in the study were mainly females, either wives of male patients or female in-law or friend of female patients. Other individual circumstances such as speaking an indigenous language ($p < 0.001$) and very low educational level (none+complete elementary school vs. complete secondary school+complete high school+higher) ($p < 0.001$) were identified as risk factors for TB disease in our study population.

3.1. Association of SNPs in TLR Genes with Active Tuberculosis. The allele and genotype frequencies of the *TLR1*, *TLR2*, *TLR4*, *TLR6*, and *TLR10* polymorphisms investigated are shown in Table 2. The genotype frequency distribution of all the seven TLR SNPs was consistent with the Hardy-Weinberg equilibrium in the control group. To determine if any of these polymorphisms could be a risk or a protective factor for developing active TB disease, we determined the odds ratios (OR). The allele and genotype frequencies of the *TLR1* (rs4833095), *TLR2* (rs5743708), *TLR6* (rs5743810), and *TLR10* (rs11466649 and rs11466651) polymorphisms were found to be similar among TB cases and controls in our study population (Table 2).

In the case of the polymorphisms in *TLR4*, the results show that the overall frequencies of the minor alleles of both

Table 2: Distribution of *TLR1*, *TLR2*, *TLR4*, *TLR6*, and *TLR10* allele and genotype polymorphisms in TB patients and controls of the state of Veracruz.

Gene SNP		TB patients $n = 279$		Controls $n = 569$		OR (95% CI)	p value*
		n	Frequency	n	Frequency		
TLR1	CC	84	30.1	176	30.9	1	
N248S (rs4833095)	CT	135	48.4	277	48.7	1.02 (0.72- 1.48)	0.88
	TT	60	21.5	116	20.4	1.06 (0.68-1.64)	0.80
Dominant	TT+TC	195		393		1.03 (0.74-1.45)	0.84
	C	303	54.3	629	55.3	1	
	T	255	45.7	509	44.7	1.04 (0.84- 1.28)	0.82
TLR2	GG	279	100	568	99.8	1	
R753Q (rs5743708)	GA	0	0	1	0.2	—	ND
	G	558	100	1137	99.9	1	
	A	0	0.00	1	0.1	—	ND
TLR4	AA	275	98.6	537	94.4	1	
D299G (rs4986790)	AG	3	1.1	32	5.6	0.20 (0.06-0.69)	**0.01**
	GG	1	0.4	0	0	—	ND
Dominant	GG+AG	4		32		0.26 (0.09-0.77)	**0.02**
	A	553	99.1	1106	97.2	1	
	G	5	0.9	32	2.8	0.31 (0.09-0.81)	**0.01**
TLR4	CC	275	98.6	543	95.4	1	
T399I (rs4986791)	CT	4	1.4	26	4.6	0.35 (0.13-0.90)	**0.03**
	C	554	99.3	1112	97.7	1	
	T	4	0.7	26	2.3	0.29 (0.10-0.90)	**0.03**
TLR6	GG	236	84.6	488	85.8	1	
S249P (rs5743810)	GA	42	15.1	78	13.7	1.15 (0.76-1.74)	0.30
	AA	1	0.4	3	0.5	0.40 (0.04-4.29)	0.45
Dominant	AA+GA	43		81		1.22 (0.79-1.88)	0.38
	G	514	92.1	1054	92.6	1	
	A	44	7.9	84	7.4	1.08 (0.72-1.60)	0.70
TLR10	CC	192	68.8	374	65.7	1	
A163S (rs11466649)	CA	77	27.6	178	31.3	0.84 (0.60-1.18)	0.32
	AA	10	3.6	17	3.0	1.09 (0.45-2.57)	0.86
Dominant	AA+CA	87		195		0.86 (0.62-1.20)	0.38
	C	461	82.6	926	81.4	1	
	A	97	17.4	212	18.6	0.92 (0.70-1.20)	0.53
TLR10	CC	192	68.8	374	65.7	1	
V298I (rs11466651)	CT	77	27.6	178	31.3	0.83 (0.60-1.18)	0.33
	TT	10	3.6	17	3.0	1.08 (0.45-2.57)	0.86
Dominant	TT+CT	87		195		0.86 (0.62-1.20)	0.38
	C	461	82.6	926	81.4	1	
	T	97	17.4	212	18.6	0.92 (0.70-1.20)	0.53

Abbreviations: TB: tuberculosis; OR: odds ratio; CI: confidence interval; ND: not determined. $*p$ values adjusted for gender, speaking an indigenous language, and educational level were determined by multivariate logistic regression.

SNPs D299G (rs4986790) and T399I (rs4986791) were low in the study population, as the allelic frequencies of the variant allele were only 2.8% for *TLR4* (D299G) and 2.3% for *TLR4* (T399I) in the control population. The minor allele (G) of rs4986790 in *TLR4* is associated with a decreased risk of TB disease in the study population (A vs. G, OR = 0.31, 95%CI = 0.09-0.81, $p = 0.01$). In the dominant genetic model, the G allele is also associated with decreased risk of TB (AA vs. GG+AG, OR = 0.26, 95%CI = 0.09-0.77, $p = 0.02$). Similarly, the minor allele (T) of rs4986791 in *TLR4* (T399I) was also found to be associated with a decreased risk of TB (C vs. T, OR = 0.29, 95%CI = 0.10-0.90, $p = 0.03$). All significant results were adjusted by gender, speaking an indigenous language, and

Table 3: Analysis of haplotypes association with active TB.

Haplotype	Frequency	Case frequency	Control frequency	OR (95% CI)	*p* value
Chromosome 4					
CCCG	0.53	0.53	0.53	1.02 (0.83-1.26)	0.85
CCTG	0.21	0.22	0.21	1.02 (0.79-1.31)	0.88
TATG	0.17	0.16	0.17	0.98 (0.74-1.30)	0.91
CCTA	0.06	0.07	0.06	1.27 (0.83-1.93)	0.25
TACG	0.01	0.01	0.01	0.73 (0.20-2.15)	0.54
Chromosome 9					
AC	0.98	0.99	0.97	3.51 (1.36-11.53)	0.006
GT	0.02	0.01	0.02	0.35 (0.09-1.03)	0.043

Chromosome 4: *TLR10* A163S, *TLR10* V298I, *TLR1* N248S, *TLR6* S249P; chromosome 9: *TLR4* D299G, *TLR4* T399I.

educational level (Table 2). Our results suggest that the variant allele (G) in the polymorphism TLR4 D299G (A/G) and the variant allele (T) in polymorphism TLR4 T399I (C/T) on *TLR4* gene confer a protective effect against active TB in this population.

Since the polymorphisms in *TLR1* (N248S), *TLR2* (R753Q), *TLR6* (S249P), and *TLR10* (A163S and V298I) are located in chromosome 4, we performed an analysis to determine if different haplotypes could be associated with an increased risk of active TB. The polymorphism in *TLR2* (R753Q) was not included in the haplotype analysis because of the very low frequency found in the population studied (overall frequency 1/1696 alleles). The results are shown in Table 3. None of the five different haplotypes present in the population showed a significant association with TB. Of the four theoretically possible haplotypes generated by the polymorphisms in the *TLR4* gene (D299G (A/G) and T399I (C/T)) located in chromosome 9, only two haplotypes are present in the population studied. This is consistent with the fact that these two SNPs have been reported to cosegregate [14]. The haplotype AC (299D/399T) is the most frequent one and is associated with an increased risk of active TB (OR = 3.51, $p = 0.006$), whereas the less frequent haplotype GT (299G/399I) is associated with a diminished risk of developing the active disease (OR = 0.35, $p = 0.043$) (Table 3).

Figure 1 shows the graphical representation of linkage disequilibrium between the four SNPs of TLR genes in chromosome 4 (*TLR1*, *TLR6*, and two in *TLR10*) and the two SNPs in *TLR4* in chromosome 9. The *TLR2* polymorphism R753Q was excluded from this analysis because of its insignificant expression in the study population. As reflected by the D' values observed, both SNPs in *TLR10* are in strong linkage disequilibrium ($D' = 97$), and LD for the other two polymorphisms studied are lower ($89 > D' > 81$). As has been found in different populations, the two polymorphisms in *TLR4* (T399I and D299G) are in strong LD in the study population.

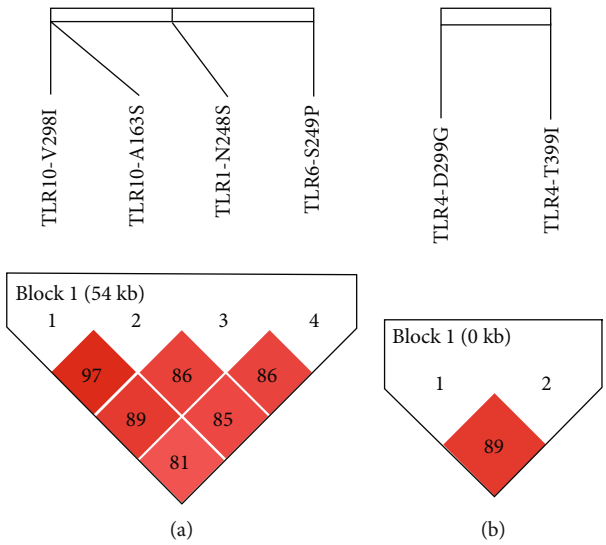

Figure 1: Haploview plot illustrating the linkage disequilibrium (LD) of TLR variants in the Mexican population from the state of Veracruz. (a) Haplotype-based association plot of the I298V, A163S (*TLR10*), S248N (*TLR1*), and S249P (*TLR6*) variants with TB disease. (b) Linkage disequilibrium plot of D299G and T399I SNPs from the TLR4 gene in the TB patients. The degree of pairwise LD (r^2) is shown in each block.

4. Discussion

TB remains a worldwide health problem, and several lines of reasoning indicate that along with environmental and bacterial factors, genetic factors of the host contribute to susceptibility for suffering active TB. Thus, to identify genes involved, many studies have been conducted to uncover the influence of polymorphisms in different immune response genes in the individual susceptibility to TB. Several polymorphisms in various genes have been shown to be associated with an increased risk of developing active TB, including genes of the innate immune response (reviewed in [3]). However, as expected for a multifactorial disease, different and sometimes contradictory results have been reported in distinct populations, emphasizing the need for additional studies analyzing larger numbers of subjects from diverse populations, as the effect of variations in one gene could differ according to the overall genetic background of the population.

Toll-like receptors are pattern recognition receptors of great relevance in the initiation and shaping of the adaptive immune response. Thus, the effects of genetic variation in TLRs have been studied in the context not only of infectious diseases but also in autoimmune and inflammatory diseases as well [15]. Different TLRs expressed in antigen-presenting cells can detect both pathogen- and danger-associated molecular patterns and induce a signaling cascade for activation of these cells for secretion of cytokines and the expression of costimulatory molecules that shape, both quantitatively and qualitatively, the activation of T cells. We analyzed single nucleotide polymorphisms in various TLRs genes, for its association with the risk for developing active TB in population from the state of Veracruz, Mexico. The state of Veracruz has a relatively high prevalence of TB (27.4 cases/100,000 people), ranking 8th among the 32 states in Mexico, where the overall rate was 17.3 TB cases/100,000 people in 2016 [11]. Thus, it is important to identify factors which affect the susceptibility to TB in this population. The SNPs that we analyzed were chosen because some of them have shown an association with TB in different populations. The studied SNPs are located in *TLR1* (rs4833095), *TLR2* (rs5743708), *TLR4* (rs4986790 and rs4986791), *TLR6* (rs5743810), and *TLR10* (rs11466649 and rs11466651). TLR1-TLR2 and TLR2-TLR6 heterodimers, as well as TLR4, have been shown to recognize mycobacterial PAMPs and mediate activation of dendritic cells and macrophages [7–10]. TLR10 is still an orphan receptor, as no ligands for it have been described, although it has been shown to have a role in some infectious diseases [16] and to modulate responses mediated by TLR2 [17].

The SNP rs4833095 in *TLR1* is a nonsynonymous polymorphism that results in an asparagine to serine change of residue 248 in the extracellular domain of the receptor (N248S). This change has been proposed to affect both ligand binding and the ability to form heterodimers with TLR2, and thus, it has been studied in association with the risk of developing TB. It has been described that PBMCs from healthy controls expressing TLR1-248N exhibited an increased secretion of TNF in response to Mtb lysates and that HEK cell lines transfected with *TLR1*-248N showed an increased activation of NF-κB in response to stimulation with Mtb [18]. In the same study, TLR1-248N SNP was found to be associated with protection from TB in a cohort from India. Several studies of the association of this SNP with TB susceptibility have been reported in different populations [19, 20]. In a recently published meta-analysis, Schurz et al. analyzed six studies that investigated this polymorphism and found a protective effect of the TLR1 248N variant (T allele) in both the heterozygous comparison (TC vs. CC: OR = 0.77, $p = 0.0031$) and in a dominant model (TT+TC vs. CC: OR = 0.78, $p = 0.0021$) [21]. The meta-analysis comprises populations from Asian, African, European, and Hispanic origin. The results of Ma et al. in a Hispanic population living in the USA (included in the meta-analysis) showed a tendency towards a protective effect of the T allele (248N) in the dominant model but without statistical significance (OR = 0.73, $p = 0.22$) [22]. We found no tendency of association of this polymorphism with susceptibility to TB in the studied population. The allelic frequency of the minor allele in our control population was 44.7%, very similar to the frequency in the patients' group (45.7%). Thus, it is possible that while in other populations variations in this SNP are relevant for susceptibility for TB, in a genetic background of American mestizos, such as our population and the Hispanic group of Ma et al. [22], the protective effect of the minor allele (coding for the 248N variant) could be countervailed by other genetic factors.

The SNP rs5743708 in *TLR2* results in an arginine to glutamine substitution in position 753 of the cytoplasmic portion of the receptor (R753Q). This change has been proposed to affect signaling by this receptor [23]. Although this is a rare polymorphism, several studies have reported the association of the variant allele A (753Q) with an increased risk for tuberculosis. Thus, Guo and Xia recently published the results of a meta-analysis of studies on the association of TLR2 (R753Q) with TB [24]. The analysis included 22 studies in Caucasian and Asian populations. They found a significant relationship between the allele A (753Q) and TB disease in the allelic genetic model (A vs. G, OR: 2.801, 95% CI: 2.130-3.683, $p < 0.001$), as well as in the homozygous model (AA vs. GG, OR: 5.795, 95% CI: 1.982-16.941, $p = 0.001$), heterozygous model (AG vs. GG, OR: 2.628, 95% CI: 1.888-3.569, $p < 0.001$), dominant genetic model (AA+AG vs. GG, OR: 2.786, 95% CI: 2.003-3.877, $p < 0.001$), and recessive genetic model (AA vs. AG+GG, OR: 5.568, 95% CI: 1.907-16.255, $p = 0.002$). In subgroup analysis based on ethnicity, significance was observed in both the Caucasian and Asian groups. Similarly, in a different meta-analysis, association of this SNP with increased risk for tuberculosis was found in the Asian population [25] but not in the white population. The meta-analysis of Schurz et al. [21] also analyzed studies of this polymorphism. While the global analysis showed no association, the subgroup analysis by ethnicity revealed the association of the A allele (753Q) with susceptibility in the Asian group, while in the Hispanic population (3 studies), it conferred protection against TB disease. However, not all studies have been able to show this association in Asian populations, as in a hospital-based case-control study in Chinese population, no association of this SNP with TB was detected [26]. We found only one mutant allele for this polymorphism in our study population (from a total of 1696 alleles). The frequency of this polymorphism in our population is close to zero. This result agrees with a previous report in a Mexican population in which analyzing 90 cases of TB and 90 controls, they found no single A allele [27].

The D299G (rs4986790) and T399I (rs4986791) SNPs in *TLR4* are among the most studied genetic variants of all TLRs, and their possible association with TB has been analyzed in various populations. Different results have been found in different cohorts, with some studies reporting significant associations [28–31] and others reporting no association. Distinct meta-analyses of published studies have been reported [21, 32]. Tian et al. [32] included six case-control studies in their analysis, involving 1587 controls and 2110 patients from diverse ethnicities (Caucasian, African American, Hispanics, and subjects from India and

Colombia). Overall, no significant associations (all $p > 0.05$) were found between the D299G and T399I SNPs in *TLR4* gene and TB. Schurz et al. [21] included in their meta-analysis twelve studies of the D299G SNP and found no significant association with TB. They also analyzed nine studies of T399I, again finding no significant association with TB in the overall analysis. In the subgroup analysis, however, they found that in the Asian population (4 studies), the minor allele (T) and the TC and TT genotypes (in the T399I polymorphism) were associated with increased susceptibility to TB. In contrast, we found that in our study population, the minor allele G in *TLR4* (299G) and the minor allele T in *TLR4* (399I) were less frequent in patients than in controls, and thus, these polymorphisms were significantly associated with decreased susceptibility to TB (Table 2; *TLR4* 299G OR = 0.31, $p = 0.01$; *TLR4* 399I OR = 0.29, $p = 0.03$). The opposite effects of *TLR4* 299G and 399I variants on susceptibility to TB between our population and the Asian subgroup of Schurz et al. [21] illustrate, as has been found for TLR2 (R753Q) (see above), that the same SNP could show different effects in different studies depending on several factors, including sample size and power of the study, differences in criteria for definition of cases, and genetic differences among study populations.

Two previous studies have analyzed the possible association of polymorphism *TLR4* (D299G) with TB in populations from México. Torres-Garcia et al. [27] analyzed 90 patients with TB and 90 controls from the state of Oaxaca and did not detect an association, while Rosas-Taraco et al. [33] analyzed the same SNP in 104 patients and 114 healthy controls from the state of Nuevo León, also finding no association. Apart from the fact that our study included a significantly higher number of participants (279 cases and 569 controls), and thus have more statistical power, the disparity of our results with these studies might reside in the populations analyzed. Although the subjects in the three studies were from México, the populations living in the three areas from which the samples were obtained could have differences in their ethnic background. In the study of Torres-Garcia et al., all participants (TB patients and controls) were from the Mazatecan ethnic group and were all living in the town called Temascal, a rural area in Oaxaca, and all were descendants of parents and grandparents born in the Mazatecan area in the state of Oaxaca, Mexico. Subjects of the Rosas-Taraco study have been residents of the state of Nuevo León for 2–3 generations and were from white and mestizo ethnic groups. In the northern part of Mexico, including the Nuevo León state, the population has overall less indigenous genes than in the central and southern parts of the country. Our population came from three different geographic areas of the state of Veracruz. Subjects from the northern part (Poza Rica) could have Totonacan ancestry (25% of our subjects from this area considered themselves as Totonacans), while in the central (Veracruz) and southern parts (Coatzacoalcos), less than 3% of the subjects declared themselves as belonging to an ethnic group, although most probably have some indigenous ancestry. It is known that there is great genetic diversity among indigenous ethnic groups in México [34], and although the study participants can be characterized as mestizos, genetic differences could exist among mestizos from different parts of the country. The low frequency of the minor alleles of these two polymorphisms in *TLR4* is also a factor that complicates a definitive conclusion as to their association with TB, since the number of homozygous individuals for the minor alleles is practically null in populations from Mexico and Hispanics living in the USA. Overall, only one homozygous individual for the minor allele in *TLR4* (D299G) was detected out of more than 1700 participants ([22, 27, 33] and this study).

In *in vitro* experiments, it has been demonstrated that heat shock protein Hsp 65 from Mtb activates TLR4-mediated proinflammatory pathways [35] and that the (D299G) polymorphism in TLR4 functionally affects the receptor, resulting in a blunted response to inhaled LPS in humans [14]. However, the influence of *TLR4* polymorphisms in susceptibility of humans to different infectious diseases has not been definitively established, suggesting that other environmental or genetic factors can potentiate or counteract the effects of these variations in *TLR4* for susceptibility to different conditions or diseases. In this regard, Ziakas et al. published a review of studies of the association of the two *TLR4* SNPs (D299G and T399I) and infections by numerous agents [36]. They concluded that the polymorphisms had been reported to be associated with increased, decreased, or no difference in susceptibility to infectious disease depending both in the population and the type of infection. Also, it should be kept in mind that the expression level, more than genetic variations in *TLR4*, has been proposed to be more critical for responsiveness to LPS [37]. Regarding the type of infection, it is interesting to note that the two SNPs in *TLR4* which we found associated with resistance to TB were reported to be associated with resistance to Legionnaires' disease [38] (caused by infection with *Legionella pneumophila*, an intracellular Gram-negative bacterium) and to be associated with a protective effect against leprosy caused by *Mycobacterium leprae* [39].

The SNP rs5743810 in *TLR6* is a nonsynonymous polymorphism that results in a serine to proline change of residue 249 in the extracellular domain of the receptor (S249P). This change has functional consequences, as it was shown that the variant allele (249P) was associated with lower NF-κB signaling in response to diacylated lipopeptide ($p = 0.019$) or Mtb lysate ($p = 0.026$) in a HEK293 cell line reconstitution assay, compared with the ancestral allele (S249) [40]. This polymorphism has been studied for its association with TB, and results from these studies have been included in meta-analyses. Zhang et al. [41] included 4 case-control studies from two papers (1093 cases vs. 620 controls) and found that the minor allele (A) is associated with protection from TB (OR = 0.66, $p = 0.04$). Similarly, Schurz et al. [21] included four articles (7 studies) in their meta-analysis and also found a protective effect of the minor allele in the allelic model (A vs. G, OR = 0.66, $p = 0.0001$) and of the AA and AG genotypes in the heterozygote, homozygote, recessive, and dominant models. No studies have been reported of association of this SNP with TB in Mexican populations. Our results show no differences in allelic or genotypic frequency of this SNP between cases and controls (Table 2).

Human TLR10 is a receptor for which no ligands have been identified. Nevertheless, it has been shown that TLR10 is a modulatory receptor that can inhibit the TLR2-mediated release of proinflammatory cytokines [17]. An SNP in *TLR10* (different from the ones studied here) was found to be associated with TB in a Croatian population [42]. More recently, the influence of distinct polymorphisms in*TLR10* on susceptibility for TB has been reported [43, 44]. Although these studies did not analyze the SNPs included in our study, they nevertheless provide support for the possible participation of *TLR10* in TB. We studied two SNPs in this receptor for their possible association with susceptibility to TB: rs11466649 (A163S) and rs11466651 (V298I). These nonsynonymous SNPs, which are in strong linkage disequilibrium, have been studied for its association with TB only by Ma et al. [22] in three populations: African American, Caucasians, and Hispanics living in the state of Texas, USA. They found that the minor alleles in each of the SNPs are associated with increased susceptibility for TB (OR = 2.3, $p = 0.003$ for rs11466649 and OR = 2.07, $p = 0.009$ for rs11466651) in the Hispanic population. In our study population, however, none of the studied SNPs in *TLR10* were associated with the risk of developing active TB.

In summary, our results in a large cohort of 279 TB patients and 569 controls from the state of Veracruz, Mexico, has revealed that two cosegregating SNPs in *TLR4* are associated with a decreased susceptibility for active TB in this population. Our results suggest that the variants (299G) and (399I) in *TLR4* confer a protective effect against active TB disease in this population. Previously reported studies in different populations have found these polymorphisms were either not associated or associated with increased susceptibility for active TB. The reasons for these apparently contradictory results are not known but are probably related to the overall genetic background of the populations and/or to possible differences in pathogenicity of the circulating Mtb strains. In any event, and considering that despite efforts in several laboratories, it has not yet been possible to predict the phenotypic effect of *TLR4* variants in Mtb infections *in vivo* (reviewed in [45]), and the effect of polymorphisms in any given gene might be confounded by the overall genetic background of the studied population; our data contributes a piece of original information that should be taken into account in future meta-analyses and reviews.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

E.O., B. S-H., I. L-L., and J. F-D. conceived and designed the experiments. S. H-B, B. S-H., and I. L-L performed the experiments. E.O., B. S-H., and S. H-B. analyzed the data. J. F-D. contributed logistic support. E.O. and B. S-H. wrote the paper.

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References

[1] WHO, "WHO | Global tuberculosis report 2018," in *WHO, Volume Licence: CC BY-NC-SA 3.0 IGO*, World Health Organization, Geneva, 2019.

[2] J. J. Yim and P. Selvaraj, "Genetic susceptibility in tuberculosis," *Respirology*, vol. 15, no. 2, pp. 241–256, 2010.

[3] A. K. Azad, W. Sadee, and L. S. Schlesinger, "Innate immune gene polymorphisms in tuberculosis," *Infection and Immunity*, vol. 80, no. 10, pp. 3343–3359, 2012.

[4] T. K. Means, S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton, "Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis," *Journal of Immunology*, vol. 163, pp. 3920–3927, 1999.

[5] S. Tsuji, M. Matsumoto, O. Takeuchi et al., "Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guérin: involvement of toll-like receptors," *Infection and Immunity*, vol. 68, no. 12, pp. 6883–6890, 2000.

[6] T. Kawai and S. Akira, "The role of pattern-recognition receptors in innate immunity: update on toll-like receptors," *Nature Immunology*, vol. 11, no. 5, pp. 373–384, 2010.

[7] N. Reiling, C. Holscher, A. Fehrenbach et al., "Cutting edge: toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis," *Journal of Immunology*, vol. 169, no. 7, pp. 3480–3484, 2002.

[8] M. Lopez, L. M. Sly, Y. Luu, D. Young, H. Cooper, and N. E. Reiner, "The 19-kDa Mycobacterium tuberculosis protein induces macrophage apoptosis through toll-like receptor-2," *Journal of Immunology*, vol. 170, no. 5, pp. 2409–2416, 2003.

[9] V. Quesniaux, C. Fremond, M. Jacobs et al., "Toll-like receptor pathways in the immune responses to mycobacteria," *Microbes and Infection*, vol. 6, no. 10, pp. 946–959, 2004.

[10] J. Uehori, K. Fukase, T. Akazawa et al., "Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation," *Journal of Immunology*, vol. 174, no. 11, pp. 7096–7103, 2005.

[11] CENAPRECE, "Secretaria *de* Salud, Mexico," *Cifras Oficiales de Tuberculosis*, 2019.

[12] S. A. Miller, D. D. Dykes, and H. F. Polesky, "A simple salting out procedure for extracting DNA from human nucleated cells," *Nucleic Acids Research*, vol. 16, no. 3, p. 1215, 1988.

[13] D. L. Streiner and G. R. Norman, "Correction for multiple testing: is there a resolution?," *Chest*, vol. 140, no. 1, pp. 16–18, 2011.

[14] N. C. Arbour, E. Lorenz, B. C. Schutte et al., "TLR4 mutations are associated with endotoxin hyporesponsiveness in humans," *Nature Genetics*, vol. 25, no. 2, pp. 187–191, 2000.

[15] M. G. Netea, C. Wijmenga, and L. A. O'Neill, "Genetic variation in toll-like receptors and disease susceptibility," *Nature Immunology*, vol. 13, no. 6, pp. 535–542, 2012.

[16] S. M. Lee, K. H. Kok, M. Jaume et al., "Toll-like receptor 10 is involved in induction of innate immune responses to influenza virus infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 10, pp. 3793–3798, 2014.

[17] M. Oosting, S.-C. Cheng, J. M. Bolscher et al., "Human TLR10 is an anti-inflammatory pattern-recognition receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 42, pp. E4478–E4484, 2014.

[18] N. Dittrich, L. C. Berrocal-Almanza, S. Thada et al., "Toll-like receptor 1 variations influence susceptibility and immune response to *Mycobacterium tuberculosis*," *Tuberculosis*, vol. 95, no. 3, pp. 328–335, 2015.

[19] E. Sinha, S. K. Biswas, M. Mittal et al., "Toll-like receptor 1 743 A>G, 1805 T>G & toll-like receptor 6 745 C>T gene polymorphism and tuberculosis: a case control study of north Indian population from Agra (India)," *Human Immunology*, vol. 75, no. 8, pp. 880–886, 2014.

[20] H. Qi, L. Sun, X. Wu et al., "*Toll-like receptor 1(TLR1)* Gene SNP rs5743618 is associated with increased risk for tuberculosis in Han Chinese children," *Tuberculosis*, vol. 95, no. 2, pp. 197–203, 2015.

[21] H. Schurz, M. Daya, M. Moller, E. G. Hoal, and M. Salie, "TLR1, 2, 4, 6 and 9 variants associated with tuberculosis susceptibility: a systematic review and meta-analysis," *PLoS One*, vol. 10, no. 10, article e0139711, 2015.

[22] X. Ma, Y. Liu, B. B. Gowen, E. A. Graviss, A. G. Clark, and J. M. Musser, "Full-exon resequencing reveals toll-like receptor variants contribute to human susceptibility to tuberculosis disease," *PLoS One*, vol. 2, no. 12, article e1318, 2007.

[23] E. Lorenz, J. P. Mira, K. L. Cornish, N. C. Arbour, and D. A. Schwartz, "A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection," *Infection and Immunity*, vol. 68, no. 11, pp. 6398–6401, 2000.

[24] X. G. Guo and Y. Xia, "The rs5743708 gene polymorphism in the TLR2 gene contributes to the risk of tuberculosis disease," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 9, pp. 11921–11928, 2015.

[25] H. Wu and L. Yang, "Arg753Gln polymorphisms in toll-like receptor 2 gene are associated with tuberculosis risk: a meta-analysis," *Medical Science Monitor*, vol. 21, pp. 2196–2202, 2015.

[26] M. J. Ma, L. P. Xie, S. C. Wu et al., "Toll-like receptors, tumor necrosis factor-α, and interleukin-10 gene polymorphisms in risk of pulmonary tuberculosis and disease severity," *Human Immunology*, vol. 71, no. 10, pp. 1005–1010, 2010.

[27] D. Torres-García, A. Cruz-Lagunas, M. García-Sancho Figueroa et al., "Variants in toll-like receptor 9 gene influence susceptibility to tuberculosis in a Mexican population," *Journal of Translational Medicine*, vol. 11, no. 1, article 1479-5876-11-220, p. 220, 2013.

[28] N. Najmi, G. Kaur, S. K. Sharma, and N. K. Mehra, "Human toll-like receptor 4 polymorphisms TLR4 Asp299Gly and Thr399Ile influence susceptibility and severity of pulmonary tuberculosis in the Asian Indian population," *Tissue Antigens*, vol. 76, no. 2, pp. 102–109, 2010.

[29] I. Pulido, M. Leal, M. Genebat, Y. M. Pacheco, M. E. Saez, and N. Soriano-Sarabia, "The TLR4 ASP299GLY polymorphism is a risk factor for active tuberculosis in Caucasian HIV-infected patients," *Current HIV Research*, vol. 8, no. 3, pp. 253–258, 2010.

[30] M. Jafari, M. R. Nasiri, R. Sanaei et al., "The NRAMP1, VDR, TNF-α, ICAM1, TLR2 and TLR4 gene polymorphisms in Iranian patients with pulmonary tuberculosis: A case-control study," *Infection, Genetics and Evolution*, vol. 39, pp. 92–98, 2016.

[31] J. M. Cubillos-Angulo, M. B. Arriaga, E. C. Silva et al., "Polymorphisms in TLR4 and TNFA and risk of Mycobacterium tuberculosis infection and development of active disease in contacts of tuberculosis cases in Brazil: a prospective cohort study," *Clinical Infectious Diseases*, vol. 69, no. 6, pp. 1027–1035, 2019.

[32] T. Tian, S. Jin, J. Dong, and G. Li, "Lack of association between toll-like receptor 4 gene Asp299Gly and Thr399Ile polymorphisms and tuberculosis susceptibility: a meta-analysis," *Infection, Genetics and Evolution*, vol. 14, pp. 156–160, 2013.

[33] A. G. Rosas-Taraco, A. Revol, M. C. Salinas-Carmona, A. Rendon, G. Caballero-Olin, and A. Y. Arce-Mendoza, "CD14 C(-159)T polymorphism is a risk factor for development of pulmonary tuberculosis," *The Journal of Infectious Diseases*, vol. 196, no. 11, pp. 1698–1706, 2007.

[34] A. Moreno-Estrada, C. R. Gignoux, J. C. Fernandez-Lopez et al., "The genetics of Mexico recapitulates Native American substructure and affects biomedical traits," *Science*, vol. 344, no. 6189, pp. 1280–1285, 2014.

[35] Y. Bulut, K. S. Michelsen, L. Hayrapetian et al., "Mycobacterium tuberculosis heat shock proteins use diverse toll-like receptor pathways to activate pro-inflammatory signals," *The Journal of Biological Chemistry*, vol. 280, no. 22, pp. 20961–20967, 2005.

[36] P. D. Ziakas, M. L. Prodromou, J. El Khoury, E. Zintzaras, and E. Mylonakis, "The role of TLR4 896 A>G and 1196 C>T in susceptibility to infections: a review and meta-analysis of genetic association studies," *PLoS One*, vol. 8, no. 11, article e81047, 2013.

[37] A. M. Hajjar, R. K. Ernst, J. Yi, C. S. Yam, and S. I. Miller, "Expression level of human TLR4 rather than sequence is the key determinant of LPS responsiveness," *PLoS One*, vol. 12, no. 10, article e0186308, 2017.

[38] T. R. Hawn, A. Verbon, M. Janer, L. P. Zhao, B. Beutler, and A. Aderem, "Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 7, pp. 2487–2489, 2005.

[39] P. Y. Bochud, D. Sinsimer, A. Aderem et al., "Polymorphisms in toll-like receptor 4 (TLR4) are associated with protection against leprosy," *European Journal of Clinical Microbiology & Infectious Diseases*, vol. 28, no. 9, article 746, pp. 1055–1065, 2009.

[40] M. S. Shey, A. K. Randhawa, M. Bowmaker et al., "Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide- and mycobacteria-induced interleukin-6 secretion," *Genes and Immunity*, vol. 11, no. 7, pp. 561–572, 2010.

[41] Y. Zhang, T. Jiang, X. Yang et al., "Toll-like receptor -1, -2, and -6 polymorphisms and pulmonary tuberculosis susceptibility: a systematic review and meta-analysis," *PLoS One*, vol. 8, no. 5, article e63357, 2013.

[42] L. J. Bulat-Kardum, G. E. Etokebe, P. Lederer, S. Balen, and Z. Dembic, "Genetic polymorphisms in the toll-like receptor 10, interleukin (IL)17A and IL17F genes differently affect the risk for tuberculosis in Croatian population," *Scandinavian Journal of Immunology*, vol. 82, no. 1, pp. 63–69, 2015.

[43] C. Uren, B. M. Henn, A. Franke et al., "A post-GWAS analysis of predicted regulatory variants and tuberculosis susceptibility," *PLoS One*, vol. 12, no. 4, article e0174738, 2017.

[44] Y. Wang, M. M. Zhang, W. W. Huang et al., "Polymorphisms in toll-like receptor 10 and tuberculosis susceptibility: evidence from three independent series," *Frontiers in Immunology*, vol. 9, p. 309, 2018.

[45] B. Ferwerda, M. B. B. McCall, K. Verheijen et al., "Functional consequences of toll-like receptor 4 polymorphisms," *Molecular Medicine*, vol. 14, no. 5-6, article 1405346, pp. 346–352, 2008.

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Review Article

Toll-Like Receptors in Natural Killer Cells and Their Application for Immunotherapy

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Innate immunity represents the first barrier for host defense against microbial infection. Toll-like receptors (TLRs) are the most well-defined PRRs with respect to PAMP recognition and induction of innate immune responses. They recognize pathogen-associated molecular patterns (PAMPs) and trigger innate immune responses by inducing inflammatory cytokines, chemokines, antigen-presenting molecules, and costimulatory molecules. TLRs are expressed either on the cell surface or within endosomes of innate immune cells. NK cells are one of the innate immune cells and also express TLRs to recognize or respond to PAMPs. TLRs in NK cells induce the innate immune responses against bacterial and viral infections via inducing NK cytotoxicity and cytokine production. In this review, we will discuss the expression and cellular function of TLRs in NK cells and also introduce some therapeutic applications of TLR agonists for NK cell-mediated immunotherapy.

1. Introduction

Cells involved in the innate immune response were initially speculated to nonspecifically eliminate microbes without presensitization; however, studies have reported that innate immune cells recognize microbial-associated or pathogen-associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs) [1–3]. In particular, the discovery of TLRs in the mid-1990s indicated that pathogen recognition by the innate immune system actually depended on PRRs [1]. TLRs are the most well-defined PRRs with respect to PAMP recognition and induction of innate immune responses. TLRs are expressed either on the cell surface or within endosomes [4–6]. The interaction of different PAMPs with their cognate TLRs induces numerous intracellular signal transduction resulting in the activation of innate immune-related genes including those encoding inflammatory cytokines, costimulatory molecules, adhesion molecules, and antimicrobial mediators [6].

Innate immunity is coordinated by epithelial barriers, plasma proteins, and tissue-resident or circulating leukocytes including macrophages and neutrophils, dendritic cells (DCs), natural killer (NK) cells, and innate lymphoid cells [7]. Cells involved in the innate immune response recognize and prevent potential pathogen invasion that could result in infectious diseases [8, 9]. During an infection, cells involved in the innate immune response rapidly recognize and activate complex responses by recognizing such pathogens. Among these, NK cells are lymphocytes that mediate multiple effector functions and detect and eliminate transformed or virus-infected cells. However, NK cells reportedly express cell surface TLRs and directly recognize or respond to pathogens [6, 10]. TLR expression and function in NK cells were revealed owing to their potential involvement in the

innate immune response to bacterial and viral infections via induction of NK cell-mediated cytotoxicity and cytokine production [6, 8, 11, 12].

Recent studies have furthered the current understanding of TLR expression and their critical role in NK cell-mediated innate immune responses against infections. This review is focused on recent advancements in studies on the expression and cellular function of TLRs in NK cell-induced antiviral and antibacterial responses. Furthermore, the potential applications of TLR agonists as potential boosters in stimulating immunological effector function of NK cells for cancer immunotherapy and infectious disease therapy are discussed herein.

2. General Features of TLRs and Their Ligands/Agonists

TLRs recognize conserved PAMPs, which serve as TLR agonists/ligands (TLRLs) [13, 14]. Some recent studies reported that endogenous, host-derived components, including fibrinogen, heat shock proteins, RNA, and DNA, also serve as TLRLs [14–16]. TLRs are expressed on cells involved in the innate immune response (myeloid and NK cells) and some cells of the adaptive immune system (regulatory and activated T cells) and mediate innate immune responses against microbial pathogens and induce adaptive immune responses [16, 17].

Ten and 13 TLRs have been identified in humans and mice, respectively, with TLR1–TLR9 being conserved in both species. Mouse TLR10 is not functional owing to retrovirus insertion, and TLR11, TLR12, and TLR13 have been lost from the human genome [1, 18]. TLRs are type I transmembrane proteins with ectodomains containing leucine-rich repeats (LRR) that mediate PAMP recognition, transmembrane domains, and a conserved region of ~200 aa intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains required for downstream signal transduction [1, 13, 19]. All TLRs induce the myeloid differentiation primary response protein 88- (MyD88-) dependent pathways except TLR3 [20]. These sensors, TLRs, are differentially expressed among immune cells and have distinct functions in terms of PAMP recognition and immune responses. Based on subcellular localization, TLRs are of two types: cell surface types (TLR1, 2, 4, 5, 6, 10, and 11) and endosomal types (TLR3, 7, 8, 9, 12, and 13) [18]. TLR2 heterodimerizes with TLR1 or TLR6, and they share an m-shaped structure. The TLR2-TLR1 heterodimer recognizes triacyl lipopeptides from Gram-negative bacteria and mycoplasma, whereas the TLR2-TLR6 heterodimer recognizes diacyl lipopeptides from Gram-positive bacteria and mycoplasma. For example, in the TLR2-TLR1 heterodimer, TLR2 interacts with two of the three lipid chains of Pam3CSK4 (a triacylated lipopeptide) and the third chain binds the hydrophobic channel of TLR1 [1, 17, 21, 22]. TLR3 was previously reported to recognize double-stranded RNA (dsRNA) produced by numerous viruses during replication or a synthetic analog of dsRNA, polyinosinic-polycytidylic acid (poly(I:C)), which mimics viral infection and induces antiviral immune responses by inducing type I interferons (IFNs) and inflammatory cytokines through the interaction of its ectodomain with dsRNA [23–25]. TLR4 was identified as the long-sought receptor that responds to bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria that can cause septic shock [18, 26]. TLR4 heterodimerizes with cell surface MD2, and the complex serves as an LPS-binding component [27]. TLR5 recognizes the flagellin in bacterial flagella [18]. TLR7 reportedly recognizes imidazoquinoline derivatives, guanine analogs including loxoribine; ssRNA derived from RNA viruses such as vesicular stomatitis virus, influenza A virus, and HIV; and certain siRNAs [18, 28, 29]. Mouse TLR8 shares the highest homology with TLR7; however, it is potentially nonfunctional, and human TLR8 recognizes imidazoquinolines and ssRNA. TLR8 is upregulated in monocytes upon bacterial infection [1, 30–32]. TLR9 recognizes unmethylated $2'$-deoxyribo CpG DNA motifs in bacteria and viruses, and the sugar backbone of DNA is important for TLR9 recognition [33–35]. TLR9 directly recognizes the insoluble crystal hemozoin, which is generated as a byproduct of the detoxification process after digestion of host hemoglobin by *Plasmodium falciparum* [36, 37]. PAMP recognition by TLRs triggers intracellular signaling pathways to produce inflammatory cytokines, type I IFNs, and chemokines for innate immune responses (Figure 1).

3. Cellular Functions of TLRs in NK Cells

3.1. Expression of TLRs on NK Cells. NK cells were previously (in the 1970s) reported as large granular circulating lymphocytes accounting for approximately 10–15% of the total blood cells and exhibiting "natural cytotoxicity" against tumor cells by releasing perforin- and granzyme-containing cytotoxic granules [38–40]. Furthermore, they protect the host by limiting viral and bacterial infections before the initiation of the adaptive immune response via activating macrophages, DCs, and neutrophils [12, 38]. Although the expression and cellular functions of TLRs have been extensively studied in macrophages, numerous recent studies have reported that TLRs are the first-line defense in NK cells via TLR-mediated signaling pathways against bacterial, viral, and fungal pathogens [38, 41–43]. Different TLRs are expressed in NK cells, and TLR ligands can activate NK cells directly or indirectly. In human NK cells, *TLR1–TLR9* mRNA was reportedly expressed, *TLR1* mRNA levels peaking, followed by *TLR2*, *TLR3*, *TLR5*, and *TLR6* mRNA at moderate levels, while *TLR9* mRNA expression levels were low or undetectable [6, 44, 45].

3.2. TLR-Induced Cellular Signaling Pathways. The presence of TLRs has been directly demonstrated through the activation of purified NK cells by TLR ligands and agonists. TLRs are expressed on NK cells independently and can cooperate with chemokines or cytokines to activate NK cell functions including cytokine production and cytotoxicity [11, 45]. As shown in Figure 1, TLRs are activated through specific PAMPs and then differentially induce signaling pathways in NK cells. After ligand or agonist binding to TLRs, TLRs dimerize and undergo conformational changes to recruit downstream adaptor proteins [13] including myeloid

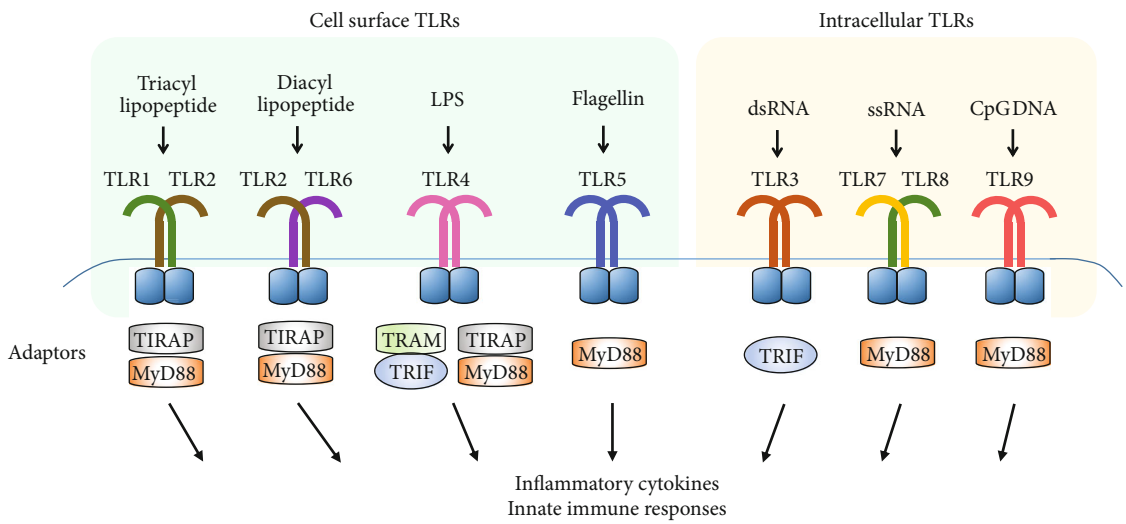

Figure 1: PAMP recognition by TLRs and adaptor proteins to mediate cellular signaling pathways. TLR members can be divided into cell surface types (TLR1, 2, 4, 5, and 6) and endosome types (TLR3, 7, 8, and 9). TLRs form homo- or heterodimer and have their respective ligands to be activated. After ligand binding to TLRs, TLRs dimerize and undergo the conformational change to recruit downstream adaptor proteins including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM).

differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM). MyD88 mediates intracellular signaling downstream of all TLRs except for TLR3 [38] (Figure 1). Interaction of adaptor proteins with TLRs is influenced by both the coligation of TLRs with their ligands and oligomerization of TLRs. TLRs activate nuclear factor κB- (NF-κB-) dependent and NF-κB-independent pathways to generate cytokines and chemokines [38]. Interaction of MyD88 with IL-1R-associated kinases (IRAKs) activates a complex containing TNF receptor-associated factor 6 (TRAF6) and TAB2, thus activating TGFβ-activated kinase 1 (TAK1). TAK1 is critical to determine the differential pathways to activate the NF-κB signaling pathway and mitogen-activated protein kinase pathways [45–47]. Briefly, MyD88 contains an N-terminal death domain (DD), which is separated from its C-terminal TIR domain by a short linker sequence [13, 48–50]. TIRAP is a second TIR-domain-containing adapter. Unlike MyD88, TIRAP does not contain a DD [51, 52]. TRIF was a third TIR-domain-containing adaptor and was identified as a TLR3-binding molecule, also referred to as TICAMI [53, 54]. TRAM is a fourth TIR-domain-containing adaptor identified on the basis of sequence homology in database searches [55]. TRAM interacts with TRIF and TLR4 but not TLR3 [13, 56].

The IRAK family comprises IRAK1, 2, 3, and 4 and IRAK-M. IRAKs contain an N-terminal DD and a central serine/threonine-kinase domain. IRAK1 and 4 exert kinase activity, whereas IRAK2 and IRAK-M have no detectable kinase activity [57]. TRAF6 comprises six members of the TRAF family in mammals, and they comprise an N-terminal coiled-coil domain and a conserved C-terminal domain. TAK1 and TAB1/2 regulate TRAF6-induced activation of NF-κB and activator protein 1 (AP1) transcription factor. Finally, transcription factors are activated to transcribe their target cytokines, chemokines, and mediators of immune responses (Figure 2).

In addition, TLR ligands or agonists differentially regulate TLR-mediated signaling pathways in NK cells. Numerous studies have demonstrated stimulation of TLRs by TLR ligands or agonists and reported the differential activation of NK cells by them. *K. pneumoniae* OmpA and flagellin reportedly stimulated TLR2 and 5 and induced IFN-γ and α-defensin production in human NK cells [44]. *M. bovis* and *H. pylori* (HpaA lipoprotein) stimulated TLR2 and induced CD69 and CD25 expression and IFN-γ and TNF production and IFN-γ production, respectively, in human NK cells [58, 59]. Moreover, diacyl lipopeptide reportedly induced IFN-γ production and cytotoxicity in mouse NK cells via TLR2 stimulation [60]. Poly (I:C) stimulated TLR3 to induce cytotoxicity and CXCL10 and IFN-γ production in human NK cells [61]. Another study reported that Poly (I:C) and loxoribine stimulate TLR3 and 7 and induce IFN-γ production and cytotoxicity in human NK cells [62]. Poly (I:C) and CpG stimulated TLR3 and 9 in human NK cells and upregulated CD69 and CD25 and increased cytotoxicity and IFN-γ and TNF production [63]. Peptidoglycan, Poly (I:C), LPS, and flagellin stimulated TLR2, 3, 4, and 5 and induced cytotoxicity and IFN-γ production in human NK cells [45]. Peptidoglycan and Poly (I:C) stimulated TLR2, 3, and 7 to induce IFN-γ production and cytotoxicity in mouse NK cells [64]. The CpG oligonucleotide reportedly serves as a TLR9 agonist and induces CD69 expression, thus suppressing bacterial growth in human and mouse NK cells, respectively [65, 66]. Although TLR agonists can directly activate NK cells, the microenvironment plays a potential role in activating their cytotoxicity and regulatory functions during

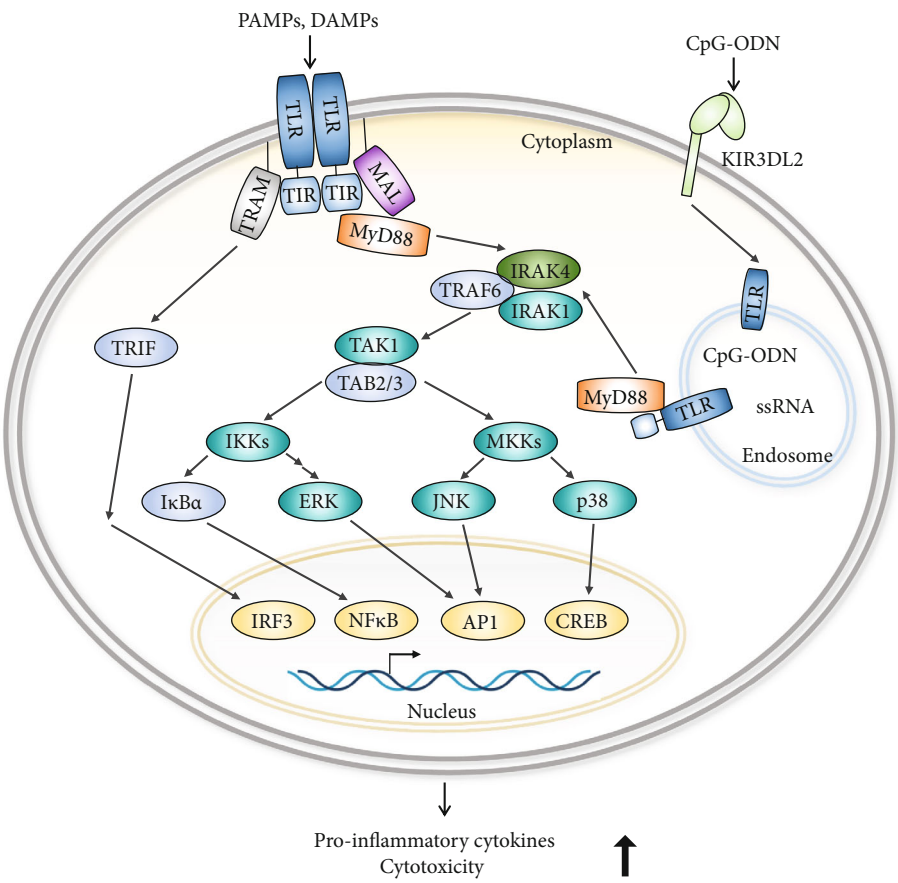

Figure 2: Overview of TLR-mediated signaling pathways. Activated TLRs trigger the association of adaptor proteins and activate their downstream molecules to induce the production of cytokines and cytotoxicity of NK cells.

TLR-mediated activation to induce subsequent immune responses [1, 67, 68].

4. Application of TLR Agonists for NK Cell-Mediated Therapy

TLR-mediated signaling pathways efficiently activate the effector functions of NK cells *in vitro* and *in vivo*. A number of clinical trials investigated the immunotherapeutic anticancer property of NK cells in various patient populations [69]. Interestingly, TLR agonists are potentially applicable to enhance the therapeutic effector function of NK cells for caner immunotherapy.

Trastuzumab is a humanized anti-HER2 monoclonal antibody (mAb) and is the first HER2-targeted therapy approved by the Food and Drug Administration. Trastuzumab has significantly advanced the clinical management of patients with HER2$^+$ breast cancer by prolonging disease-free survival and overall survival in patients with early-stage breast cancer, and progression-free survival and overall survival in patients with metastatic breast cancer [70, 71]. The therapeutic effect of trastuzumab therapy is partially dependent on functional NK cells. NK cell recognition of antibody-coated tumor cells through surface FcγRIII/CD16 provides a potent activation signal leading to antibody-dependent cell-mediated cytotoxicity (ADCC) [72, 73]. A polysaccharide krestin (PSK), a natural product extracted from medicinal mushroom *Trametes versicolor*, has recently been considered a potent TLR2 agonist. The effect of PSK on human NK cells and the potential of PSK to enhance HER2-targeted mAb therapy has been investigated. PSK activates human NK cells to produce IFN-γ and to lyse K562 target cells, enhances trastuzumab-mediated ADCC against SKBR3 and MDA-MB-231 breast cancer cells, and activates human NK cells and potentiates trastuzumab-mediated ADCC. Concurrently, PSK and trastuzumab therapy is a potentially novel method to induce the antitumor effect of trastuzumab [74].

TLR3 is an endosomal receptor that senses viral dsRNA [75]. Sensing of viral dsRNA by TLR3 leads to the secretion of type I IFN and other proinflammatory cytokines [23]. The TLR3 agonist Poly (I:C) reportedly suppressed tumor growth in mice [76, 77], and TLR3 agonists have been assessed in phase I/II trials as adjuvants for therapeutic vaccination against melanoma and breast cancer [78]. TLR3 reportedly limited experimental B16F10 lung metastasis, an immunologic constraint dependent on both IFN-γ secretion and NK cells, and NK cells derived from Tlr3 null mice were hyporesponsive to cytokine stimulation, thus suggesting a pivotal role of endogenous TLR3 stimulation in the acquisition of complete NK cell functions and immune protection against experimental metastasis [79].

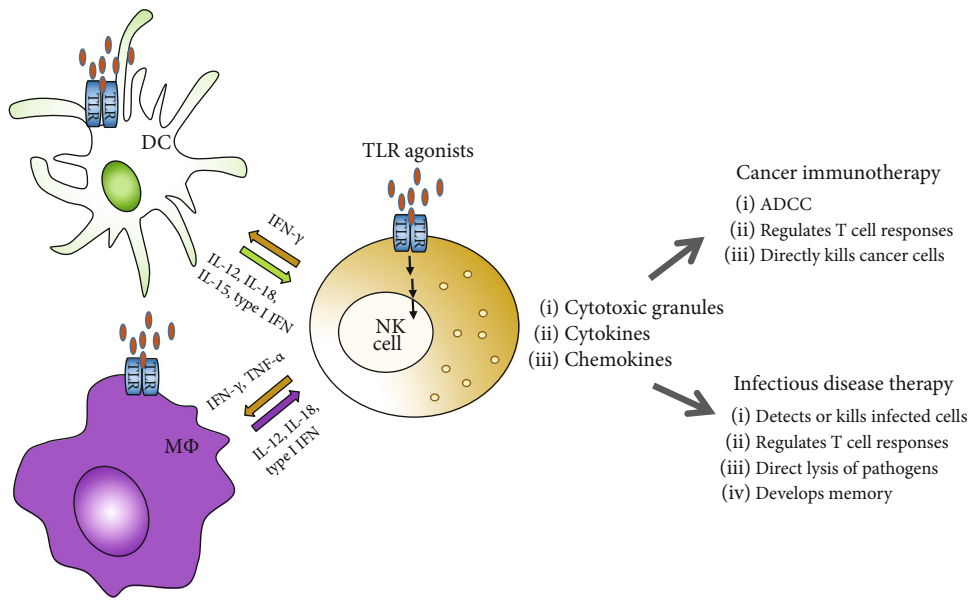

Figure 3: Application of TLR agonists for NK cell-mediated therapy. NK cells are activated directly by TLR agonists through TLRs or indirectly by NK cell-activating cytokines released by dendritic cells (DCs) and macrophages (MΦ). NK cells also activate DCs and macrophages by secretion of IFN-γ and TNF-α.

Synthetic TLR7 ligands induced a type 1 interferon response along with the secretion of proinflammatory cytokines including IL-1b, IL-6, and IL-12 by recruiting MyD88, interferon regulatory factors, and NF-κB [80–82]. A novel small-molecule agonist, SC1, has been developed for TLR7, and *in vivo* studies have attempted to determine the mode of action of SC1. Mice bearing the NK cell-sensitive lymphoma RMA-S were cured via repeated s.c. SC1 administration. SC1 treatment reportedly activated NK cells in a TLR7- and IFN-α-dependent manner, and SC1 thus reverses NK cell anergy leading to efficient tumor cell lysis [83].

The anti-CD20 monoclonal antibody (mAb) rituximab reportedly significantly improved patient survival; however, numerous patients ultimately experience relapse, thus necessitating the development of novel therapies and improved anti-CD20 mAbs [84, 85]. Immune stimulation through TLR7 activation in combination with obinutuzumab is hypothesized to further enhance lymphoma clearance and the generation of long-term antitumor immune responses. In syngeneic human CD20- (hCD20-) expressing models of lymphoma, systemic administration of a TLR7 agonist (R848) reportedly augmented responses upon combinatorial administration with obinutuzumab, thus preventing tumor recurrence. Furthermore, primary antitumor activity depended on both NK cells and $CD4^+$ T cells but not on $CD8^+$ T cells, suggesting that combinatorial treatment with TLR7 agonists potentially improves the outcome of obinutuzumab treatment [86].

ADCC is a well-established effector pathway that contributes to the mAb-mediated therapies including cetuximab, an epidermal growth factor receptor- (EGFR-) specific mAb approved for treating squamous cell carcinoma of the head and neck (SCCHN). VTX-2337 is a selective TLR8 agonist that is more potent than either resiquimod (R848) or 3M-002 (CL075), which is currently in phase II clinical trials for multiple oncological indications [87]. Cetuximab, a clinically approved, epidermal growth factor receptor-specific monoclonal antibody, activates NK cells through interactions with FcγRIII and facilitates ADCC in tumor cells. A phase I open-label, dose escalation trial including 13 patients with recurrent or metastatic SCCHN reported that patient NK cells become more responsive to stimulation by NKG2D or FcγRIII after VTX-2337 treatment, suggesting that TLR8 stimulation and inflammasome activation by VTX-2337 potentially complements FcγRIII engagement and augments clinical responses in SCCHN patients treated with cetuximab [88].

NK cells play an important role in the host response against various pathogens. NK cells can detect and damage various viral, bacterial, and fungal pathogens and also modulate or activate a variety of cells in the innate and adaptive immune system. NK cells are active against pathogens, and animal studies suggested that NK cells could be applied in the antimicrobial immunotherapy [69].

Over the past decade, the effect of NK cells in controlling HIV-1 infections *in vivo* has been reported [89, 90]. TLR agonists are potent enhancers of innate antiviral immunity and potentially reverse HIV-1 latency. Studies have attempted to improve NK cell function, using TLR9 agonists, suggesting that a novel TLR9 agonist, MGN1703, is potentially effective in an HIV-1 eradication trial [91]. Incubation of peripheral blood mononuclear cells with MGN1703 reportedly resulted in NK cell activation and increased NK cell function, thus significantly inhibiting the spread of HIV in a culture of autologous $CD4^+$ T cells. MGN1703 induced strong antiviral innate immune responses, enhanced HIV-1 transcription, and boosted NK cell-mediated suppression of HIV-1 infections in autologous $CD4^+$ T cells, suggesting that the preclinical basis for an HIV eradication clinical trial is the inclusion of MGN1703 [92].

NK cell activation during TLR stimulation by TLR agonists including bacteria-associated peptidoglycan, LPS, virus-derived dsRNA, and DNA with CpG motifs can be potently and indirectly induced by cytokines released by coexisting dendritic cells (DCs) and macrophages at sites of infection [93–95]. The activation of NK cells by DCs is dependent on both cell-to-cell interaction and soluble factors [96, 97]. DC-derived IL-12, IL-15, IL-18, and type I IFN are crucial for the production of IFN-γ in NK cells, and NK cell-derived IFN-γ then facilitates the activation of DCs. They have a positive feedback loop that amplifies TLR-induced activation of NK cells and DCs [95, 98–100]. Macrophages secrete IL-12, IL-18, and type I IFN to activate NK cells during microbial infection through TLR signaling pathways. Activated NK cells induce antimicrobial functions of macrophages by producing IFN-γ and TNF-α [94, 95]. Although these positive feedback loops between NK cells and DCs or macrophages facilitate beneficial functions of microbial clearance, the excessive production of cytokines can induce systemic inflammation *in vivo* [95] (Figure 3).

5. Conclusion

NK cells play an important role in the host response against various pathogens. TLRs are expressed on innate immune cells or some adaptive immune cells and mediate innate immune responses against microbial pathogens and induce adaptive immune responses. TLRs are also expressed in NK cells, and TLR ligands can activate NK cells directly or indirectly. Recent studies have reported that TLRs perform the first-line defense in NK cells against bacterial and viral infections by inducing NK cytotoxicity and cytokine production. TLR agonists were suggested as potential boosters in stimulating immunological effector function of NK cells for cancer immunotherapy and infectious disease therapy. However, to develop new drugs targeting TLRs, we should understand the complex mechanisms underlying TLR localization and function in NK cells. It will provide data for novel therapeutic tools involving TLRs and their agonists, and these approaches may be promising and have an important clinical impact for immunotherapy using NK cells in the future.

Conflicts of Interest

The authors declare no conflict of interest.

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References

[1] T. Kawai and S. Akira, "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors," *Nature Immunology*, vol. 11, no. 5, pp. 373–384, 2010.

[2] K. Takeda and S. Akira, "Toll-like receptors in innate immunity," *International Immunology*, vol. 17, no. 1, pp. 1–14, 2005.

[3] M. Yoneyama and T. Fujita, "RNA recognition and signal transduction by RIG-I-like receptors," *Immunological Reviews*, vol. 227, no. 1, pp. 54–65, 2009.

[4] C. C. Lee, A. M. Avalos, and H. L. Ploegh, "Accessory molecules for Toll-like receptors and their function," *Nature Reviews Immunology*, vol. 12, no. 3, pp. 168–179, 2012.

[5] L. A. O'Neill, D. Golenbock, and A. G. Bowie, "The history of Toll-like receptors - redefining innate immunity," *Nature Reviews Immunology*, vol. 13, no. 6, pp. 453–460, 2013.

[6] M. Adib-Conquy, D. Scott-Algara, J. M. Cavaillon, and F. Souza-Fonseca-Guimaraes, "TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals," *Immunology and Cell Biology*, vol. 92, no. 3, pp. 256–262, 2014.

[7] R. Nowarski, N. Gagliani, S. Huber, and R. A. Flavell, "Innate immune cells in inflammation and cancer," *Cancer Immunology Research*, vol. 1, no. 2, pp. 77–84, 2013.

[8] F. Souza-Fonseca-Guimaraes, M. Adib-Conquy, and J. M. Cavaillon, "Natural killer (NK) cells in antibacterial innate immunity: angels or devils?," *Molecular Medicine*, vol. 18, pp. 270–285, 2012.

[9] D. C. Hargreaves and R. Medzhitov, "Innate sensors of microbial infection," *Journal of Clinical Immunology*, vol. 25, no. 6, pp. 503–510, 2005.

[10] B. Zitti and Y. T. Bryceson, "Natural killer cells in inflammation and autoimmunity," *Cytokine & Growth Factor Reviews*, vol. 42, pp. 37–46, 2018.

[11] S. Sivori, S. Carlomagno, S. Pesce, A. Moretta, M. Vitale, and E. Marcenaro, "TLR/NCR/KIR: which one to use and when?," *Frontiers in Immunology*, vol. 5, p. 105, 2014.

[12] E. Vivier, D. H. Raulet, A. Moretta et al., "Innate or adaptive immunity? The example of natural killer cells," *Science*, vol. 331, no. 6013, pp. 44–49, 2011.

[13] S. Akira and K. Takeda, "Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 4, no. 7, pp. 499–511, 2004.

[14] B. Beutler, "Inferences, questions and possibilities in Toll-like receptor signalling," *Nature*, vol. 430, no. 6996, pp. 257–263, 2004.

[15] H. Wagner, "Endogenous TLR ligands and autoimmunity," *Advances in Immunology*, vol. 91, pp. 159–173, 2006.

[16] I. Vaknin, L. Blinder, L. Wang et al., "A common pathway mediated through Toll-like receptors leads to T- and natural killer-cell immunosuppression," *Blood*, vol. 111, no. 3, pp. 1437–1447, 2008.

[17] T. Kawai and S. Akira, "TLR signaling," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 816–825, 2006.

[18] S. Akira, S. Uematsu, and O. Takeuchi, "Pathogen recognition and innate immunity," *Cell*, vol. 124, no. 4, pp. 783–801, 2006.

[19] Y. Xu, X. Tao, B. Shen et al., "Structural basis for signal transduction by the Toll/interleukin-1 receptor domains," *Nature*, vol. 408, no. 6808, pp. 111–115, 2000.

[20] H. Patel, S. G. Shaw, X. Shi-Wen, D. Abraham, D. M. Baker, and J. C. Tsui, "Toll-like receptors in ischaemia and its potential role in the pathophysiology of muscle damage in critical limb ischaemia," *Cardiology Research and Practice*, vol. 2012, Article ID 121237, 13 pages, 2012.

[21] M. S. Jin, S. E. Kim, J. Y. Heo et al., "Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide," *Cell*, vol. 130, no. 6, pp. 1071–1082, 2007.

[22] J. Y. Kang, X. Nan, M. S. Jin et al., "Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer," *Immunity*, vol. 31, no. 6, pp. 873–884, 2009.

[23] L. Alexopoulou, A. C. Holt, R. Medzhitov, and R. A. Flavell, "Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3," *Nature*, vol. 413, no. 6857, pp. 732–738, 2001.

[24] J. Choe, M. S. Kelker, and I. A. Wilson, "Crystal structure of human toll-like receptor 3 (TLR3) ectodomain," *Science*, vol. 309, no. 5734, pp. 581–585, 2005.

[25] J. K. Bell, J. Askins, P. R. Hall, D. R. Davies, and D. M. Segal, "The dsRNA binding site of human Toll-like receptor 3," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 23, pp. 8792–8797, 2006.

[26] K. Hoshino, O. Takeuchi, T. Kawai et al., "Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product," *Journal of Immunology*, vol. 162, pp. 3749–3752, 1999.

[27] S. Akashi-Takamura and K. Miyake, "TLR accessory molecules," *Current Opinion in Immunology*, vol. 20, no. 4, pp. 420–425, 2008.

[28] T. Kawai and S. Akira, "Innate immune recognition of viral infection," *Nature Immunology*, vol. 7, no. 2, pp. 131–137, 2006.

[29] V. Hornung, M. Guenthner-Biller, C. Bourquin et al., "Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7," *Nature Medicine*, vol. 11, no. 3, pp. 263–270, 2005.

[30] F. Heil, H. Hemmi, H. Hochrein et al., "Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8," *Science*, vol. 303, no. 5663, pp. 1526–1529, 2004.

[31] F. Heil, P. Ahmad-Nejad, H. Hemmi et al., "The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily," *European Journal of Immunology*, vol. 33, no. 11, pp. 2987–2997, 2003.

[32] M. Jurk, F. Heil, J. Vollmer et al., "Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848," *Nature Immunology*, vol. 3, no. 6, p. 499, 2002.

[33] H. Hemmi, O. Takeuchi, T. Kawai et al., "A Toll-like receptor recognizes bacterial DNA," *Nature*, vol. 408, no. 6813, pp. 740–745, 2000.

[34] A. Krug, A. R. French, W. Barchet et al., "TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function," *Immunity*, vol. 21, no. 1, pp. 107–119, 2004.

[35] J. Lund, A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki, "Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells," *The Journal of Experimental Medicine*, vol. 198, no. 3, pp. 513–520, 2003.

[36] C. Coban, K. J. Ishii, T. Kawai et al., "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin," *The Journal of Experimental Medicine*, vol. 201, no. 1, pp. 19–25, 2005.

[37] C. Coban, Y. Igari, M. Yagi et al., "Immunogenicity of whole-parasite vaccines against plasmodium falciparum involves malarial hemozoin and host TLR9," *Cell Host & Microbe*, vol. 7, no. 1, pp. 50–61, 2010.

[38] Z. Yang, B. Kong, D. M. Mosser, and X. Zhang, "TLRs, macrophages, and NK cells: our understandings of their functions in uterus and ovary," *International Immunopharmacology*, vol. 11, no. 10, pp. 1442–1450, 2011.

[39] R. Kiessling, E. Klein, and H. Wigzell, ""Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype," *European Journal of Immunology*, vol. 5, no. 2, pp. 112–117, 1975.

[40] R. B. Herberman, M. E. Nunn, H. T. Holden, and D. H. Lavrin, "Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells," *International journal of cancer*, vol. 16, no. 2, pp. 230–239, 1975.

[41] A. Cerwenka and L. L. Lanier, "Natural killer cells, viruses and cancer," *Nature Reviews Immunology*, vol. 1, no. 1, pp. 41–49, 2001.

[42] M. Della Chiesa, E. Marcenaro, S. Sivori, S. Carlomagno, S. Pesce, and A. Moretta, "Human NK cell response to pathogens," *Seminars in Immunology*, vol. 26, no. 2, pp. 152–160, 2014.

[43] E. Bar, P. G. Whitney, K. Moor, C. R. e Sousa, and S. LeibundGut-Landmann, "IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells," *Immunity*, vol. 40, no. 1, article S1074761313005529, pp. 117–127, 2014.

[44] A. Chalifour, P. Jeannin, J. F. Gauchat et al., "Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production," *Blood*, vol. 104, no. 6, pp. 1778–1783, 2004.

[45] N. M. Lauzon, F. Mian, R. MacKenzie, and A. A. Ashkar, "The direct effects of Toll-like receptor ligands on human NK cell cytokine production and cytotoxicity," *Cellular Immunology*, vol. 241, no. 2, pp. 102–112, 2006.

[46] K. Takeda, T. Kaisho, and S. Akira, "Toll-like receptors," *Annual Review of Immunology*, vol. 21, pp. 335–376, 2003.

[47] A. Dunne and L. A. O'Neill, "The interleukin-1 receptor/-Toll-like receptor superfamily: signal transduction during inflammation and host defense," *Science Signaling*, vol. 2003, no. 171, p. re3, 2003.

[48] M. Muzio, J. Ni, P. Feng, and V. M. Dixit, "IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling," *Science*, vol. 278, no. 5343, pp. 1612–1615, 1997.

[49] H. Wesche, W. J. Henzel, W. Shillinglaw, S. Li, and Z. Cao, "MyD88: an adapter that recruits IRAK to the IL-1 receptor complex," *Immunity*, vol. 7, no. 6, pp. 837–847, 1997.

[50] K. Burns, F. Martinon, C. Esslinger et al., "MyD88, an adapter protein involved in interleukin-1 signaling," *The Journal of Biological Chemistry*, vol. 273, no. 20, pp. 12203–12209, 1998.

[51] T. Horng, G. M. Barton, and R. Medzhitov, "TIRAP: an adapter molecule in the Toll signaling pathway," *Nature Immunology*, vol. 2, no. 9, pp. 835–841, 2001.

[52] K. A. Fitzgerald, E. M. Palsson-McDermott, A. G. Bowie et al., "Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction," *Nature*, vol. 413, no. 6851, pp. 78–83, 2001.

[53] M. Yamamoto, S. Sato, K. Mori et al., "Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor

signaling," *Journal of Immunology*, vol. 169, no. 12, pp. 6668–6672, 2002.

[54] H. Oshiumi, M. Matsumoto, K. Funami, T. Akazawa, and T. Seya, "TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction," *Nature Immunology*, vol. 4, no. 2, pp. 161–167, 2003.

[55] M. Yamamoto, S. Sato, H. Hemmi et al., "TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway," *Nature Immunology*, vol. 4, no. 11, pp. 1144–1150, 2003.

[56] K. A. Fitzgerald, D. C. Rowe, B. J. Barnes et al., "LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF," *The Journal of Experimental Medicine*, vol. 198, no. 7, pp. 1043–1055, 2003.

[57] S. Janssens and R. Beyaert, "Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members," *Molecular Cell*, vol. 11, no. 2, pp. 293–302, 2003.

[58] E. Marcenaro, B. Ferranti, M. Falco, L. Moretta, and A. Moretta, "Human NK cells directly recognize Mycobacterium bovis via TLR2 and acquire the ability to kill monocyte-derived DC," *International Immunology*, vol. 20, no. 9, pp. 1155–1167, 2008.

[59] A. Lindgren, V. Pavlovic, C. F. Flach, A. Sjoling, and S. Lundin, "Interferon-gamma secretion is induced in IL-12 stimulated human NK cells by recognition of helicobacter pylori or TLR2 ligands," *Innate Immunity*, vol. 17, no. 2, pp. 191–203, 2011.

[60] M. Azuma, R. Sawahata, Y. Akao et al., "The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation," *PLoS One*, vol. 5, no. 9, article e12550, 2010.

[61] S. Pisegna, G. Pirozzi, M. Piccoli, L. Frati, A. Santoni, and G. Palmieri, "p38 MAPK activation controls the TLR3-mediated up-regulation of cytotoxicity and cytokine production in human NK cells," *Blood*, vol. 104, no. 13, pp. 4157–4164, 2004.

[62] M. V. Girart, M. B. Fuertes, C. I. Domaica, L. E. Rossi, and N. W. Zwirner, "Engagement of TLR3, TLR7, and NKG2D regulate IFN-gamma secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12," *Journal of Immunology*, vol. 179, no. 6, pp. 3472–3479, 2007.

[63] S. Sivori, M. Falco, M. Della Chiesa et al., "CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells," *Proceedings of the National Academy of Sciences*, vol. 101, no. 27, pp. 10116–10121, 2004.

[64] J. Sawaki, H. Tsutsui, N. Hayashi et al., "Type 1 cytokine/-chemokine production by mouse NK cells following activation of their TLR/MyD88-mediated pathways," *International Immunology*, vol. 19, no. 3, pp. 311–320, 2007.

[65] V. Hornung, S. Rothenfusser, S. Britsch et al., "Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides," *Journal of Immunology*, vol. 168, no. 9, pp. 4531–4537, 2002.

[66] K. L. Elkins, S. M. Colombini, A. M. Krieg, and R. De Pascalis, "NK cells activated in vivo by bacterial DNA control the intracellular growth of Francisella tularensis LVS," *Microbes and Infection*, vol. 11, no. 1, pp. 49–56, 2009.

[67] E. Marcenaro, S. Carlomagno, S. Pesce, A. Moretta, and S. Sivori, "Bridging innate NK cell functions with adaptive immunity," *Advances in Experimental Medicine and Biology*, vol. 780, pp. 45–55, 2011.

[68] E. Marcenaro, M. Della Chiesa, F. Bellora et al., "IL-12 or IL-4 prime human NK cells to mediate functionally divergent interactions with dendritic cells or tumors," *Journal of Immunology*, vol. 174, no. 7, pp. 3992–3998, 2005.

[69] S. Schmidt, L. Tramsen, B. Rais, E. Ullrich, and T. Lehrnbecher, "Natural killer cells as a therapeutic tool for infectious diseases-current status and future perspectives," *Oncotarget*, vol. 9, no. 29, pp. 20891–20907, 2018.

[70] C. A. Hudis, "Trastuzumab–mechanism of action and use in clinical practice," *The New England Journal of Medicine*, vol. 357, no. 1, pp. 39–51, 2007.

[71] J. Baselga, E. A. Perez, T. Pienkowski, and R. Bell, "Adjuvant trastuzumab: a milestone in the treatment of HER-2-positive early breast cancer," *The oncologist*, vol. 11, Supplement 1, pp. 4–12, 2006.

[72] A. Diefenbach and D. H. Raulet, "The innate immune response to tumors and its role in the induction of T-cell immunity," *Immunological Reviews*, vol. 188, pp. 9–21, 2002.

[73] T. Kaifu, B. Escaliere, L. N. Gastinel, E. Vivier, and M. Baratin, "B7-H6/NKp30 interaction: a mechanism of alerting NK cells against tumors," *Cellular and molecular life sciences*, vol. 68, no. 21, pp. 3531–3539, 2011.

[74] H. Lu, Y. Yang, E. Gad et al., "TLR2 agonist PSK activates human NK cells and enhances the antitumor effect of HER2-targeted monoclonal antibody therapy," *Clinical cancer research*, vol. 17, no. 21, pp. 6742–6753, 2011.

[75] M. Matsumoto and T. Seya, "TLR3: interferon induction by double-stranded RNA including poly(I:C)," *Advanced Drug Delivery Reviews*, vol. 60, no. 7, pp. 805–812, 2008.

[76] H. B. Levy, L. W. Law, and A. S. Rabson, "Inhibition of tumor growth by polyinosinic-polycytidylic acid," *Proceedings of the National Academy of Sciences*, vol. 62, no. 2, pp. 357–361, 1969.

[77] G. Forte, A. Rega, S. Morello et al., "Polyinosinic-polycytidylic acid limits tumor outgrowth in a mouse model of metastatic lung cancer," *Journal of Immunology*, vol. 188, no. 11, pp. 5357–5364, 2012.

[78] S. Sharma, L. Zhu, M. Davoodi et al., "TLR3 agonists and pro-inflammatory antitumor activities," *Expert Opinion on Therapeutic Targets*, vol. 17, no. 5, pp. 481–483, 2013.

[79] C. Guillerey, M. T. Chow, K. Miles et al., "Toll-like receptor 3 regulates NK cell responses to cytokines and controls experimental metastasis," *Oncoimmunology*, vol. 4, no. 9, article e1027468, 2015.

[80] H. Hemmi, T. Kaisho, O. Takeuchi et al., "Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway," *Nature Immunology*, vol. 3, no. 2, pp. 196–200, 2002.

[81] C. Bourquin, L. Schmidt, A. L. Lanz et al., "Immunostimulatory RNA oligonucleotides induce an effective antitumoral NK cell response through the TLR7," *Journal of Immunology*, vol. 183, no. 10, pp. 6078–6086, 2009.

[82] M. Gilliet, W. Cao, and Y. J. Liu, "Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases," *Nature Reviews Immunology*, vol. 8, no. 8, pp. 594–606, 2008.

[83] G. M. Wiedemann, S. J. Jacobi, M. Chaloupka et al., "A novel TLR7 agonist reverses NK cell anergy and cures RMA-S lymphoma-bearing mice," *OncoImmunology*, vol. 5, no. 7, article e1189051, 2016.

[84] B. Coiffier, E. Lepage, J. Briere et al., "CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma," *The New England Journal of Medicine*, vol. 346, no. 4, pp. 235–242, 2002.

[85] M. Hallek, K. Fischer, G. Fingerle-Rowson et al., "Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial," *The Lancet*, vol. 376, no. 9747, pp. 1164–1174, 2010.

[86] E. J. Cheadle, G. Lipowska-Bhalla, S. J. Dovedi et al., "A TLR7 agonist enhances the antitumor efficacy of obinutuzumab in murine lymphoma models via NK cells and CD4 T cells," *Leukemia*, vol. 31, no. 7, pp. 1611–1621, 2017.

[87] H. Lu, G. N. Dietsch, M. A. Matthews et al., "VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC," *Clinical cancer research*, vol. 18, no. 2, pp. 499–509, 2012.

[88] G. N. Dietsch, H. Lu, Y. Yang et al., "Coordinated activation of Toll-like receptor8 (TLR8) and NLRP3 by the TLR8 agonist, VTX-2337, ignites tumoricidal natural killer cell activity," *PloS one*, vol. 11, no. 2, article e0148764, 2016.

[89] G. Alter, D. Heckerman, A. Schneidewind et al., "HIV-1 adaptation to NK-cell-mediated immune pressure," *Nature*, vol. 476, no. 7358, pp. 96–100, 2011.

[90] M. Sips, G. Sciaranghella, T. Diefenbach et al., "Altered distribution of mucosal NK cells during HIV infection," *Mucosal Immunology*, vol. 5, no. 1, pp. 30–40, 2012.

[91] M. Schmidt, N. Hagner, A. Marco, S. A. Konig-Merediz, M. Schroff, and B. Wittig, "Design and structural requirements of the potent and safe TLR-9 agonistic immunomodulator MGN1703," *Nucleic Acid Therapeutics*, vol. 25, no. 3, pp. 130–140, 2015.

[92] R. Offersen, S. K. Nissen, T. A. Rasmussen et al., "A novel Toll-like receptor 9 agonist, MGN1703, enhances HIV-1 transcription and NK cell-mediated inhibition of HIV-1-infected autologous CD4+ T cells," *Journal of Virology*, vol. 90, no. 9, pp. 4441–4453, 2016.

[93] F. Souza-Fonseca-Guimaraes, M. Parlato, F. Philippart, B. Misset, J. M. Cavaillon, and M. Adib-Conquy, "Toll-like receptors expression and interferon-γ production by NK cells in human sepsis," *Critical care*, vol. 16, no. 5, p. R206, 2012.

[94] T. K. Varma, C. Y. Lin, T. E. Toliver-Kinsky, and E. R. Sherwood, "Endotoxin-induced gamma interferon production: contributing cell types and key regulatory factors," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 3, pp. 530–543, 2002.

[95] Y. Guo, N. K. Patil, L. Luan, J. K. Bohannon, and E. R. Sherwood, "The biology of natural killer cells during sepsis," *Immunology*, vol. 153, no. 2, pp. 190–202, 2018.

[96] N. C. Fernandez, A. Lozier, C. Flament et al., "Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo," *Nature Medicine*, vol. 5, no. 4, pp. 405–411, 1999.

[97] Y. Yu, M. Hagihara, K. Ando et al., "Enhancement of human cord blood CD34+ cell-derived NK cell cytotoxicity by dendritic cells," *Journal of Immunology*, vol. 166, no. 3, pp. 1590–1600, 2001.

[98] G. Ferlazzo, M. Pack, D. Thomas et al., "Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs," *Proceedings of the National Academy of Sciences*, vol. 101, no. 47, pp. 16606–16611, 2004.

[99] S. Anguille, H. H. Van Acker, J. Van den Bergh et al., "Interleukin-15 dendritic cells harness NK cell cytotoxic effector function in a contact- and IL-15-dependent manner," *PLoS One*, vol. 10, no. 5, article e0123340, 2015.

[100] C. Semino, G. Angelini, A. Poggi, and A. Rubartelli, "NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1," *Blood*, vol. 106, no. 2, pp. 609–616, 2005.

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Research Article

TLR7 Modulated T Cell Response in the Mesenteric Lymph Node of *Schistosoma japonicum*-Infected C57BL/6 Mice

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Toll-like receptors (TLRs) play an important role in regulating immune responses during pathogen infection. However, roles of TLRs on T cells reside in the mesenteric lymph node (MLN) were not be fully elucidated in the course of *S. japonicum* infection. In this study, T lymphocytes from the mesenteric lymph node (MLN) of *S. japonicum*-infected mice were isolated and the expression and roles of TLR2, TLR3, TLR4, and TLR7 on both $CD4^+$ and $CD8^+$ T cells were compared. We found that the expression of TLR7 was increased in the MLN cells of *S. japonicum*-infected mice, particularly in $CD4^+$ and $CD8^+$ T cells ($P < 0.05$). R848, a TLR7 agonist, could enhance the production of IFN-γ from MLN T cells of infected mice ($P < 0.05$), especially in $CD8^+$ T cells ($P < 0.01$). In TLR7 gene knockedout (KO) mice, the *S. japonicum* infection caused a significant decrease ($P < 0.05$) of the expression of CD25 and CD69, as well as the production of IFN-γ and IL-4 inducted by PMA plus ionomycin on both $CD4^+$ and $CD8^+$ T cells. Furthermore, the decreased level of IFN-γ and IL-4 in the supernatants of SEA- or SWA-stimulated mesenteric lymphocytes was detected ($P < 0.05$). Our results indicated that *S. japonicum* infection could induce the TLR7 expression on T cells in the MLN of C57BL/6 mice, and TLR7 mediates T cell response in the early phase of infection.

1. Introduction

Schistosomiasis is a chronic, parasitic disease caused by blood flukes with significant morbidity and mortality, especially in vertebrates, including humans [1]. Immunopathological studies have shown that schistosomiasis results predominantly from the evoked host immune response to schistosome eggs and the granulomatous reaction [2]. After infection, schistosomula and its eggs migrate through a variety of tissues, such as the skin, lung [3], liver [4, 5], and intestinal and vesical mucosa [6]. Schistosoma eggs must migrate from the mesenteric vessels, across the intestinal wall and into the feces. A vast proportion of eggs fail to leave their definite host, instead becoming lodged within intestinal or hepatic tissue, where they could evoke potentially life-threatening pathology [7].

The mesenteric lymph node (MLN) is the main draining lymph node in mouse enterocoelia which contains many types of immune cells [8]. MLN has been associated with initiation of immunological responses to bacterial translocation

and inflammatory bowel diseases (IBDs) [9]. Moreover, it was reported that MLN $CD4^+$ T lymphocytes could migrate to liver and contribute to nonalcoholic fatty liver disease [10]. Our previous study have found that *S. japonicum* infection could stimulate the responses of multiple immune cells, including Th cells, NK cells, NKT cells, and $\gamma\delta$T cells in the B6 mouse MLN [11, 12].

$CD4^+$ Th cells could modulate the immune response by secreting many kinds of cytokines. According to the different cytokine production profiles, divide into different subtypes, such as Th1, Th2, Th9, and Th17 [13]. It was reported that $CD4^+$ Th2 cell is the main effector T cell response to *S. japonicum* infection by producing produce IL-4, IL-5, and IL-13 [14]. IL-17-secreting Th17 cell was reported playing an important roles in *S. japonicum* infection inducing liver granuloma damage [4, 5]. Th9 cells could influence the progress of *S. japonicum* infection-induced liver damage, too [15]. IFN-γ and IL-4 were classic Th1 and Th2 cytokines, respectively. IFN-γ could mediate cellular immune response, including the activity of $CD8^+$ cytotoxin T cell and macrophages. On the contrary, IL-4 is the most important cytokine in induced B cell activation and antibody production. IFN-γ and IL-4 were the most important cytokines secreting by Th cell which influence the progress *S. japonicum* infection-induced disease [16].

TLRs are the best characterized class of pattern recognition receptors (PRRs) that prevent pathogen invasion by recognizing pathogen-associated molecular patterns (PAMPs), which are highly conserved components derived from bacteria, viruses, fungi, and parasites [17]. Studies show that TLRs are the most important sensors to parasite components during *Schistosoma mansoni* infection [18, 19]. Although TLRs are predominantly expressed in innate immune cells, such as dendritic cells, macrophages, and natural killer (NK) cells [20]. TLRs have also been detected in T cells [21] and were found to be able to modulate the function of T lymphocytes [22]. For example, the report by Lee et al. indicated that TLR2 was constitutively expressed on *Listeria*-specific memory $CD8^+$ T cells [23]. In addition, Caron et al. reported that effector memory T cells exhibit an enhanced response to TLR activation and are more sensitive to TLR-mediated activation than naive $CD4^+$ T cells [24].

Among all the TLRs identified, TLR7 is an intracellular member of the innate immune receptor that recognizes intracellular single-stranded and double-stranded RNA [25]. It was reported that TLR7 was involved in the progress of autoimmune disease [26], graft-versus-host disease [27], and infectious diseases [28]. For example, TLR7 could be detected on different $CD4^+$ and $CD8^+$ T cell subpopulations from blood of hepatitis C virus infected patients by flow cytometry [29]. Resiquimod (R848), a TLR7 and TLR8 agonist, could not only induce immune response as an adjuvant [30], but also blocks virus replication by inducing the antiviral protein viperin [31]. To date, however, the exact role of the TLR7 in *S. japonicum* infection remains elusive. In this study, we utilized both *in vivo* TLR7 gene knockedout (KO) mice and *in vitro* schistosome worm (SWA)- and egg (SEA)-stimulated mesenteric lymphocytes to investigate the roles of TLR7 on T cells residue in the mesenteric lymph node (MLN) in the course of *S. japonicum* infection.

2. Materials and Methods

2.1. Ethics Statement. Animal experiments were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (1988.11.1). All protocols for animal use were approved to be appropriate and humane by the Institutional Animal Care and Use Committee of Guangzhou Medical University (2012-11).

2.2. Mice, Parasites, and Infection. Sixty female C57BL/6 mice, 6 to 8 weeks old, weighted 20-25 g, were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China), and TLR7 KO mice were purchased from the Jackson Laboratory (B6.129S1-Tlr7^{tm1Fl}v/J, strains: 008380). All mice were maintained in a specific pathogen-free microenvironment (SPF) at the Laboratory Animal Centre, Guangzhou Medical University. Mice were fed with standard diet, allowed ad libitum access to food and water and taken care of on a 12 h light-dark cycle. *S. japonicum* cercariae were shed from naturally infected *Oncomelania hupensis* snails, which were purchased from Jiangsu Institute of Parasitic Disease (Wuxi, China). There are 3 groups of mice in this study. 40 C57BL/6 mice were divided into normal and infected group randomly, twenty mice per group. 20 C57BL/6 mice in the infected group and 10 TLR7 KO mice (TLR7 KO group) were infected percutaneously with 40 ± 5 cercariae and sacrificed at 6 weeks after infection; 10 uninfectedTLR7 KO mice were served as control, too. The animal experiments were performed in strict accordance with the regulations for the Administration of Affairs Concerning Experimental Animals, and all efforts were made to minimize suffering. The bodies of the mice were frozen in -20°C and sent to the Laboratory Animal Centre of Guangzhou Medical University after the experiment.

2.3. Antibodies. FITC-conjugated anti-mouse CD8 (53-6.7), PerCP-cy5.5-conjugated anti-mouse CD4 (RM4-5), PE-conjugated anti-mouse CD25 (3C7), APC-conjugated anti-mouse CD69 (H1.2F3), APC-conjugated anti-mouse CD3 (145-2C11), APC-cy7-conjugated anti-mouse CD3 (145-2C11), PE-conjugated anti-mouse TLR4 (MTS510), APC-conjugated anti-mouse IFN-γ (XMG1.2), PE-conjugated anti-mouse IL-4 (11B11), and APC-conjugated anti-mouse TLR3 (11F8) were purchased from BD Pharmingen (San Diego, CA, USA). FITC-conjugated anti-mouse TLR2 (T2.5) and FITC-conjugated anti-mouse CD127 (ATR34) were purchased from Bio-Legend (San Diego, CA, USA). Purified anti-mouse CD3 (145-2C11) and anti-mouse CD28 (37.51) were purchased from BD Pharmingen (San Diego, CA, USA).

2.4. SEA and SWA Preparation. SEA and SWA were obtained from the Jiangsu Institute of Parasitic Diseases as previously described [32]. In brief, SEA and SWA were sterile filtered and the endotoxin was removed with polymyxin B agarose beads (Sigma-Aldrich). A Limulus amebocyte lysate assay kit (Lonza, Basel, Switzerland) was used to confirm the removal of the endotoxin from SEA and SWA.

2.5. Isolation of Lymphocytes. At 6 weeks after infection, the mice were sacrificed by cervical dislocation in laboratory, and the mesenteric lymph nodes (MLN) were harvested [11, 12]. A 100 μm cell strainer (BD, CA, USA) was used for preparing the single cell suspensions. The isolated cells were washed twice in Hanks' balanced salt solution, stained by 0.4% trypan blue (Guangzhou chemical reagent factory), and counted under a microscope (the rate of ling cell >98%). The cells were resuspended and adjusted to 2×10^6 cells/ml in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mm glutamine, and 50 μm 2-mercaptoethanol.

2.6. Total RNA Isolation and Quantitative Real-Time PCR (qRT-PCR). 2×10^6 cells from LN of both infected and normal groups were collected. Total RNA was isolated from the MLN cells of infected and normal mice using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. The relative expression of each TLR mRNA was determined by real-time PCR using the ABI Prism 7500 Real-Time PCR System (Life Technologies) with SYBR ® Premix Ex Taq II (Tli RNaseH Plus) (Takara), according to the manufacturer's instructions. The cycle threshold (Ct) numbers were derived from the exponential phase of PCR amplification. The cDNAs were amplified under conditions of initial denaturation at 95°C for 10 minutes, followed by 40 cycles with denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The levels of TLR2, TLR3, TLR4, and TLR7 transcripts were normalized to β-actin transcripts, using the relative quantity $(\mathrm{RQ}) = 2^{-\triangle \mathrm{Ct}}$ method.

The primers were synthesized from Invitrogen (Shanghai, China) as follows: for β-actin, 5-CCGTAAAGA CCTCTATGCCAAC-3 (forward) and 5-GGGTGTAAAAC GCAGCTCAGTA-3 (reverse); for TLR2, 5-AAGATGTCG TTCAAGGAGGTGCG-3 (forward) and 5-ATCCTCTGA GATTTGACGCTTTG-3 (reverse); for TLR3, 5-CCTCTT CATAATCAGCACCAG-3 (forward) and 5-CCAAGAATC CGATGCACTGA-3 (reverse); for TLR4, 5-ACCTGGAAT GGGAGGACAATC-3 (forward) and 5-AGGTCCAAGTT GCCGTTTCT-3 (reverse); and for TLR7, 5-CCACATTCA CTCTCTTCATTGG-3 (forward) and 5-GGTCAAGAACT TCCAGCCTG-3 (reverse).

2.7. ELISA Detection of Cytokines. Single cell suspensions from the normal, infected, and TLR7 KO group were prepared, respectively. Cells were plated in 96-well plates at 4×10^5 cells/200 μl medium per well and cultured for 72 h at 37°C with 5% CO_2 in the presence or absence of anti-CD3 mAb (1 μg/ml) plus PAMPs (PGN, 10 μg/ml, Poly I:C 25 μg/ml, LPS 1 μg/ml, or R848 2 μg/ml) or not. The supernatants were collected 72 h later and the released cytokines were measured using mouse ELISA kits for IFN-γ (R&D Systems Inc., Minneapolis, MN, USA) and IL-4 (BD Pharmingen, Franklin Lakes, NJ, USA). ELISAs were performed in accordance with the manufacturer's instructions. The optical density of each well was read at 450 nm using a microplate reader (Model ELX-800; BioTek Instruments Inc., Winooski, VT, USA).

2.8. Cell Surface and Intracellular Cytokine Staining (ICS). For cell surface staining, single cell suspensions from the MLN of the normal group, infected group, and TLR KO group were washed twice in PBS contained 0.5% BSA and then stained for 30 min at 4°C in the dark with conjugated antibodies specific for the cell surface antigens CD3, CD4, CD8 CD25, CD69, TLR2, and TLR4. Cells were washed twice in PBS, fixed with 4% paraformaldehyde, and permeabilized overnight at 4°C in PBS buffer containing 0.1% saponin (Sigma), 0.1% BSA, and 0.05% NaN_3. The cells were then stained for 30 min at 4°C in the dark with conjugated antibodies specific for TLR3 and TLR7. Stained cells were washed twice and detected by using flow cytometry (Cytoflex, Beckman Coulter, USA) and data were analyzed by the program CytExpert 1.1 (Beckman Coulter, USA).

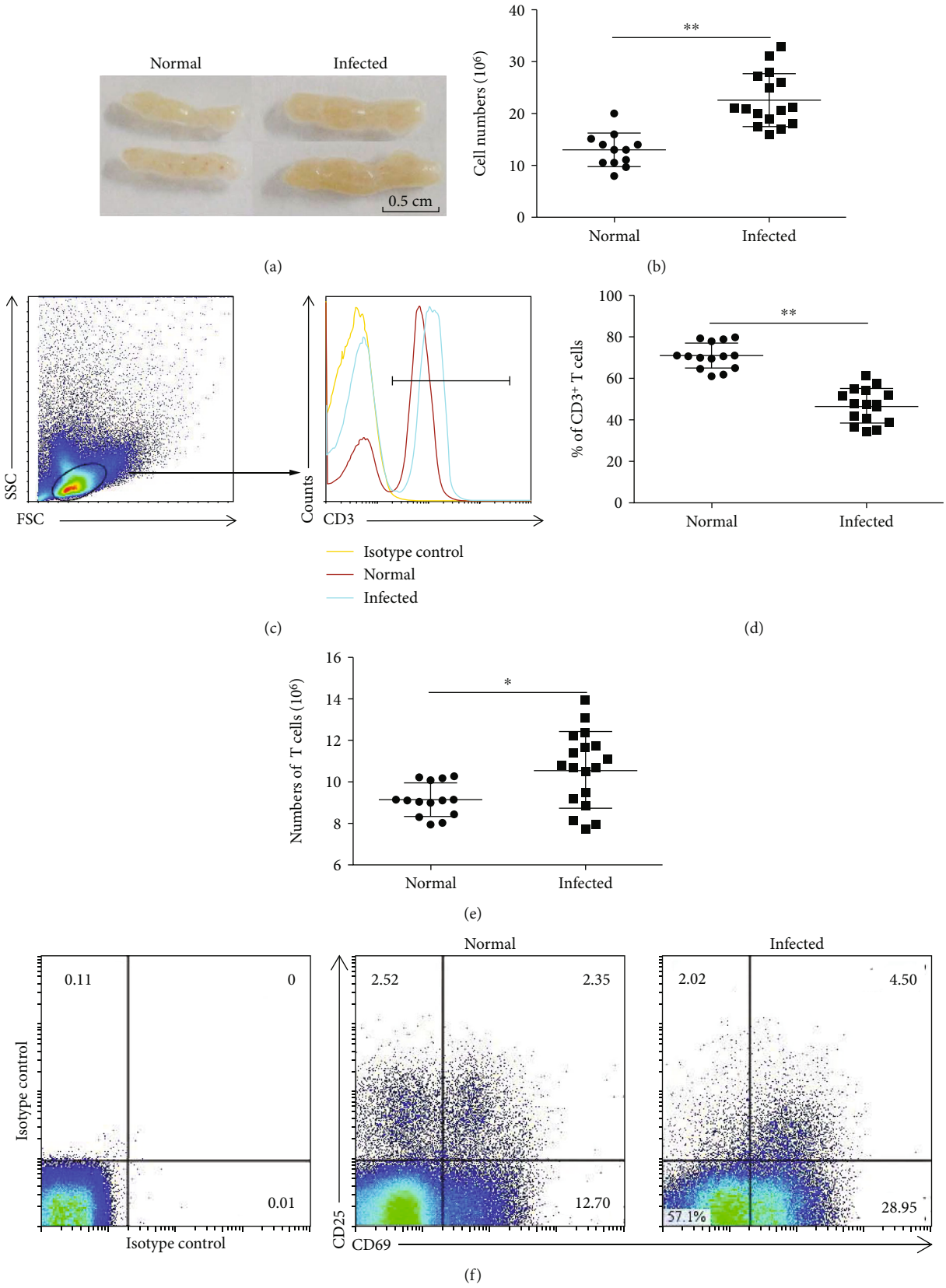
For intracellular cytokine staining, single cell suspensions from the MLN of control mice and mice infected with *S. japonicum* were stimulated with TLR ligands (PGN 10 μg/ml, Poly I:C 25 μg/ml, LPS 1 μg/ml, or R848 2 μg/ml) plus 1 μg/ml anti-CD3 for 5 h at 37°C under a 5% CO_2 atmosphere. Brefeldin A (1 μg/ml, Sigma) was added during the last 4 h of incubation. The cells were washed twice in PBS and stained for 30 min at 4°C in the dark with conjugated antibodies specific for the cell surface antigens CD3, CD4, and CD8. The cells were washed twice in PBS, fixed with 4% paraformaldehyde, and permeabilized overnight at 4°C in PBS buffer containing 0.1% saponin (Sigma), 0.1% BSA, and 0.05% NaN_3. The cells were then stained for 30 min at 4°C in the dark with conjugated antibodies specific for the intracellular IFN-γ and IL-4. Stained cells were washed twice and detected by using flow cytometry (Cytoflex, Beckman Coulter, USA), and data were analyzed by the program CytExpert 1.1 (Beckman Coulter, USA).

2.9. Statistics. Data from each group were analysed using SPSS (v11.0). Statistical evaluation of the difference between means was performed by unpaired, two-tailed Student's t-tests; $P < 0.05$ was considered to be significant.

3. Results

3.1. Accumulation of $CD3^+$ T Cells in Infected Mesenteric Lymph Nodes. Six weeks after infection, the mice were sacrificed, and MLNs were harvested. Compared to the normal group, the infected MLN had significantly increased in size (Figure 1(a)). Single mononuclear cell solutions were prepared and stained by trypan blue; the living cells were counted. The average number of cells in nontreated MLN was $(12.88 \pm 3.26) \times 10^6$. This number significantly increased to $(22.54 \pm 5.90) \times 10^6$ after 6 weeks of infection (Figure 1(b), $P < 0.01$).

To investigate whether MLN T cells were involved in the host response to *S. japonicum* infection, mononuclear cells from normal or infected mouse MLN were stained by fluorescence-labeled anti-CD3 antibody and were detected by FACS (Figure 1(c)). As shown in Figure 1(d), the



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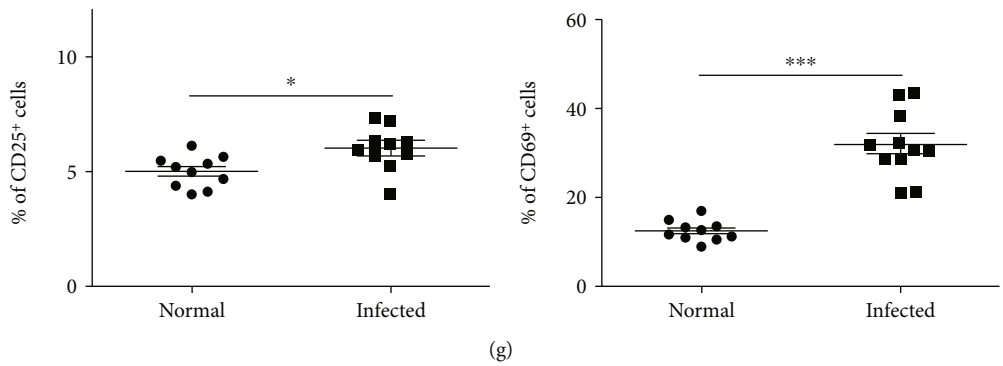


Figure 1: *Schistosoma japonicum* infection promotes $CD3^+$ T cell accumulation in mesenteric lymph nodes. Female C57BL/6 mice were infected with 40 ± 5 S. *japonicum* cercariae per mouse. The mice were sacrificed 6 weeks after infection. The tissues and single cell suspensions were harvested. (a) Representative images of mesenteric lymph nodes. (b) Single mononuclear cell solutions were stained by trypan blue, and the absolute numbers were counted under a microscope (15/20). (c) Flow cytometric analysis of CD3 expression in MLN cells of normal and infected mice is shown. (d) Average percentages of $CD3^+$ T cells were calculated from the FACS analysis (15/20). (e) The absolute number of $CD3^+$ T cells evaluated by flow cytometry after staining with specific antibodies (15/20). (f) Flow cytometric analysis of CD25 and CD69 expression in MLN cells is shown. The numbers represent the expression of cells in each subset. (g) Average percentages of CD25 and CD69 expressions in the $CD3^+$ T cells were calculated from FACS data. Data was from three independent experiments with 5 mice per group and shown as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

percentage of $CD3^+$ T cells in infected mouse MLN was $46.76 \pm 8.43\%$, which was lower compared to normal mice ($70.9 \pm 6.31\%$, $P < 0.01$). However, because of the number of MLN mononuclear cells in response to infection dramatically increased, the absolute number of MLN $CD3^+$ T cells was obviously increased after infection ($P < 0.05$, Figure 1(e)).

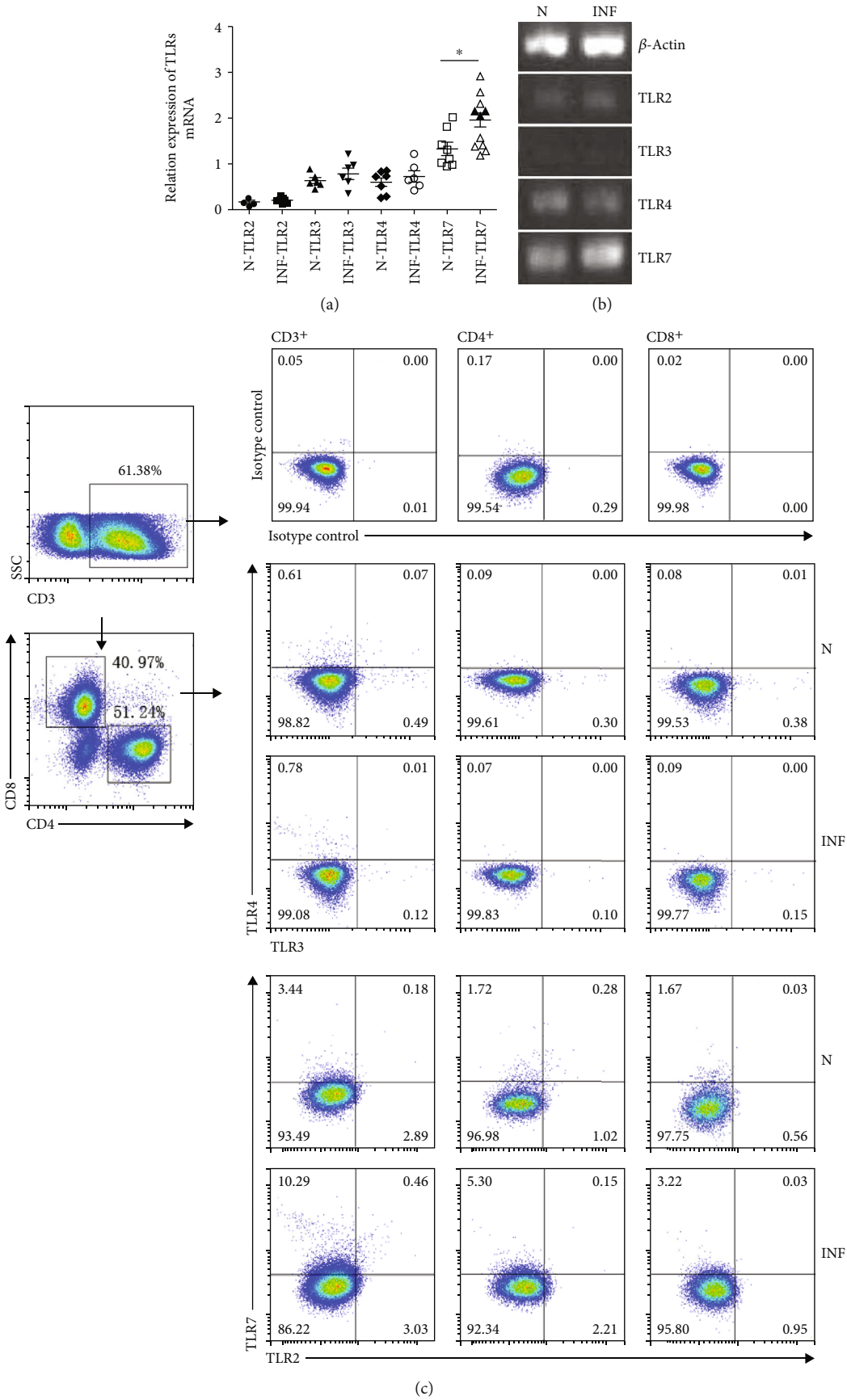
CD25 and CD69 were classic markers for T cell activation [33]. To detect the degree of activation, the expression of CD25 and CD69 on $CD3^+$ T cells was detected by the cell surface staining. As shown in Figure 1(f), the expression of CD69 on the $CD3^+$ cells after infection ($31.87 \pm 9.58\%$) was significantly higher than normal mice ($12.26 \pm 2.54\%$, $P < 0.05$, Figure 1(g)). However, no significant change was found in the population of $CD3^+CD25^+$ T cells ($P > 0.05$, Figure 1(g)).

3.2. Expression of TLRs in S. Japonicum-Infected Mouse MLN. To explore the expression changes of TLRs in the *S. japonicum*-infected mouse *MLN*, we isolated MLN from both normal and infected mice and performed the qRT-PCR. The expression of TLR2, TLR3, TLR4, TLR7, and β-actin genes was detected as described in Materials and Methods. As shown in Figures 2(a) and 2(b), the amount of TLR7 mRNA in infected mice (2.040 ± 0.2062) was higher than that in nontreated mice (1.327 ± 0.1436, $P < 0.05$). Although there were also changes in the expression of TLR2, TLR3, and TLR4, the difference was not statistically significant ($P > 0.05$).

Moreover, the frequency of TLR2, TLR3, TLR4, and TLR7 on $CD3^+$, $CD4^+$, and $CD8^+$ T cells was detected by flow cytometry after staining with specific antibodies as described in Materials and Methods. Results (Figures 2(c) and 2(d)) showed that the percentages of $TLR7^+$ cells in the infected mice were higher than normal on $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes (CD3: $9.9 \pm 0.86\%$ *vs.* $5.25 \pm 0.91\%$; CD4: $6.62 \pm 0.96\%$ *vs.* 3.21 ± 0.43; CD8: $3.12 \pm 0.25\%$ *vs.* $1.65 \pm 0.7\%$, $P < 0.05$). There was no significant difference on frequency of the rest of TLRs between the two groups, except TLR2 on $CD8^+$ T lymphocytes ($1.51 \pm 0.26\%$ *vs.* $0.63 \pm 0.18\%$, $P < 0.05$).

3.3. IFN-γ and IL-4 Induced by TLR Agonists. To explore the roles of TLRs in the function of T cells, the suspensions of single mononuclear cells from the MLN of normal and infected mice were cultured with PGN, Poly I:C, LPS, or R848, respectively, with or without anti-CD3 Ab, and the expression of cytokines was detected by ELISA. As shown in Figure 3(a), R848 showed a strong effect in promoting the production of IFN-γ and IL-4 from infected mouse MLN cells (IFN-γ: 19.59 ± 1.00 pg/ml *vs.* 5.54 ± 0.21 pg/ml, IL-4: 70.39 ± 6.82 pg/ml *vs.* 33.37 ± 5.52 pg/ml, $P < 0.05$). This effect was obvious in the presence of anti-CD3 Ab (IFN-γ: 826.31 ± 54.07 pg/ml *vs.* 365.36 ± 52.31 pg/ml, IL-4: 132.02 ± 32.40 pg/ml *vs.* 65.37 ± 11.71 pg/ml, $P < 0.05$, Figure 3(b). When the cells were stimulated by LPS, PGN, or Poly I:C alone, little IFN-γ and IL-4 were induced (Figure 3(a)). With the stimulation by the CD3 antibody, LPS could induce a higher level of both IFN-γ (167.55 ± 40.94 pg/ml) and IL-4 (165.34 ± 22.23 pg/ml) in infected mouse cells than in the normal control (76.50 ± 16.26 pg/ml; 34.73 ± 6.69 pg/ml, $P < 0.05$, Figure 3(b)). PGN or Poly I:C-stimulated T cells could induce IFN-γ in infected mouse MLN cells, compared to normal control (PGN: 37.38 ± 6.14 pg/ml *vs.* 9.43 ± 0.06 pg/ml; Poly I:C: 32.98 ± 2.12 pg/ml *vs.* 21.30 ± 3.09 pg/ml, $P < 0.05$, Figure 3(a)). The same trend was observed in IL-4 production (PGN: 68.43 ± 5.44 pg/ml *vs.* 33.00 ± 9.90 pg/ml; Poly I:C: 57.72 ± 6.02 pg/ml *vs.* 33.03 ± 0.73 pg/ml, $P < 0.05$, Figure 3(b)).

Furthermore, MLN lymphocytes isolated from normal and infected C57BL/6 mice were stimulated by anti-CD3 Ab plus TLR agonists for 5 hours, and the IFN-γ and IL-4 expression on $CD4^+$ T cells or $CD8^+$ T cells was detected by FACS (Figure 3(c)). As shown in Figure 3(d), the percentage of IL-4^+CD4^+ T cells and IFN-γ^+CD8^+ T cells in infected



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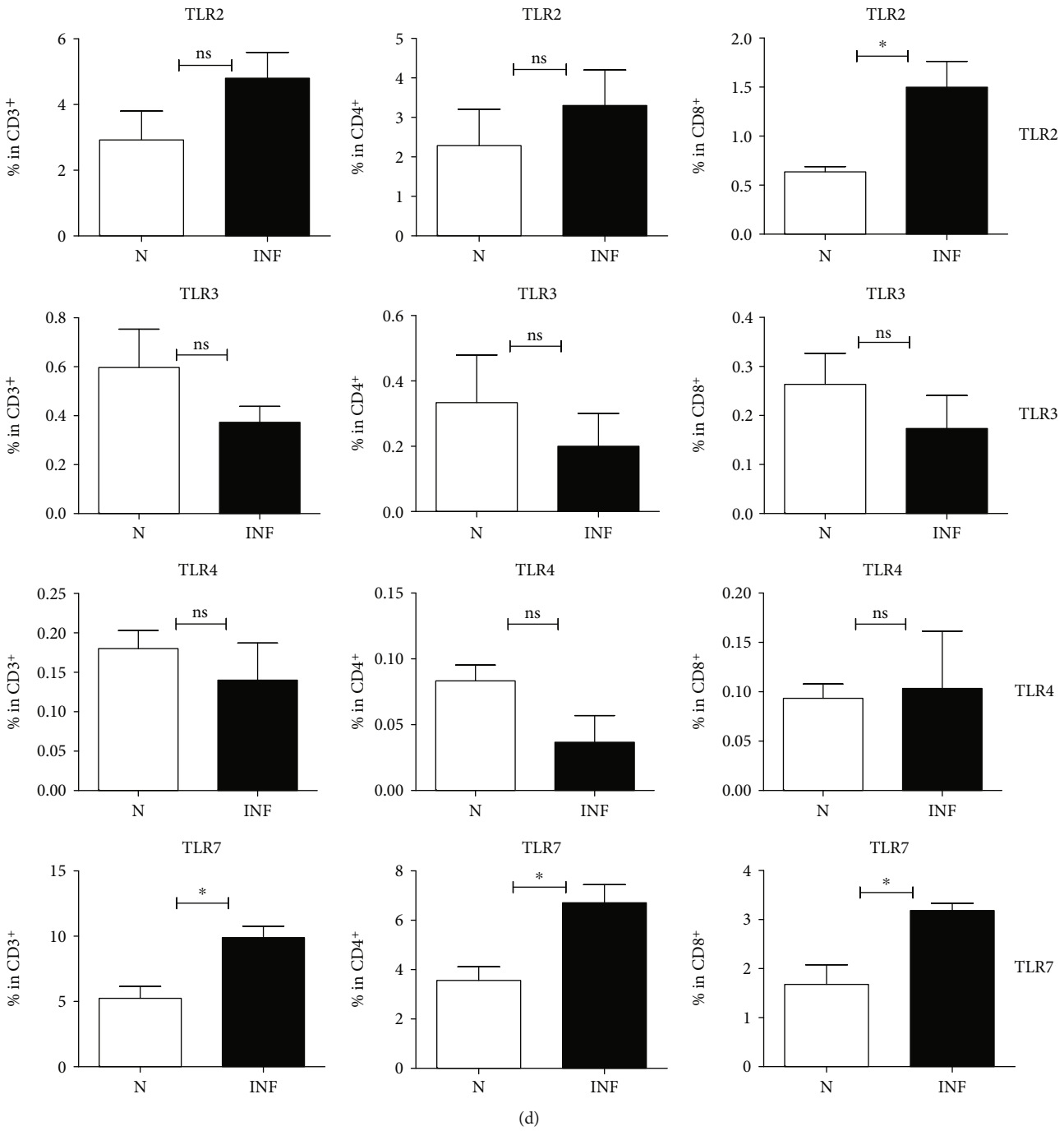
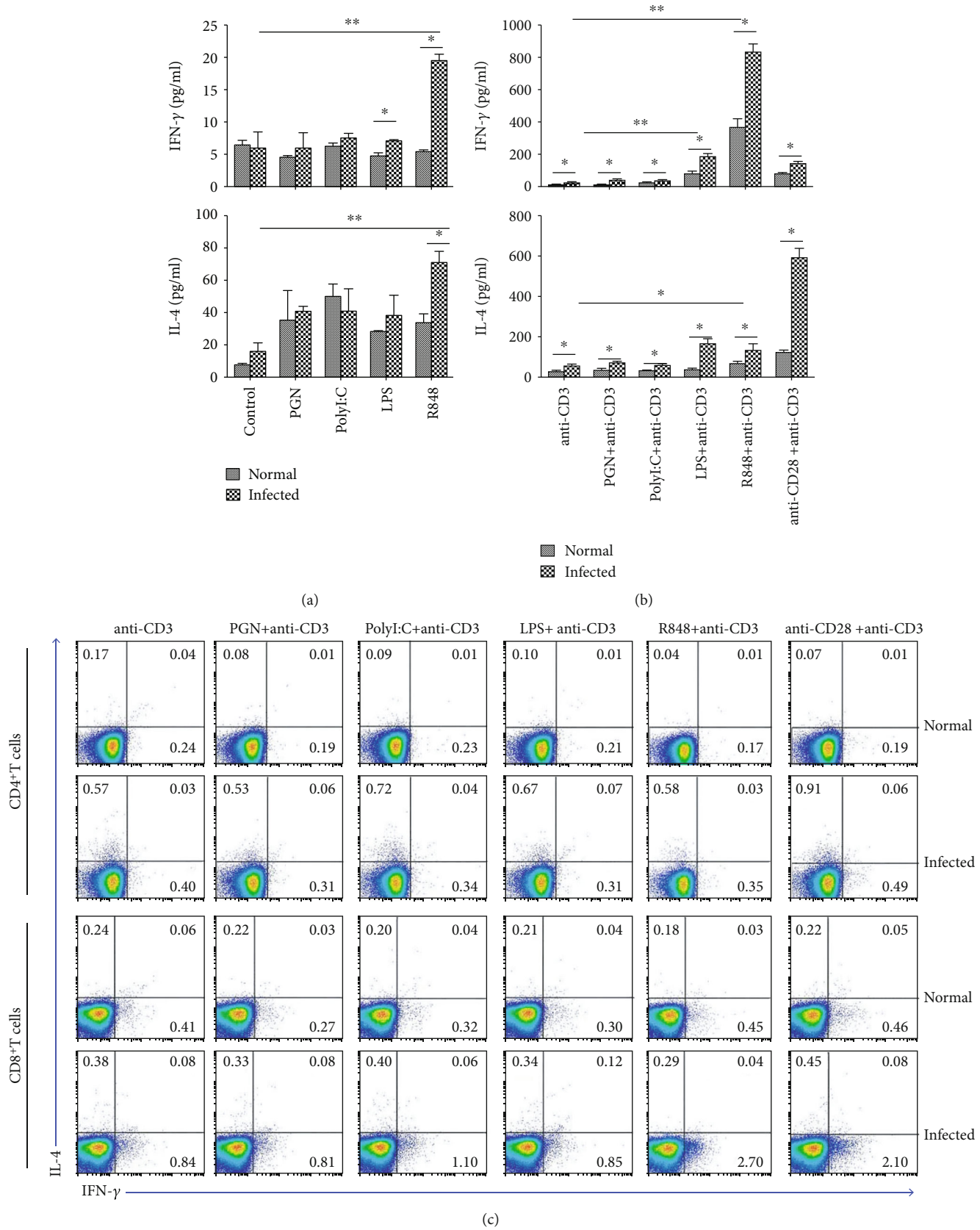


Figure 2: Expression of TLRs in the MLN of *S. japonicum*-infected mouse. Six weeks after *S. japonicum* infection, the mice were sacrificed. Single mononuclear cell suspensions from normal and infected mice were prepared as described in Materials and Methods. Total RNA was collected and purified, and cDNA was synthesized. (a, b) The relative mRNA expression of TLRs and β-actin genes was detected. Data was from three independent experiments with 5 mice per group and shown as the mean ± SEM. $^{*}P < 0.05$, $^{ns}P > 0.05$. (c, d) Single cell suspensions of MLN cells were prepared, and the expression of TLR2, TLR3, TLR4, and TLR7 on $CD4^{+}$ or $CD8^{+}$ T cells was detected by flow cytometry after staining with specific antibodies. (c) The numbers represent the expression of cells in each subset. (d) Average percentages of TLR2, TLR3, TLR4, and TLR7 on $CD4^{+}$ or $CD8^{+}$ T cells were calculated from FACS data. Three independent experiments (5–6 mice per group) were performed, and one representative result is shown. $^{*}P < 0.05$, $^{ns}P > 0.05$.

mice was higher than that in normal mice, significantly ($P < 0.05$). After infection, $CD4^{+}$ T cells displayed an increased capacity in producing IFN-γ also. The expression of IL-4 in $CD8^{+}$ T cells from the infected MLNs was slightly increased compared with the normal MLNs. However, the difference was not significant ($P > 0.05$, Figure 3(e)).



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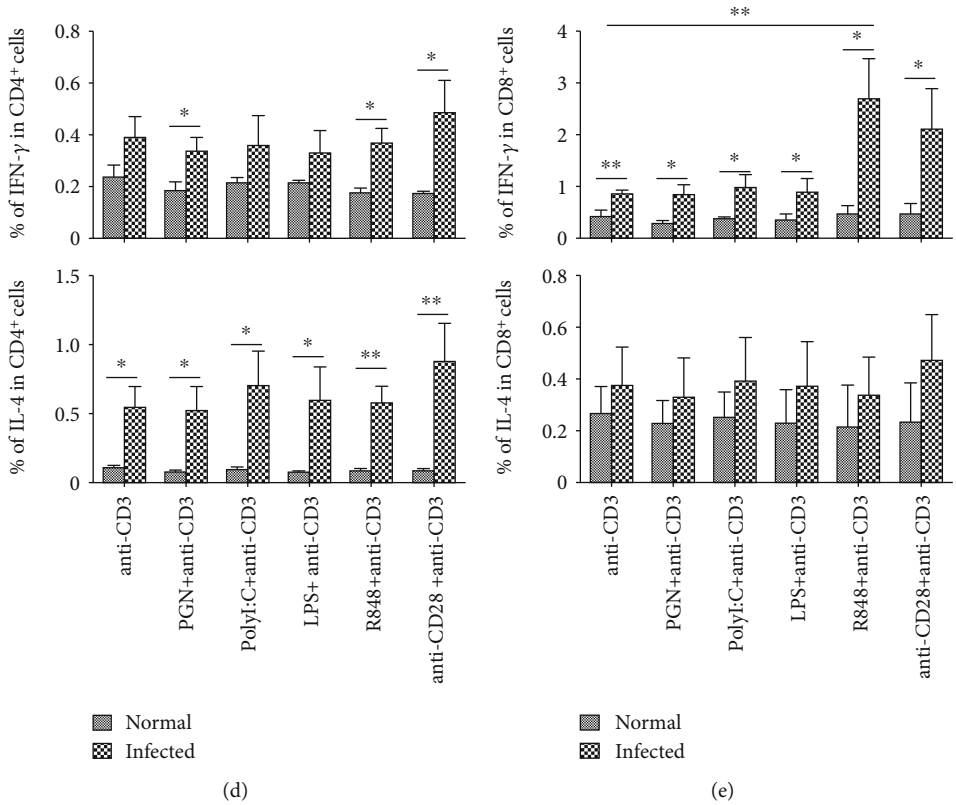


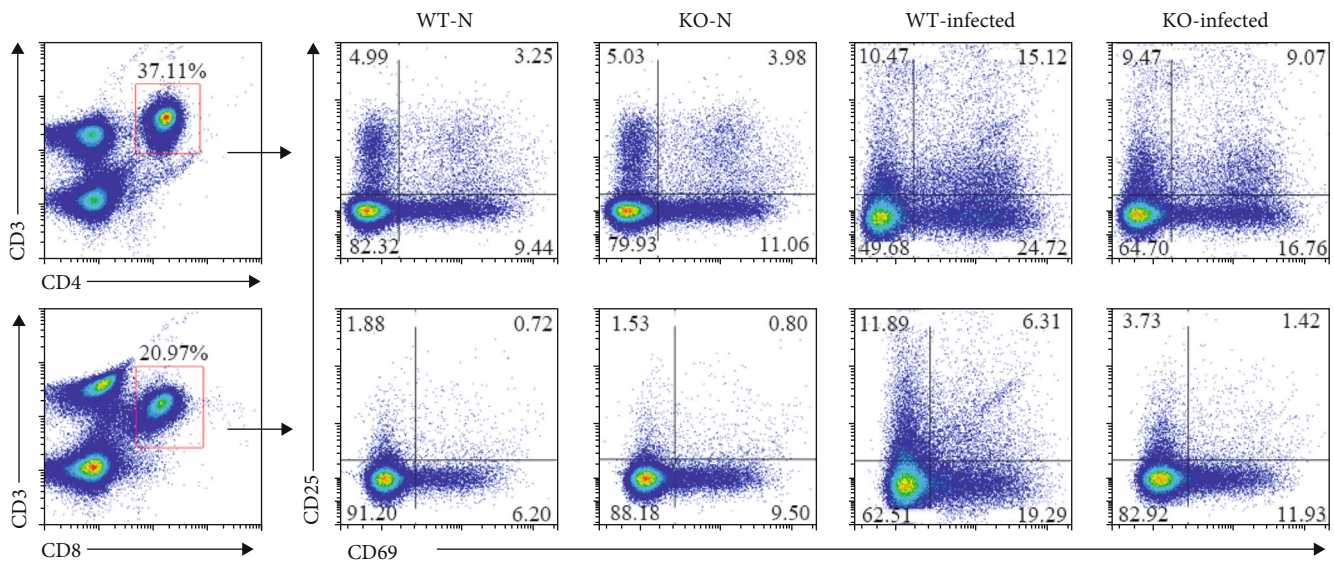
Figure 3: Role of TLR agonists in inducing IFN-γ and IL-4. Single mononuclear MLN cell suspensions of normal and infected mouse were prepared and cultured in vitro with PGN, PolyI:C, LPS, and R848, with or without anti-CD3 Ab. 72 h later, the concentration of IFN-γ (a) and IL-4 (b) in the supernatants of cultured cells was detected by ELISA. The MLN lymphocytes isolated from normal and infected mice were stimulated by PGN, Poly I:C, LPS, and R848, with anti-CD3 Ab. The expression of INF-γ and IL-4 on $CD4^+$ or $CD8^+$ T cells in normal and infected mouse was detected by flow cytometry. (c) The numbers represent the expression of cells in each subset. The average percentages of INF-γ and IL-4 in $CD4^+$ (d) or $CD8^+$ (e) T cells were calculated from the FACS analysis. Three independent experiments (5–6 mice per group) were performed, and one representative result is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{ns}P > 0.05$.

When compared to cells stimulated by anti-CD3 Ab alone, R848 induced a significant increase in the percentage of IFN-γ^+CD8^+ T cells (Figure 3(e), $P < 0.05$).

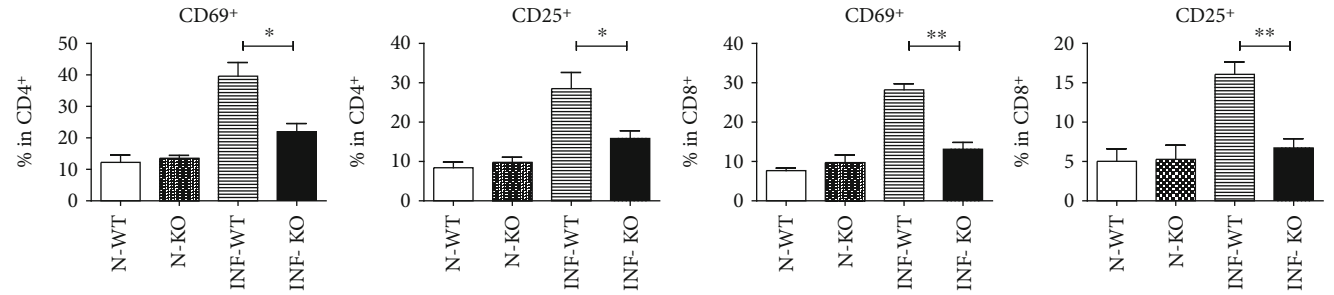
3.4. Phenotypic and Functional Changes of $CD4^+$ and $CD8^+$ T Cells from MLN of S. Japonicum-Infected TLR7 KO Mice. T lymphocytes were isolated from wild-type normal (WT-N), TLR7 knockout normal (TLR7-N), wild-type infected (WT-INF), and TLR7 knockout infected (TLR7-Inf) mice separately. The single cell solutions were prepared. The expression of CD25 and CD69 on both $CD4^+$ and $CD8^+$ T cells was detected by the means of cell surface staining as shown in Figure 4(a). The expressions of CD25 (CD4: WT-INF: 28.53 ± 4.08%, TLR7-INF: 15.85 ± 1.97%, $P < 0.05$; CD8: WT-INF: 16.07 ± 1.57%, TLR7-INF: 6.72 ± 1.17%, $P < 0.01$) and CD69 (CD4: WT-INF: 39.61 ± 4.33%, TLR7-INF: 21.94 ± 2.6%, $P < 0.05$; CD8: WT-INF: 28.2 ± 1.562%, TLR7-INF: 13.12 ± 1.74%, $P < 0.01$) from infected TLR7 KO mice were much lower than the WT-INF group on both $CD4^+$ and $CD8^+$ T lymphocytes (Figure 4(b)). It is suggesting the knockout of TLR7 influenced activation of T lymphocytes.

In the same time, cells were stimulated by PMA plus ionomycin; the expression of IFN-γ and IL-4 on both $CD4^+$ and $CD8^+$ T cells was detected by the means of intracellular cytokine staining as showed in Figure 4(c). Production of IFN-γ secreted by CD4 and CD8 T lymphocytes from wild-type infected mice were 3.4 ± 0.6% and 18.57 ± 1.45%, which were much higher than TLR7-INF (CD4: 1.27 ± 0.15%; CD8: 8.38 ± 1.72%, $P < 0.05$). Secretion of IL-4 of T lymphocytes from the WT-INF group (CD4: 8.01 ± 1.23%; CD8: 2.1 ± 0.23%) was also higher than TLR7-INF (CD4: 1.43 ± 0.48%; CD8: 0.94 ± 0.14%, $P < 0.05$) after infection (Figure 4(d)).

Moreover, lymphocytes from MLN were cultured with stimulation of SEA, SWA, or CD3, respectively, with CD28 for 72 hours. The concentration of IFN-γ (Figure 4(e)) and IL-4 (Figure 4(f)) in the supernatant of cultured cells was detected by ELISA. Results are shown in Figure 4(c); the production of IFN-γ (SEA: WT-INF: 61.78 ± 15.86 pg/ml, TLR7-INF: 21.8 ± 3.42 pg/ml, $P < 0.05$; SWA: WT-INF: 44.42 ± 3.33 pg/ml, TLR7-INF: 32.02 ± 2.35 pg/ml, $P < 0.05$) and IL-4 (SEA: WT-INF: 148.8 ± 19.41 pg/ml, TLR7-INF: 73.49 ± 14.27 pg/ml, $P < 0.05$; SWA: WT-INF: 98.92 ± 19.17 pg/ml, TLR7-INF: 42.09 ± 9.35 pg/ml, $P < 0.05$) from T lymphocytes of infected TLR7 knockout mice stimulated with SEA and SWA was less than the infected wild-type mice.



(a)



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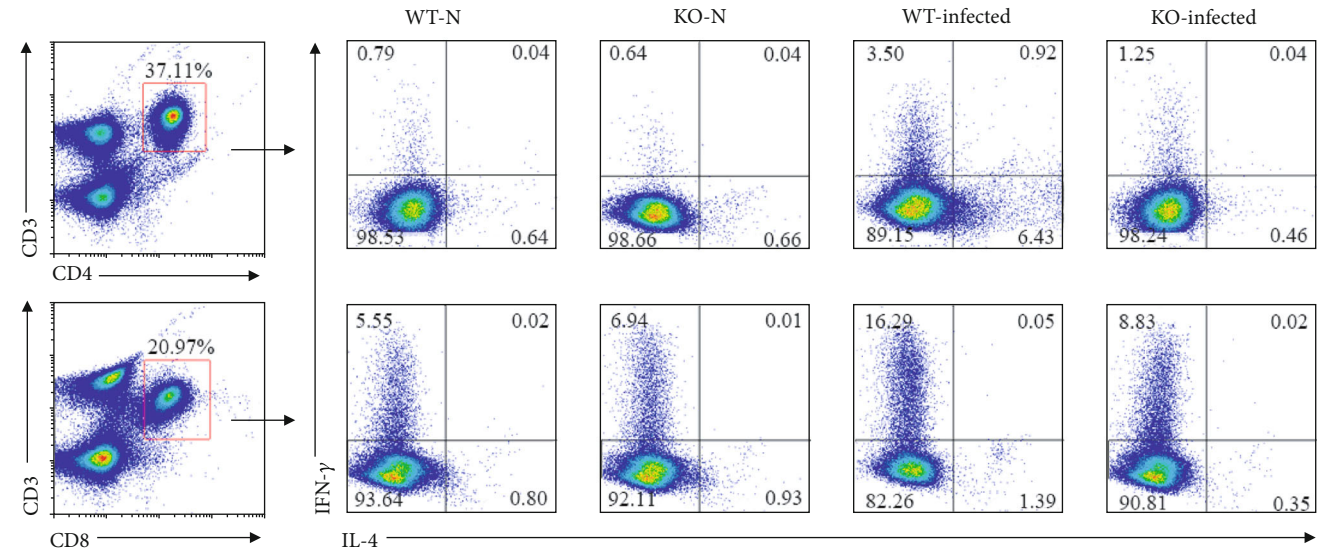

(c)

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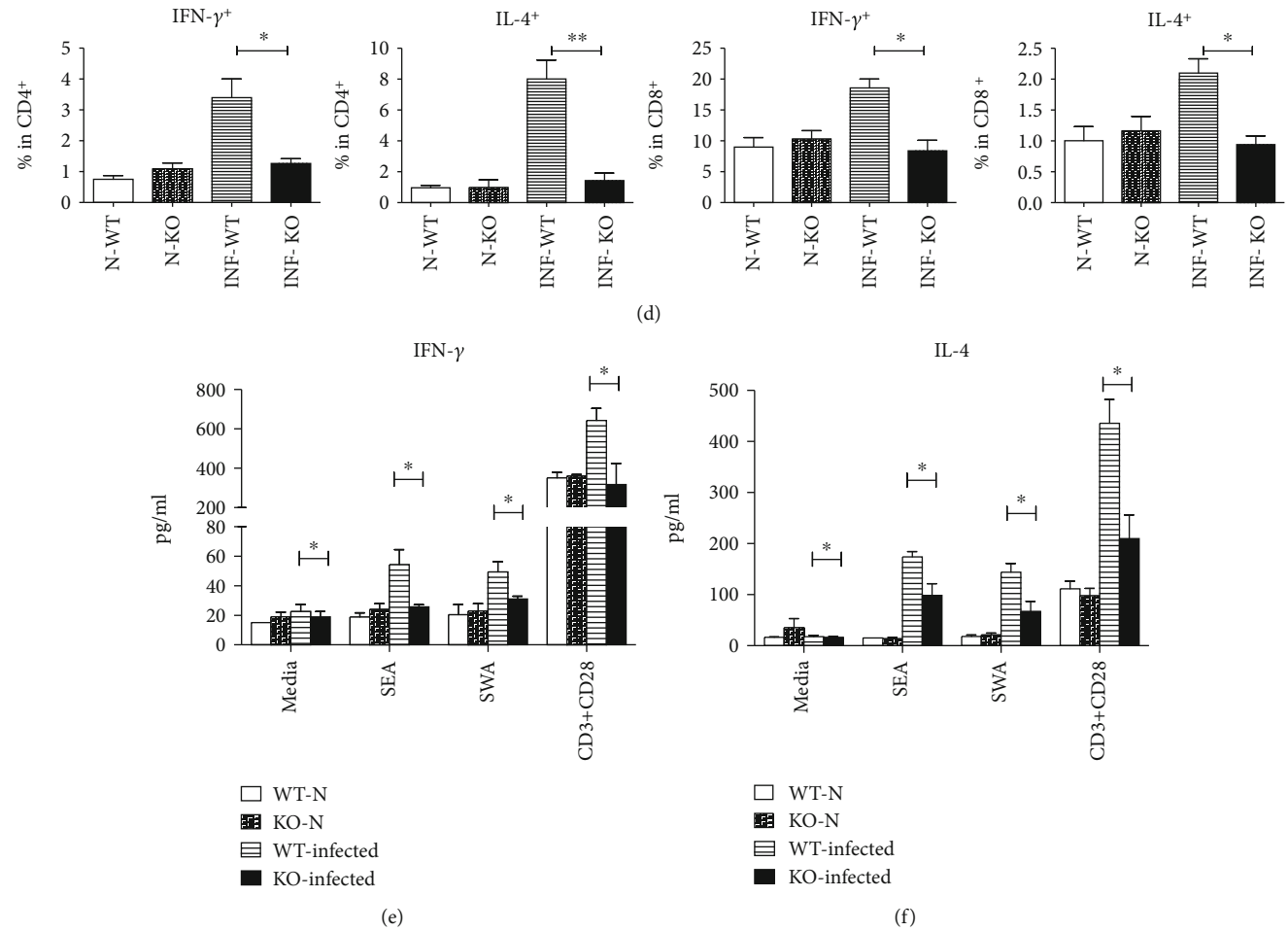

Figure 4: Change in activation and function of T lymphocytes of MLN after TLR7 knockout. T lymphocytes were isolated from wild-type normal (WT-N), TLR7 knockout normal (KO-N), wild-type infected (WT-infected), and TLR7 knockout infected (KO-infected) mice separately. The single cell solutions were prepared. (a, b) The expression of CD25 and CD69 on both $CD4^+$ and $CD8^+$ T cells was detected by the means of cell surface staining. (c, d) Cells were stimulated by PMA plus ionomycin; the expression of IFN-γ and IL-4 was detected by the means of intracellular cytokines staining as described in Materials and Methods. (e, f) Cells were cultured with plus SEA, SWA, and CD3, respectively, with CD28 for 72 hours. The concentration of IFN-γ and IL-4 was detected by the means of ELISA. Three independent experiments (5–6 mice per group) were performed, and one representative result is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{ns}P > 0.05$.

4. Discussion

In *S. japonicum* infection, T cells, stimulated mainly by the soluble adult worm antigens and soluble egg antigens of *S. japonicum*, were believed to play an important role in the infection-induced pathogenic immune response [11, 34]. In this study, the bigger size of MLN, which contained a greater number of $CD3^+$ T cells, was found in the *S. japonicum*-infected B6 mouse. These results suggested that *S. japonicum* infection could induce strong immune response in intestinal tract. CD25 and CD69 were classic markers for T cell activation [33], though $CD4^+CD25^+foxp3^+$ T cells were served as nature regulatory T cells (Treg) [35], and CD69 was seem to be a marker for tissue resident memory T cells (TRM) [36]. Higher percentages of CD25 and CD69-expressing $CD3^+$ T were found in the infected mouse MLN. It further indicated that $CD3^+$ T lymphocytes might be a component of the immune response during *S. japonicum* infection, as our previous study reported [12].

TLRs are the most well-described PRRs, which promote both innate defense mechanisms and adaptive immune responses to invasive pathogen infection [37]. Previous studies showed that TLR4 might be involved in the protection against *S. japonicum* infection [19], and the absence of TLR7 could influence the immune response against *S. japonicum* infection [19]. In this study, higher expression levels of TLR7 mRNA were found in infected MLN lymphocytes ($P < 0.05$), which suggested that TLR7 might involved in the infected-induced immune response in the lymph nodes. Recently, Applequist and Mac Leod et al. reported the detection of TLRs expression on T cells [21] and the functional roles of TLRs on the modulation of both $CD4^+$ and $CD8^+$ T lymphocytes [22]. Our FACS results showed that the expressions of TLR2 in $CD4^+$ T cells and TLR7 in $CD4^+$

and $CD8^+$ T cells from infected mouse were higher than that in normal T cells ($P < 0.05$), especially the expression of TLR7 ($P < 0.05$). This finding suggested that TLR7 might have important effects directly on T cells in response to *S. japonicum* infection.

The binding of TLRs with their specific ligands could initiate a signaling cascade that results in the secretion of cytokines, which subsequently drives an inflammatory response and activates the adaptive immune system [38]. As shown in Figure 3, significant higher levels of IFN-γ and IL-4 could be induced by R848-stimulated lymphocytes from infected mice. It further confirmed that TLR7 played an important role in *S. japonicum* infection-induced immune response. It implied that many kinds of TLR7-expressing innate immune cells played an important role in this progress. Moreover, the ELISA and FACS results showed that TLR7 could help anti-CD3 antibody inducing IFN-γ releasing and promoting the percentage of IFN-γ^+CD8^+ T cells during *S. japonicum* infection. It implied that *S. japonicum* infection could induce a Th1 immune response and CTL activity through TLR7. Consistent with our results, the levels of Th1 cytokines, TNF-α, and INF-γ in the supernatant of cultured spleen cells from $TLR7^{-/-}$ infected mice were found lower than those of WT mice [19]. Similarly, TLR7 was confirmed to promote Th1 polarization and may thus contribute to the pathogenesis of immune thrombocytopenia [39].

In the same time, we found that in the infected mice, the percentage of IFN-γ and IL-4 producing both $CD4^+$ and $CD8^+$ T cells induced by CD3 plus R848 was similar to that induced by CD3 plus CD28. However, significantly higher level of IFN-γ and lower level of IL-4 were induced by CD3 plus R848 in the supernatant of cultured cells from infected mice ($P < 0.05$). It meant that *S. japonicum* infection induce TLR7-expressing innate cells in the mesenteric lymph nodes apt to induce Th1 response.

Previous study, however, showed *S. japonicum* infection could induce a Th2-dominant immune response in the body [40]. To further evaluate the role of TLR7 in *S. japonicum* infection in the induction of T cell response in MLN, we performed further phenotypic and functional characterization of $CD4^+$ and $CD8^+$ T cells from both TLR7 KO mice and cultured lymphocytes. As showed in Figure 4, results indicated that decreased CD25, CD69, IFN-γ, and IL-4 expressed on $CD4^+$ and $CD8^+$ T cells from MLN of *S. japonicum-infected* TLR7 KO mice ($P < 0.05$). In the same time, ELISA results showed that both SEA- and SWA-specific IFN-γ and IL-4 decreased significantly in culture lymphocytes from MLN of *S. japonicum*-infected TLR7 KO mice ($P < 0.05$). Together, these findings imply that the effect of TLR7 might only play an early or limited effect on T cell responses in the course of *S. japonicum* infection.

In conclusion, this study indicated that *S. japonicum* infection could induce TLR7 expression in both $CD4^+$ and $CD8^+$ T cells of the MLN in C57BL/6 mice, and importantly, the alteration of TLR7 mediates T cell response in the early phase of infection. Further clinic investigations are warranted to define the roles of TLR7 in human host infection of *S. japonicum*.

Abbreviations

TLR:	Toll-like receptor
S. japonicum:	*Schistosoma japonicum*
MLN:	Mesenteric lymph node
RT-PCR:	Real-time PCR
FACS:	Fluorescence-activated cell sorter
KO:	Knockedout
PGN:	Peptidoglycan
Poly I:C:	Polyinosinic-polycytidylic acid
LPS:	Lipopolysaccharide
R848:	Resiquimod
CD:	Cluster of differentiation
ELISA:	Enzyme-linked immunosorbent assay
ICS:	Intracellular cytokine staining
Ab:	Antibody
SEA:	Soluble egg antigen
SWA:	Soluble worm antigen.

Data Availability

The datasets used in the current study are available from the corresponding authors on reasonable request.

Ethical Approval

All protocols for animal use were approved to be appropriate and humane by the institutional animal care and use committee of Guangzhou Medical University (2012-11). Every effort was made to minimize suffering.

Disclosure

We declare that the partial funder did not participate in the design of the study, data collection, analysis, interpretation, and in the manuscript preparation.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

JH and YZ conceived and designed the experiments. JQ, XY, and CJ performed the experiments. HX and YF analyzed the data. QY, YQ, HQ, and HC provided the data interpretation. JH, JM, and YZ wrote the manuscript. All authors reviewed, revised, and approved the final manuscript. JQ, XY, and CJ share equal first authorship.

Acknowledgments

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References

[1] H. F. Liu, W. Li, M. B. Lu, and L. J. Yu, "Pharmacokinetics and risk evaluation of DNA vaccine against Schistosoma japonicum," *Parasitology Research*, vol. 112, no. 1, pp. 59–67, 2013.

[2] M. S. Wilson, M. M. Mentink-Kane, J. T. Pesce, T. R. Ramalingam, R. Thompson, and T. A. Wynn, "Immunopathology of schistosomiasis," *Immunology and Cell Biology*, vol. 85, no. 2, pp. 148–154, 2007.

[3] D. Chen, H. Xie, X. Luo et al., "Roles of Th17 cells in pulmonary granulomas induced by Schistosoma japonicum in C57BL/6 mice," *Cellular Immunology*, vol. 285, no. 1-2, pp. 149–157, 2013.

[4] D. Chen, X. Luo, H. Xie, Z. Gao, H. Fang, and J. Huang, "Characteristics of IL-17 induction by *Schistosoma japonicum* infection in C57BL/6 mouse liver," *Immunology*, vol. 139, no. 4, pp. 523–532, 2013.

[5] H. Xie, D. Chen, X. Luo, Z. Gao, H. Fang, and J. Huang, "Some characteristics of IL-5-producing T cells in mouse liver induced by *Schistosoma japonicum* infection," *Parasitology Research*, vol. 112, no. 5, pp. 1945–1951, 2013.

[6] E. J. Pearce and A. S. MacDonald, "The immunobiology of schistosomiasis," *Nature Reviews Immunology*, vol. 2, no. 7, pp. 499–511, 2002.

[7] A. H. Costain, A. S. MacDonald, and H. H. Smits, "Schistosome egg migration: mechanisms, pathogenesis and host immune responses," *Frontiers in Immunology*, vol. 9, 2018.

[8] Y. Chung, W. S. Chang, S. Kim, and C. Y. Kang, "NKT cell ligand alpha-galactosylceramide blocks the induction of oral tolerance by triggering dendritic cell maturation," *European Journal of Immunology*, vol. 34, no. 9, pp. 2471–2479, 2004.

[9] M. G. Kiernan, J. C. Coffey, K. McDermott et al., "The human mesenteric lymph node microbiome differentiates between Crohn's disease and ulcerative colitis," *Journal of Crohn's & Colitis*, vol. 13, no. 1, pp. 58–66, 2019.

[10] L. Su, Z. Wu, Y. Chi et al., "Mesenteric lymph node $CD4^+$ T lymphocytes migrate to liver and contribute to non-alcoholic fatty liver disease," *Cellular Immunology*, vol. 337, pp. 33–41, 2019.

[11] X. Luo, H. Xie, D. Chen et al., "Changes in NK and NKT cells in mesenteric lymph nodes after a Schistosoma japonicum infection," *Parasitology Research*, vol. 113, no. 3, pp. 1001–1009, 2014.

[12] X. Yu, X. Luo, H. Xie et al., "Characteristics of $\gamma\delta$ T cells in *Schistosoma japonicum*-infected mouse mesenteric lymph nodes," *Parasitology Research*, vol. 113, no. 9, pp. 3393–3401, 2014.

[13] T. T. Loo, Y. Gao, and V. Lazarevic, "Transcriptional regulation of $CD4^+$ T_H cells that mediate tissue inflammation," *Journal of Leukocyte Biology*, vol. 104, no. 6, pp. 1069–1085, 2018.

[14] A. Farwa, C. He, L. Xia, and H. Zhou, "Immune modulation of Th1, Th2, and T-reg transcriptional factors differing from cytokine levels in Schistosoma japonicum infection," *Parasitology Research*, vol. 117, no. 1, pp. 115–126, 2018.

[15] L. Li, H. Xie, M. Wang et al., "Characteristics of IL-9 induced by *Schistosoma japonicum* infection in C57BL/6 mouse liver," *Scientific Reports*, vol. 7, no. 1, article 2343, 2017.

[16] D. Chen, H. Xie, H. Cha et al., "Characteristics of Schistosoma japonicum infection induced IFN-γ and IL-4 co-expressing plasticity Th cells," *Immunology*, vol. 149, no. 1, pp. 25–34, 2016.

[17] L. A. O'Neill, D. Golenbock, and A. G. Bowie, "The history of Toll-like receptors - redefining innate immunity," *Nature Reviews. Immunology*, vol. 13, no. 6, pp. 453–460, 2013.

[18] D. van der Kleij, E. Latz, J. F. H. M. Brouwers et al., "A novel host-parasite lipid cross-talk: Schistosomal lysophosphatidylserine activates toll-like receptor 2 and affects immune polarization," *The Journal of Biological Chemistry*, vol. 277, no. 50, pp. 48122–48129, 2002.

[19] Y. Jiang, Y. X. Xu, Z. Y. Yuan et al., "Effect of Toll-like receptor (TLR) 7 deficiencies on the in vivo immune response against Schistosoma japonicum," *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, vol. 32, no. 3, pp. 172–175, 2014.

[20] M. Dorner, S. Brandt, M. Tinguely et al., "Plasma cell Toll-like receptor (TLR) expression differs from that of B cells, and plasma cell TLR triggering enhances immunoglobulin production," *Immunology*, vol. 128, no. 4, pp. 573–579, 2009.

[21] S. E. Applequist, R. P. Wallin, and H. G. Ljunggren, "Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines," *International Immunology*, vol. 14, no. 9, pp. 1065–1074, 2002.

[22] H. MacLeod and L. M. Wetzler, "T cell activation by TLRs: a role for TLRs in the adaptive immune response," *Science's STKE*, vol. 2007, no. 402, article pe48, 2007.

[23] S. M. Lee, Y. D. Joo, and S. K. Seo, "Expression and function of TLR2 on CD4 versus CD8 T cells," *Immune Network*, vol. 9, no. 4, pp. 127–132, 2009.

[24] G. Caron, D. Duluc, I. Fremaux et al., "Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-γ production by memory $CD4^+$ T cells," *Journal of Immunology*, vol. 175, no. 3, pp. 1551–1557, 2005.

[25] S. Pieters, D. McGowan, F. Herschke et al., "Discovery of selective 2,4-diaminoquinazoline Toll-like receptor 7 (TLR 7) agonists," *Bioorganic & Medicinal Chemistry Letters*, vol. 28, no. 4, pp. 711–719, 2018.

[26] M. G. Hasham, N. Baxan, D. J. Stuckey et al., "Systemic autoimmunity induced by the TLR7/8 agonist Resiquimod causes myocarditis and dilated cardiomyopathy in a new mouse model of autoimmune heart disease," *Disease Models & Mechanisms*, vol. 10, no. 3, pp. 259–270, 2017.

[27] M. Gaignage, R. G. Marillier, P. M. Cochez et al., "The TLR7 ligand R848 prevents mouse graft-*versus*-host disease and cooperates with anti-interleukin-27 antibody for maximal protection and regulatory T-cell upregulation," *Haematologica*, vol. 104, no. 2, pp. 392–402, 2019.

[28] Y. Zhu, Y. Shao, X. Qu et al., "Sodium ferulate protects against influenza virus infection by activation of the TLR7/9-MyD88-IRF7 signaling pathway and inhibition of the NF-κB signaling pathway," *Biochemical and Biophysical Research Communications*, vol. 512, no. 4, pp. 793–798, 2019.

[29] T. Hammond, S. Lee, M. W. Watson et al., "Toll-like receptor (TLR) expression on $CD4^+$ and $CD8^+$ T-cells in patients chronically infected with hepatitis C virus," *Cellular Immunology*, vol. 264, no. 2, pp. 150–155, 2010.

[30] S. van Aalst, M. Jansen, I. S. Ludwig, R. van der Zee, W. van Eden, and F. Broere, "Routing dependent immune responses after experimental R848-adjuvated vaccination," *Vaccine*, vol. 36, no. 11, pp. 1405–1413, 2018.

[31] B. Vanwalscappel, T. Tada, and N. R. Landau, "Toll-like receptor agonist R848 blocks Zika virus replication by inducing the

antiviral protein viperin," *Virology*, vol. 522, pp. 199–208, 2018.

[32] Y. Duan, X. Gu, D. Zhu et al., "Schistosoma japonicum soluble egg antigens induce apoptosis and inhibit activation of hepatic stellate cells: a possible molecular mechanism," *International Journal for Parasitology*, vol. 44, no. 3-4, pp. 217–224, 2014.

[33] A. K. Holbrook, H. D. Peterson, S. A. Bianchi et al., "$CD4^+$ T cell activation and associated susceptibility to HIV-1 infection in vitro increased following acute resistance exercise in human subjects," *Physiological Reports*, vol. 7, no. 18, article e14234, 2019.

[34] J. D. Turner, G. R. Jenkins, K. G. Hogg et al., "$CD4^+CD25^+$ regulatory cells contribute to the regulation of colonic Th2 granulomatous pathology caused by schistosome infection," *PLoS Neglected Tropical Diseases*, vol. 5, no. 8, article e1269, 2011.

[35] Q. F. Mao, Z. F. Shang-Guan, H. L. Chen, and K. Huang, "Immunoregulatory role of IL-2/STAT5/CD4+CD25+Foxp3 Treg pathway in the pathogenesis of chronic osteomyelitis," *Annals of Translational Medicine*, vol. 7, no. 16, p. 384, 2019.

[36] D. A. Walsh, H. Borges da Silva, L. K. Beura et al., "The functional requirement for CD69 in establishment of resident memory $CD8^+$ T cells varies with tissue location," *Journal of Immunology*, vol. 203, no. 4, pp. 946–955, 2019.

[37] J. K. Dowling and A. Mansell, "Toll-like receptors: the Swiss army knife of immunity and vaccine development," *Clinical & Translational Immunology*, vol. 5, no. 5, article e85, 2016.

[38] S. Akira, K. Takeda, and T. Kaisho, "Toll-like receptors: critical proteins linking innate and acquired immunity," *Nature Immunology*, vol. 2, no. 8, pp. 675–680, 2001.

[39] Q. Yang, B. Wang, H. Yu et al., "TLR7 promotes Th1 polarization in immune thrombocytopenia," *Thrombosis Research*, vol. 128, no. 3, pp. 237–242, 2011.

[40] Y. Zhang, D. Huang, W. Gao et al., "Lack of IL-17 signaling decreases liver fibrosis in murine schistosomiasis japonica," *International Immunology*, vol. 27, no. 7, pp. 317–325, 2015.

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Research Article

Analysis of Toll-Like Receptors in Human Milk: Detection of Membrane-Bound and Soluble Forms

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The bioactive and anti-inflammatory role of human milk components has been recognized; active milk components include soluble forms of Toll-like receptors (TLRs). Preterm babies are more susceptible to infections and may succumb to necrotizing enterocolitis (NEC), a gastrointestinal disease which is exacerbated by an excessive inflammatory response after TLR activation. Here, we investigated the presence of Toll-like receptors TLR1/2/4/6 in colostrum and mature milk of women who delivered before (preterm) or after (term) 37 weeks of gestational age, integrating classical immune-related techniques with proteomic LC-MS/MS analysis. We have detected immunoreactivity for TLRs mostly in preterm samples, even for TLR1 and TLR6, until now not described in human milk. We demonstrated the presence of only TLR2 in the milk fat globule membrane, while the immunoreactivity of TLR1/4/6 was ascribed to crossreaction with some interesting milk proteins sharing leucine-rich repeat domains. These results will provide new insights into the definition of the role of TLRs in intestinal immune regulation of the newborns.

1. Introduction

Milk is the first food of mammals, providing them nutrients but also protection via immunoglobulins and other immune-related molecules. Milk composition is extremely dynamic, changing its content in nutrient and bioactive factors through the lactation stages (colostrum-transition-mature milk) in order to fulfil the growth needs of the newborn [1]. Milk proteins are classified as caseins, whey proteins, and milk fat globule membrane (MFGM) proteins derived from the apical membrane of the milk-producing epithelial cells. The most abundant proteins are contained in whey and casein fractions, while MFGM proteins represent a minor part (2-4%) of the milk total protein content [2]. However, minor proteins include nonnutrient bioactive factors involved in organism development and immune system maturation. The benefits of human breast milk for human infants, in diminishing mortality and protecting against specific infections during the period of breastfeeding, are well documented ([1] and references therein). Anyway, the contribution of human milk molecules to the development of the newborn's innate and adaptive immune function is still a matter of study. Toll-like receptors (TLRs) are transmembrane glycoproteins, involved in the innate immune response, which recognize conserved molecular structures. TLRs are composed of an extracellular domain with leucine-rich repeats (LRRs), a single-path transmembrane domain, and an intracellular domain called TIR (Toll/IL-1 resistance). The ectodomain is involved in the recognition of ligands, which induce the dimerization of the intracellular domain. TLR2 forms heterodimers with TLR1 and TLR6 and recognizes the broadest range of pathogen-associated

molecular patterns (PAMPs) among TLRs, including diacylated and triacylated bacterial lipopeptides and glycolipids such as lipoteichoic acid from Gram-positive bacteria and lipoarabinomannan from mycobacteria. TLR4 requires the association with Myeloid Differentiation Factor 2 (MD-2), a soluble protein that associates with the extracellular domain of TLR4, to recognize the lipopolysaccharides (LPS). TLR activation starts a signaling cascade which leads to nuclear translocation of NF-κB and synthesis of proinflammatory cytokines. Physiologically, TLR signaling is modulated by negative regulators, possibly including soluble forms of the receptor itself. This mechanism avoids the perpetuation of an inflammatory response which could bring irreversible damage of the organism ([3] and references therein).

Babies born before 37 weeks of gestation are defined as "preterm" and present a multifactorial syndrome due to their limited organ development at birth. Preterm babies are more susceptible to infections and may succumb to necrotizing enterocolitis (NEC), a gastrointestinal disease which is exacerbated by an excessive inflammatory response after TLR activation. In this study, we have investigated the presence of TLR1/2/4/6 in breast milk, using both immunodetection techniques and proteomic LC-MS/MS analysis. It has been reported that immunochemical methods applied to a complex substrate such as milk are of concern due to crossreactivity or nonspecific antibody recognition [4]. Until now, soluble forms of TLR2 were immunodetected in breast milk such as 6 isoforms from 20 to 85 kDa [5]; sTLR2 can modulate TLR2 signaling and suppress inflammation [6]. We investigated if it is possible to detect membrane-bound and soluble forms of TLRs by immunoblotting and mass spectrometry. Deeper knowledge of anti-inflammatory molecules, like soluble forms of TLRs (or those present in MFGM), in human milk and their mode of action may help in the development of strategies to prevent infections in premature newborns.

2. Materials and Methods

2.1. Human Milk Samples. In this study, we investigated the presence of immune-related proteins such as TLRs in colostrum (0) and mature milk (2) of women who delivered before (preterm) or after (term) 37 weeks of gestation. Human milk samples used in this study were provided by Azienda Ospedaliera Nazionale SS. Antonio e Biagio e Cesare Arrigo, Alessandria, and the Milk Bank of Ospedale Sant'Andrea, Vercelli, Italy. The samples (5-10 mL) were not pooled, in order to keep a trace of biological variability, added with a protease inhibitor cocktail (cOmplete, Mini; Roche), aliquoted in 1.5 mL tubes, and stored at -80°C until use.

2.2. Milk Fractionation. Milk aliquots were centrifuged at $2000 \times g$ for 30 min at 10°C (Fresco 21, Heraeus), and the subsequent two fractions (milk fat globule membranes (MFGMs) and skimmed milk) were analyzed separately. The floating MFGM fraction was prepared as described in [7], transferred to a new tube, and washed three times with NaCl 0.9%, followed by a centrifugation step ($3000 \times g$, 30 min, 10°C). MFGM proteins were solubilised from membranes after 1-hour incubation at room temperature in urea 7 M, thiourea 2 M, CHAPS 4%, and DTT 100 mM, then centrifuged at $10000 \times g$ for 15 min at 10°C, and the soluble fraction was stored at −20°C until use. The liquid fraction with high protein content, termed skimmed milk, was carefully recovered in order to avoid contamination from other fractions and stored at -20°C until use.

Protein concentration of each fraction was quantified by the method of Bradford [8] at 595 nm with a Spark 10M microplate reader (Tecan). BSA was used as the protein standard. The method was optimized for absorbance readings on a 96-well microplate, mixing 10 μL of buffer/sample/standard dilution with 200 μL of Bradford reagent (Serva).

2.3. Immunoreactivity Detection. The fractions of MFGM and skimmed milk were analyzed by SDS-PAGE and immunoblotting against TLR1, TLR2, TLR4, and TLR6. The immunoreactivity to β-actin was tested as the internal reference in the same samples. TLR-reactive profiles of preterm and term samples were compared by semiquantitative analysis. Skimmed milk and MFGM fractions were also incubated with anti-TLR antibodies absorbed in a multiwell plate, using a modified ELISA. After removal of the liquid, proteins interacting with the antibody were eluted and recovered. Immunoreactive bands excised from membranes and/or the corresponding SDS-PAGE bands and eluted fractions from the ELISA were digested with trypsin and analyzed by LC-MS/MS using a DDA approach.

2.3.1. SDS-PAGE. Proteins from skimmed milk and MFGMs were analyzed by SDS-PAGE. The samples (20 μg) were mixed with an equal volume of Laemmli reducing sample buffer and boiled. Skimmed milk proteins were separated on a 12.5% polyacrylamide gel, while MFGM proteins were separated on a 10% gel. A molecular weight protein standard was also loaded (Precision Plus Protein™ Dual Color Standards, Bio-Rad, CA, USA). The gels were run in a Mini Protein Tetra System (Bio-Rad, CA, USA) at 100-120 V. The gels were run in duplicate: one was subjected to Coomassie staining and the other was used for Western blotting. In the first case, the gels were fixed in 40% methanol and 10% acetic acid and stained overnight with Blue Silver [9]. After destaining, the gel images were acquired with a GS-900 densitometer (Bio-Rad, CA, USA).

2.3.2. Western Blot Analysis of TLR1, TLR2, TLR4, and TLR6. After SDS-electrophoresis, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane in a Mini Trans-Blot cell (Bio-Rad, CA, USA) at a constant voltage of 100 V on ice for about 90 min. The membranes were incubated for one hour at room temperature with a blocking solution of PBS/BSA 5%+NaN_3, then washed for 5 min in Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris-HCl (pH 7.4)), to remove the exceeding BSA. Multiple replicates were run in order to probe different antibodies on the same sample, as reported in Table 1: anti-TLR1 (STJ25862, St John's Laboratory), anti-TLR2, anti-TLR4 (sc-10739, sc-10741, Santa Cruz Biotechnology, Inc.), and anti-TLR6 (PRS3653, Sigma-Aldrich Inc., St. Louis, MO, USA). Each

Table 1: List of antibodies used in this study.

Antibody	Source	Producer	Dilution
Anti-TLR1	Polyclonal rabbit antibody, directed against a recombinant peptide from human TLR1 (STJ25862)	St John's Laboratory	1 : 500
Anti-TLR2	Polyclonal rabbit antibody (H-175), directed against aa 180-354 of human TLR2 (sc-10739)	Santa Cruz Biotechnology, Inc.	1 : 500
Anti-TLR4	Polyclonal rabbit antibody (H-80), directed against aa 242-321 of human TLR4 (sc-10741)	Santa Cruz Biotechnology, Inc.	1 : 500
Anti-TLR6	Polyclonal rabbit antibody directed against a peptide of 13 aa near the central part of human TLR6 (PRS3653)	Sigma-Aldrich	1 : 1000
Anti-β-actin	Polyclonal rabbit antibody (20536-1-AP)	Proteintech	1 : 2000
Anti-rabbit-AP	Polyclonal goat anti-rabbit antibody conjugated with alkaline phosphatase (A3687)	Sigma-Aldrich	1 : 3000

membrane was split into two identical parts, containing the same samples, and one half was probed with an anti-β-actin antibody (20536-1-AP, Proteintech). The membranes were incubated overnight at 4°C with a primary antibody, then washed once with Tris-buffered saline-Tween (TBST: 150 mM NaCl, 10 mM Tris-HCl, and 0.1% Tween 20) for 15 min, followed by three washes with TBS (10 min). The membranes were then incubated with an alkaline phosphatase- (AP-) conjugated secondary antibody (1 : 3000 in TBS) (Sigma-Aldrich Inc., St. Louis, MO, USA) at room temperature for 1 hour. The membranes were then washed once in TBST for 15 min and 4 times in TBST for 5 min and left in AP buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$) for 5 min. The signals were detected with BCIP/NBT (5-bromo-4-chloro-3′-indolyl-phosphate p-toluidine salt/nitro blue tetrazolium chloride, Sigma-Aldrich Inc., St. Louis, MO, USA), the membrane images were acquired with a GS-900 densitometer (Bio-Rad, CA, USA), and band intensities were measured with Image Lab Software (5.2.1 version, Bio-Rad, CA, USA) and normalized to the expression of β-actin for semiquantitative analysis.

2.3.3. Modified ELISA of Skimmed Milk. In order to identify soluble components of skimmed milk bound to anti-TLR1/TLR2/TLR4/TLR6 Abs, colostral skimmed fractions from healthy preterm donors were analyzed with a modified ELISA as described in [10]. An ELISA flat-bottomed 96-well microplate (Pure Grade™, BRAND, Wertheim, Germany) was coated with 200 μL/well of the antibody solution in coating buffer (0.05 M sodium carbonate-bicarbonate (pH 9.8)). The plates were incubated for 4 h at 37°C and washed with TBST. Residual binding sites were blocked with 200 μL/well of 1% BSA and washed in TBST. Samples in TBST were added and left overnight at 4°C. After a wash with TBST, 100 μL of elution buffer (0.1 M Glycin-HCl (pH 2.7)) were added and left for 30 min. Finally, the eluate was collected and neutralized with ammonium bicarbonate 0.1 M and immediately submitted to trypsin digestion.

2.4. Trypsin Digestion of Proteins. Before mass spectrometry analysis, protein bands separated on SDS-PAGE, immunoreactive bands detected on PVDF membranes, and eluted fractions from the modified ELISA were submitted to trypsin digestion. Proteins from SDS-gels were in-gel digested as described in [11] with a few modifications. Briefly, the protein bands corresponding to the apparent MW of anti-TLR1/2/4/6 immunoreactive bands were excised from Coomassie-stained gels, destained overnight with 50% methanol and 5% acetic acid. The gel pieces were subjected to a series of shrinking steps in acetonitrile, followed by rehydration with 100 mM ammonium bicarbonate (NH_4HCO_3). Proteins were then reduced with 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 for 30 minutes at room temperature and alkylated in the dark with 100 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature for 30 minutes. The solution was removed and bands were rinsed in 100 mM NH_4HCO_3 for 5 minutes. After the final rehydrating step, gel bands were dried in Concentrator Plus (Eppendorf, Germany). Trypsin (Sequencing Grade, Roche, Germany) was reconstituted and added to gel pieces. The digestion was performed overnight at 37°C. The supernatant was collected in a new vial, and peptides were extracted twice in 50% ACN/0.1% formic acid (FA) for 10 min with an ultrasonic bath. The supernatants were pooled, dried, and stored at −20°C until mass spectrometry (MS) analysis.

Immunoreactive protein bands on PVDF membranes were excised, thoroughly washed with HPLC water, then washed once with 50 mM NH_4HCO_3, and submitted to reducing-alkylating steps as described for in-gel digestion. After two washes in 50 mM NH_4HCO_3, protein bands were covered with trypsin solution and incubated overnight at 37°C. Trypsin-digested peptides were collected as described above. In the case of fainter bands, 4-5 replicates of the same band were digested and pooled in order to increase the amount of protein to be analyzed.

Eluates from the modified ELISA were reduced, alkylated, covered with trypsin solution as described above, and incubated overnight at 37°C. The digested samples were finally dried in Concentrator Plus (Eppendorf, Germany) and stored at -20°C until mass spectrometry analyses.

2.5. Mass Spectrometry Characterization. The peptide digests were desalted on the Discovery® DSC-18 solid phase extraction (SPE) 96-well plate (25 mg/well) (Sigma-Aldrich Inc., St.

Louis, MO, USA) prior to the mass spectrometry analysis. The LC-MS/MS analyses were performed with a micro-LC Eksigent Technologies (Dublin, USA) system that included a micro LC200 Eksigent pump with flow module 5-50 μL and a programmable autosampler CTC PAL with a Peltier unit (1.0-45.0°C). The stationary phase was a Halo Fused C18 column (0.5 × 100 mm, 2.7 μm; Eksigent Technologies, Dublin, USA). The mobile phase was a mixture of 0.1% (*v*/*v*) formic acid in water (A) and 0.1% (*v*/*v*) formic acid in acetonitrile (B), eluting at a flow rate of 15.0 μL min^{-1} and at an increasing concentration of solvent B from 2% to 40% in 30 minutes. The injection volume was 4.0 μL. The oven temperature was set at 40°C.

The LC system was interfaced with a 5600^{+} TripleTOF™ system (AB Sciex, Concord, Canada) equipped with DuoSpray™ Ion Source and CDS (Calibrant Delivery System). The mass spectrometer worked in a data-dependent acquisition mode (DDA). Peptide profiling was performed using a mass range of 100–1300 Da (TOF scan with an accumulation time of 100.0 ms), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored per cycle). The ion source parameters in an electrospray positive mode were set as follows: curtain gas (N_2) at 25 psig, nebulizer gases GAS1 at 25 psig and GAS2 at 20 psig, ionspray floating voltage (ISFV) at 5000 V, source temperature at 450°C, and declustering potential at 25 V.

2.6. Protein Database Search. The DDA files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston, USA). Trypsin as a digestion enzyme was specified with 2 missed cleavages. The instrument was set to ESI-QUAD-TOF, and the following modifications were specified for the search: carbamidomethyl cysteines as fixed modification and oxidized methionine as variable modification. A search tolerance of 50 ppm was specified for the peptide mass tolerance, and 0.1 Da for the MS/MS tolerance. The charges of the peptides to search for were set to 2+, 3+, and 4+, and the search was set on monoisotopic mass. The UniProt Swiss-Prot *human* unreviewed database (version 2017.06.21, containing 43234 sequence entries) was used. Only proteins with at least two peptides with individual ion scores > 20 were considered for identification purposes.

2.7. TLR1/2/4/6 Sequence Alignment. Sequences of TLR1/2/4/6 mature forms were aligned with Clustal Omega [12] in order to evaluate their homology degree. FASTA sequences of TLR1, TLR2, TLR4, and TLR6 used for the alignment are reported in Figure 1. The alignment of mature TLR1 with TLR6 revealed an identity percentage of 69.4%. TLR4 has the lowest identity compared with the other TLRs (23-25%). TLR2 shows a higher percentage of identity with TLR1 and TLR6 (30%) than with TLR4 (22.7%).

3. Results and Discussion

3.1. SDS-PAGE Protein Profile. The protein profile of MFGM and skimmed milk (SM) fractions after SDS-PAGE is shown in Figures 2 and 3, respectively. Milk samples obtained from healthy donors who delivered at term (T) or preterm (PT) and two milk stages (colostrum and mature milk) were analyzed.

MFGM and skimmed milk fractions show a different and characteristic protein profile. Though protein profiles of samples from the same milk fraction are quite similar, it is possible to observe little individual variability, with protein bands of different intensities. The most notable difference is observed between samples of different gestational ages; the colostrum of donors who delivered at term shows the lack of a band related to casein, with regard to preterm samples. The absence of casein in the colostrum of early production is reported in literature [13, 14]. In our case, the difference between term and preterm colostrum could be related to a delayed collection of milk (between the 3rd and 5th day of lactation) in mothers who delivered at preterm.

3.2. Analysis of TLR Expression in MFGMs. Immunoblots of MFGMs obtained from colostrum and mature milk samples using anti-TLR1/TLR2/TLR4/TLR6 are shown in Figures 4 and 5, respectively. Colostrum showed immunoreactivity versus anti-TLR1 at 150 kDa and 100 kDa, especially in preterm samples. Similarly, anti-TLR2 reactivity is more evident in preterm colostrum, with the appearance of a single band at 100 kDa. We could not observe any reactive band after anti-TLR4 Ab incubation (see also Supplementary Figure S1 and Figure S3). Finally, the incubation with anti-TLR6 Ab determined the appearance of a prevalent band at 80-85 kDa and a series of bands with lower molecular weight. Among these, a 50 kDa band is especially evident in preterm milk. The presence of bands around 80-100 kDa is in accordance with the molecular weight of the analyzed TLRs.

Immunoblot analysis of mature milk MFGM proteins confirmed anti-TLR1 reactivity in both term and preterm samples at 150 kDa, but if compared with colostrum, the band at 100 kDa disappeared while a new band more evident in preterm samples came out at 30 kDa. Two bands detectable in all samples appeared at 100 and 80 kDa after incubation with an anti-TLR2 antibody, while anti-TLR4 reactivity was observed at 75 kDa (see also Supplementary Figure S2 and Figure S4). All samples tested showed two prevalent bands at 80 kDa and at 50 kDa, after anti-TLR6 antibody incubation.

Semiquantitative analysis of immunoreactive bands in MFGMs (data not shown), obtained after actin standardization, confirmed the differences observed between term and preterm mainly in colostral samples. Immunoblotting results show changes in the amount of actin; anyway, we used actin normalization assuming that the presence of actin in milk originates from cell residues, since actin is not a typical milk secreted protein and could represent proportionally the amount of cells from which TLRs are derived.

Colostrum of preterm donors shows higher immunoreactivity to anti-TLR1 Ab than that of term women, with two intense bands at 150 and 100 kDa. The band at 100 kDa disappeared in mature milk, while a new band at 30 kDa appeared, with higher intensity in preterm samples.

```
TLR1 >sp|Q15399|25-786(MW=87,414 kDa)
SEFLVDRSKNGLIHVPKDLSQKTTILNISQNYISELWTSDILSLSKLRILIISHNRIQYLDISVFKFNQELEYLDLSHNKLVKIS
CHPTVNLKHLDLSFNAFDALPICKEFGNMSQLKFLGLSTTHLEKSSVLPIAHLNISKVLLVLGETYGEKEDPEGLQDFNTE
SLHIVFPTNKEFHFILDVSVKTVANLELSNIKCVLEDNKCSYFLSILAKLQTNPKLSNLTLNNIETTWNSFIRILQLVWHTT
VWYFSISNVKLQGQLDFRDFDYSGTSLKALSIHQVVSDVFGFPQSYIYEIFSNMNIKNFTVSGTRMVHMLCPSKISPFLHL
DFSNNLLTDTVFENCGHLTELETLILQMNQLKELSKIAEMTTQMKSLQQLDISQNSVSYDEKKGDCSWTKSLLSLNMSS
NILTDTIFRCLPPRIKVLDLHSNKIKSIPKQVVKLEALQELNVAFNSLTDLPGCGSFSSLSVLIIDHNSVSHPSADFFQSCQK
MRSIKAGDNPFQCTCELGEFVKNIDQVSSEVLEGWPDSYKCDYPESYRGTLLKDFHMSELSCNITLLIVTIVATMLVLAV
TVTSLCSYLDLPWYLRMVCQWTQTRRRARNIPLEELQRNLQFHAFISYSGHDSFWVKNELLPNLEKEGMQICLHERNF
VPGKSIVENIITCIEKSYKSIFVLSPNFVQSEWCHYELYFAHHNLFHEGSNSLILILLEPIPQYSIPSSYHKLKSLMARRTYLE
WPKEKSKRGLFWANLRAAINIKLTEQAKK
TLR2 >sp|O60603|21-784(MW=87,515 kDa)
ESSNQASLSCDRNGICKGSSGSLNSIPSGLTEAVKSLDLSNNRITYISNSDLQRCVNLQALVLTSNGINTIEEDSFSSLGSLE
HLDLSYNYLSNLSSSWFKPLSSLTFLNLLGNPYKTLGETSLFSHLTKLQILRVGNMDTFTKIQRKDFAGLTFLEELEIDASD
LQSYEPKSLKSIQNVSHLILHMKQHILLLEIFVDVTSSVECLELRDTDLDTFHFSELSTGETNSLIKKFTFRNVKITDESLFQ
VMKLLNQISGLLELEFDDCTLNGVGNFRASDNDRVIDPGKVETLTIRRLHIPRFYLFYDLSTLYSLTERVKRITVENSKVFL
VPCLLSQHLKSLEYLDLSENLMVEEYLKNSACEDAWPSLQTLILRQNHLASLEKTGETLLTLKNLTNIDISKNSFHSMPET
CQWPEKMKYLNLSSTRIHSVTGCIPKTLEILDVSNNNLNLFSLNLPQLKELYISRNKLMTLPDASLLPMLLVLKISRNAITT
FSKEQLDSFHTLKTLEAGGNNFICSCEFLSFTQEQQALAKVLIDWPANYLCDSPSHVRGQQVQDVRLSVSECHRTALVSG
MCCALFLLILLTGVLCHRFHGLWYMKMMWAWLQAKRKPRKAPSRNICYDAFVSYSERDAYWVENLMVQELENFNPPF
KLCLHKRDFIPGKWIIDNIIDSIEKSHKTVFVLSENFVKSEWCKYELDFSHFRLFDENNDAAILILLEPIEKKAIPQRFCKLR
KIMNTKTYLEWPMDEAQREGFWVNLRAAIKS
TLR4 >sp|O00206|24-839(MW=93,274 kDa)
ESWEPCVEVVPNITYQCMELNFYKIPDNLPFSTKNLDLSFNPLRHLGSYSFFSFPELQVLDLSRCEIQTIEDGAYQSLSHLS
TLILTGNPIQSLALGAFSGLSSLQKLVAVETNLASLENFPIGHLKTLKELNVAHNLIQSFKLPEYFSNLTNLEHLDLSSNKIQ
SIYCTDLRVLHQMPLLNLSLDLSLNPMNFIQPGAFKEIRLHKLTLRNNFDSLNVMKTCIQGLAGLEVHRLVLGEFRNEGN
LEKFDKSALEGLCNLTIEEFRLAYLDYYLDDIIDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKL
KSLKRLTFTSNKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTTSLKYLDLSFNGVITMSSNFLGLEQLEHLDFQH
SNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQCQLEQLSP
TAFNSLSSLQVLNMSHNNFFSLDTFPYKCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFACTCEHQSFLQW
IKDQRQLLVEVERMECATPSDKQGMPVLSLNITCQMNKTIIGVSVLSVLVVSVVAVLVYKFYFHLMLLAGCIKYGRGENI
YDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIAANIIHEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQ
TWQFLSSRAGIIFIVLQKVEKTLLRQQVELYRLLSRNTYLEWEDSVLGRHIFWRRLRKALLDGKSWNPEGTVGTGCNW
QEATSI
TLR6 >sp|Q9Y2C9|32-796(MW=88,343 kDa)
NEFAVDKSKRGLIHVPKDLPLKTKVLDMSQNYIAELQVSDMSFLSELTVLRLSHNRIQLLDLSVFKFNQDLEYLDLSHNQ
LQKISCHPIVSFRHLDLSFNDFKALPICKEFGNLSQLNFLGLSAMKLQKLDLLPIAHLHLSYILLDLRNYYIKENETESLQI
LNAKTLHLVFHPTSLFAIQVNISVNTLGCLQLTNIKLNDDNCQVFIKFLSELTRGSTLLNFTLNHIETTWKCLVRVFQFLWP
KPVEYLNIYNLTIIESIREEDFTYSKTTLKALTIEHITNQVFLFSQTALYTVFSEMNIMMLTISDTPFIHMLCPHAPSTFKFLN
FTQNVFTDSIFEKCSTLVKLETLILQKNGLKDLFKVGLMTKDMPSLEILDVSWNSLESGRHKENCTWVESIVVLNLSSNM
LTDSVFRCLPPRIKVLDLHSNKIKSVPKQVVKLEALQELNVAFNSLTDLPGCGSFSSLSVLIIDHNSVSHPSADFFQSCQKM
RSIKAGDNPFQCTCELREFVKNIDQVSSEVLEGWPDSYKCDYPESYRGSPLKDFHMSELSCNITLLIVTIGATMLVLAVTV
TSLCIYLDLPWYLRMVCQWTQTRRRARNIPLEELQRNLQFHAFISYSEHDSAWVKSELVPYLEKEDIQICLHERNFVPGK
SIVENIINCIEKSYKSIFVLSPNFVQSEWCHYELYFAHHNLFHEGSNNLILILLEPIPQNSIPNKYHKLKALMTQRTYLQWP
KEKSKRGLFWANIRAAFNMKLTLVTENNDVKS
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Figure 1: Sequences of TLR1, TLR2, TLR4, and TLR6 mature forms, preceded by UniProt identifier, initial and final amino acid number, and estimated molecular weight.

Concerning colostrum, we could observe anti-TLR2 reactivity only in preterm samples, while two reactive bands at 100 and 80 kDa were detectable in each sample of mature milk tested. Interestingly, we could not observe any anti-TLR4 reactivity in colostral samples, while a band appeared in each sample of mature milk. Preterm colostral samples showed higher reactivity, at 75 and 50 kDa, to anti-TLR6 Ab too. The intensity values of these bands declined in mature milk.

3.3. Analysis of TLR Expression in Skimmed Milk. Immunoblots of skimmed fractions from colostrum and mature milk samples using the anti-TLR1/TLR2/TLR4/TLR6 antibodies are shown in Figures 6 and 7, respectively. Anti-β-actin reactivity of the same samples was used as an internal standard.

Concerning colostrum skimmed fraction, two bands around 75 kDa appeared after anti-TLR1 Ab incubation, but if compared with MFGM fraction, no bands at 150-100 kDa were observed. Anti-TLR2 and anti-TLR4 Abs determined the appearance of two bands at 75 and 50 kDa (Supplementary Figure S5 and Figure S7). However, these bands were considered aspecific since the same profile was observed in samples probed with anti-β-actin Ab. An additional band at 25 kDa was observed after anti-TLR4 Ab incubation. The immunoreactive profile after anti-TLR6 Ab incubation is quite similar, with the appearance of high MW bands (250 kDa, 150 kDa), a most intense band between 100 and 75 kDa (the only one clearly observed in 4PT), and minor bands at 50 kDa, 37 kDa, and 25 kDa. Concerning the skimmed fraction of mature milk, the immunoreactive profile observed after anti-TLR1/2/4/6 incubation is shown in Figure 7.

A 150 kDa band appeared in all samples except in 7 T after anti-TLR1 Ab incubation. As observed in colostrum,

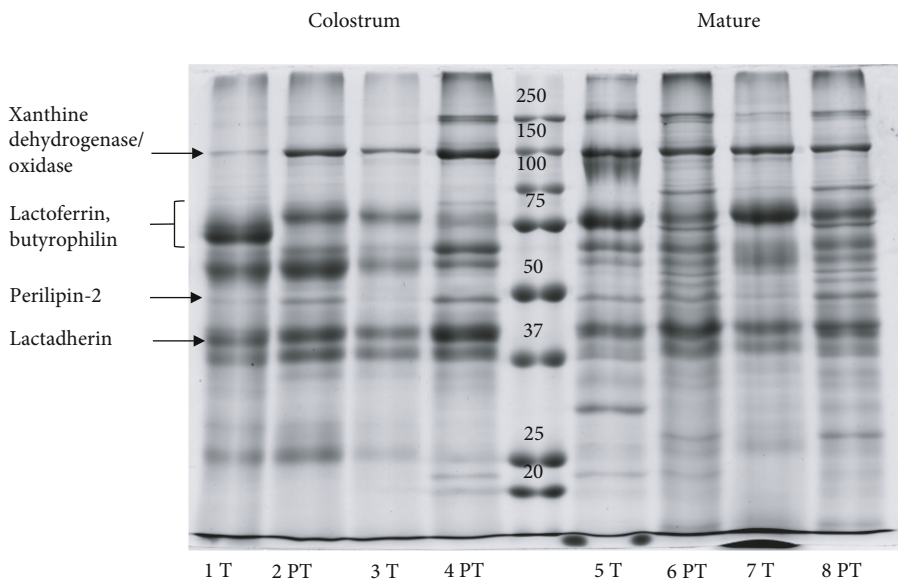

Figure 2: Electrophoretic profile of MFGM proteins in colostrum and mature milk. Gestational age is indicated as "T" (term) or "PT" (preterm). Each number refers to a different milk sample.

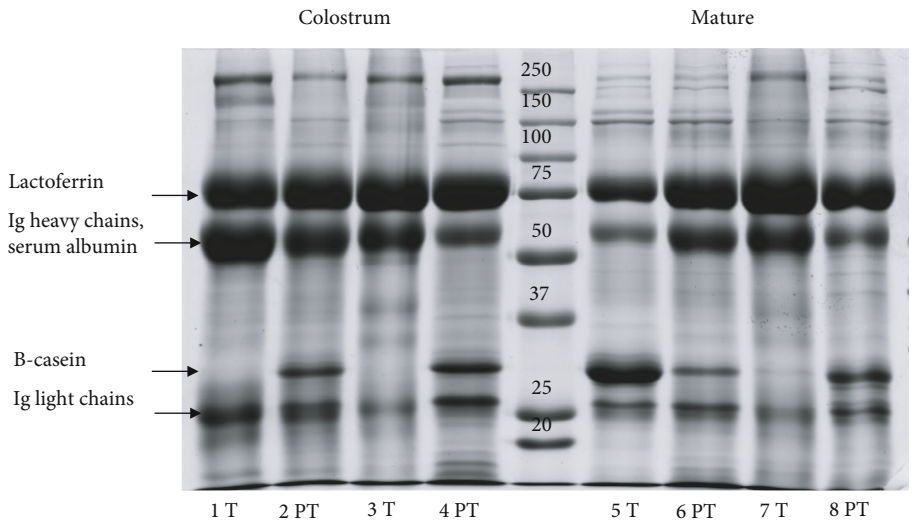

Figure 3: Electrophoretic profile of skimmed milk proteins in colostrum and mature milk. Gestational age is indicated as "T" (term) or "PT" (preterm). Each number refers to a different milk sample.

we did not detect signals relative to TLR2 and TLR4 but only aspecific bands at 75 and 50 kDa (Supplementary Figure S6 and Figure S8). Reactivity to anti-TLR6 Ab was similar to that observed for the skimmed fraction of colostrum. The main bands were observed between 75 and 100 kDa.

Immunoreactive bands against anti-TLR1 and anti-TLR4 Abs showed higher intensity in preterm than in term samples of colostrum. This trend is no more evident in mature samples, where these bands disappeared and a clear band at 150 kDa appeared only after anti-TLR1 Ab incubation. Concerning anti-TLR6 immunoreactivity, bands of higher intensity were observed in colostrum than in mature skimmed milk. Again, colostrum preterm samples revealed higher intensity bands at 75-100 kDa. This trend is no more evident in lower MW bands of both colostrum and mature skimmed milk.

The observed differences between preterm and term samples are supported by evidences reported in literature. The concentration of total protein is higher in preterm milk [15], and their expression is differently regulated. In a study on human skimmed milk [16], 28 proteins with higher expression levels and 27 proteins with lower levels were found in preterm milk compared to term milk.

Concerning TLRs, the presence of bands with apparent molecular weight lower than that of TLR mature forms (which is estimated between 87 kDa and 93 kDa considering the amino acidic sequence only) could be due to posttranslational modifications, with the production of truncated forms.

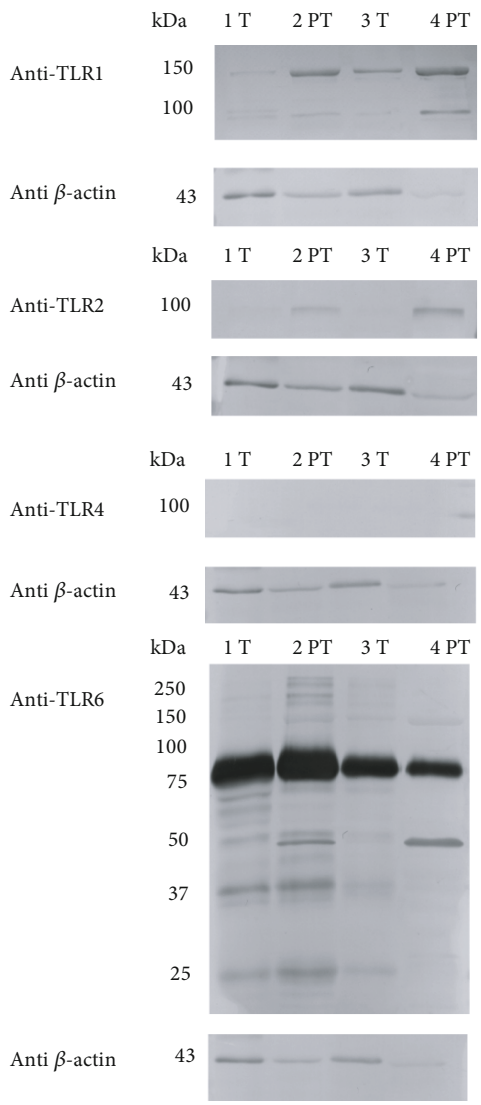

Figure 4: Immunoblot of TLR1/2/4/6 in MFGMs of colostrum. Gestational age is indicated as "T" (term) or "PT" (preterm). Each number refers to a different milk sample. Standard molecular weights are indicated on the left. Each box shows the blot reactivity to a specific anti-TLR antibody and, on the lower side, the same blot incubated with an anti-β-actin antibody.

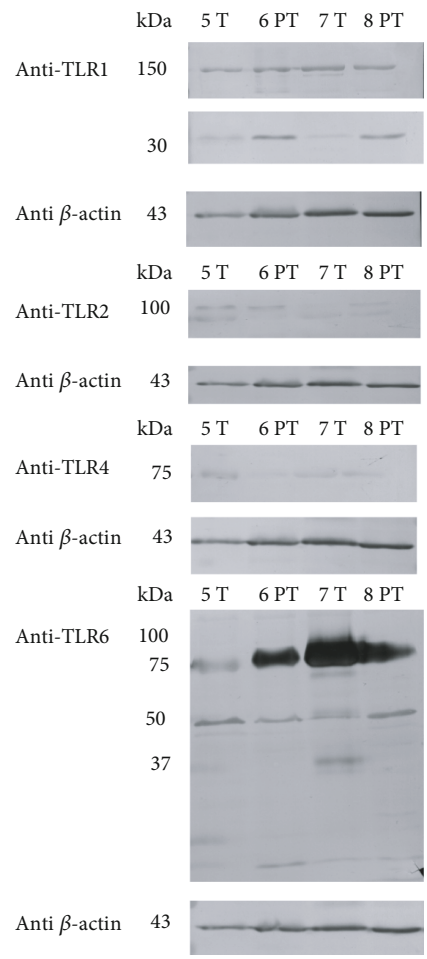

Figure 5: Immunoblot of TLR1/2/4/6 in MFGMs of mature milk. Gestational age is indicated as "T" (term) or "PT" (preterm). Each number refers to a different milk sample. Standard molecular weights are indicated on the left. Each box shows the blot reactivity to a specific anti-TLR antibody and, on the lower side, the same blot incubated with an anti-β-actin antibody.

In literature, there is some evidence of TLR2 and TLR4 soluble forms (sTLRs) revealed by Western blot analysis of biological samples such as saliva, amniotic fluid, plasma, and milk [5, 17, 18]. To our knowledge, the presence of soluble forms of TLR1 and TLR6 has never been reported. Immunoblot analysis of normal unstimulated whole saliva (UWS) revealed three sTLR2 polypeptides of 54, 40, and 30 kDa and four sTLR4 polypeptides of 90, 78, 54, and 44 kDa [17]. Two polypeptides (42 and 30 kDa), corresponding to the extracellular domain of the full-length TLR2 receptor (98 kDa), were found in the human amniotic fluid, where the 42 kDa isoform is the main sTLR2 released. In this compartment, sTLR2 forms were found to be constitutively expressed, but their expression level is regulated by gestational age, with high sTLR2 expression levels observed until the 37th week of gestation and decreasing levels in women who delivered at term [18]. LeBouder and colleagues [5] were the first to report the presence of soluble forms of TLR2 in human plasma and milk, where they observed up to six polypeptide bands of 83, 70, 66, 40, 38, and 25 kDa. Another study [19] reported variations to this pattern in breast milk, in terms of number and intensity of bands, with the absence of 70 and 40 kDa bands and the appearance of a 130 kDa band, probably due to sample variability and the type of antibody tested. In fact, the polyclonal (p) Ab used was found to be specific for the 83 and 38 kDa bands, whereas the monoclonal (m) Abs tested revealed two bands of 83 and 26 kDa. Moreover, pAb detected the presence of commercially available recombinant sTLR2 produced in mouse myeloma cell lines, while anti-TLR2 mAbs were unable to detect the recombinant protein. Our results differ from the previous reports, since we observed only a 25 kDa band after anti-TLR4 Ab incubation in the skimmed fraction of colostrum. The difference could be due to a number of reasons, such as the choice of antibody, which could recognize an N-term or

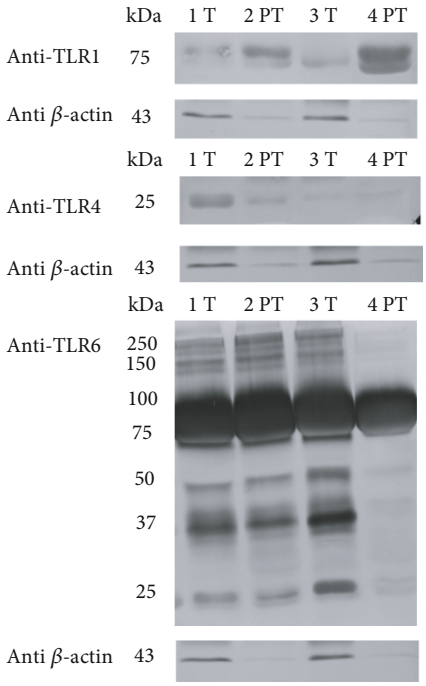

Figure 6: Immunoblot of TLR1/4/6 in colostral skimmed milk. Gestational age is indicated as "T" (term) or "PT" (preterm). Each number refers to a different milk sample. Standard molecular weights are indicated on the left. Each box shows the blot reactivity to a specific anti-TLR antibody and, on the lower side, the same blot incubated with anti-β-actin Ab.

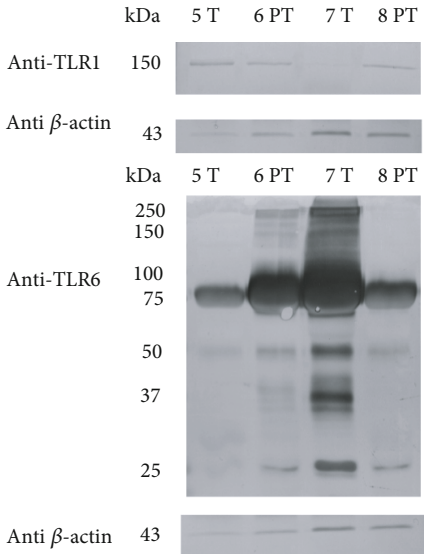

Figure 7: Immunoblot of TLR1/6 in mature skimmed milk. Gestational age is indicated as "T" (term) or "PT" (preterm). Each number refers to a different milk sample. Standard molecular weights are indicated on the left. Each box shows the blot reactivity to a specific anti-TLR antibody and, on the lower side, the same blot incubated with anti-β-actin Ab.

a C-term region of TLR. The mechanism of sTLR production is still unclear, but it was suggested that sTLR2 production does not involve new protein production and is a result of posttranslational modification, probably starting from the C-terminal side [5]. The Abs chosen in this study recognize a central region of the TLR2 and TLR4 ectodomains, but part of them could be removed during the processing of soluble forms with the consequent failure of protein binding. Another possibility could be the time-related amount of soluble forms in milk, since a decline of sTLR2 levels over time postpartum was reported, with the disappearance of some soluble forms few days after delivery [5, 19]. Finally, it is possible that some signals reflect nonspecific binding of the anti-TLR2 and anti-TLR4 polyclonal Abs to proteins constitutively present in the human milk, as previously stated in [18]. In the skimmed fraction of milk, we observed two anti-TLR2/TLR4 immunoreactive bands at 75 and 50 kDa that were considered aspecific since the same reactive profile was observed after anti-β-actin Ab incubation.

3.4. Mass Spectrometry Identifications. Mass spectrometry analysis of immunoreactive bands detected in MFGM and skimmed milk fractions (Figure 8) led to the identification of TLR2 and some TLR-related proteins, but the presence of TLR1/4/6 was not determined (Supplementary Table S1). In detail, TLR2 was identified in B2, B4, G2, and G11; antigen CD14 was identified in B6, B11, B12, G12, and G13; CD36 glycoprotein was identified in G3, G8, G12, S1, S2, and B8; leucine-rich protein was identified in S3, S4, S5, G7, and G12; tenascin was identified in S1, S3, S4, S5, S6, S8, S10, B1, B2, B6, B11, B12, and G12; and zinc alpha glycoprotein was identified in S3, S4, and S5.

TLR2 was detected by mass spectrometry in the MFGM fraction only, with peptides of six different sequences. In order to assess if TLR2 found in the MFGM fraction corresponds to the whole receptor or parts of it, the sequences of the identified peptides were overlapped to the complete sequence of TLR2. As shown in Figure 9, MS-identified peptides almost cover the entire sequence of TLR2, with four peptides belonging to the extracellular domain (SLDLSNNR: aa 56-63 corresponding to leucine-rich repeat (LRR) 1; VGNMDTFTK: aa 156-164, LRR 5; TGETLLTLK: aa 404-413, LRR 14-15; and TLEILDVSNNNLNLFSLNLPQLK: aa 458-480, LRR 17-18), one belonging to the transmembrane region (GQQVQDVR: aa 572-579, LRRCT), and one belonging to the C-terminal intracellular domain (LFDENNDAAILILLEPIEK: aa 724-742, TIR), confirming the association of the detected form of TLR2 with the MFG membrane.

Our MS results are supported by studies reporting the finding of TLR2 in MFGMs but not in serum of milk [2, 20]. Concerning other TLRs, the presence of TLR1/2/3/4/5/6/7/9 was detected at an mRNA level in cells isolated from human breast milk [21]; however, proteomic studies performed so far do not report the presence of TLR1 and TLR6 proteins in MFGM and skimmed fractions of breast milk.

It is interesting to note that our MS analysis of immunoreactive bands detected some TLR-related proteins such as CD14 and CD36 or LRR-containing proteins. Cluster of differentiation 14 (CD14) is a pattern recognition receptor with a bent solenoid structure typical of leucine-rich repeat proteins [22]. It is present in soluble form in the blood or as a GPI-anchored membrane protein on myeloid cells and

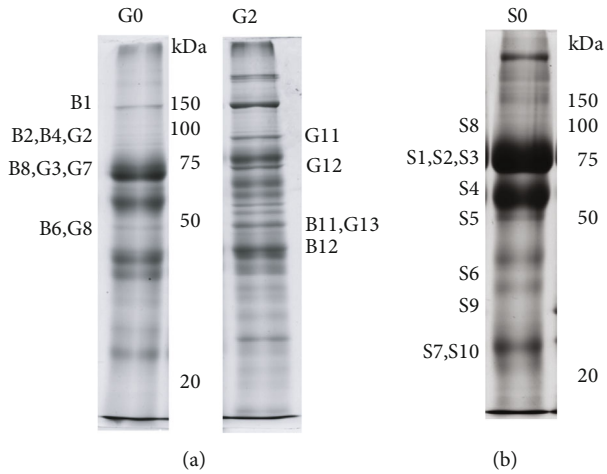

Figure 8: Representative image of proteins from MFGM (a) and skimmed milk (b) fractions of colostrum (0) and/or mature milk (2) after SDS-PAGE separation. The name of bands analyzed by mass spectrometry is reported beside each lane. Bands labelled with "B" were digested from PVDF blots, while "G" bands were digested from polyacrylamide gels. S1 was digested from polyacrylamide gels, and S2, S3, S4, S5, S6, S7, S8, S9, and S10 were digested from PVDF blots.

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MPHTLWMVWVLGVIISLSKEESSNQASLSCDRNGICKGSSGSLNSIPSGLTEAVKSLDLS
NNRITYISNSDLQRCVNLQALVLTSNGINTIEEDSFSSLGSLEHLDLSYNYLSNLSSSWF
KPLSSLTFLNLLGNPYKTLGETSLFSHLTKLQILRVGNMDTFTKIQRKDFAGLTFLEELE
IDASDLQSYEPKSLKSIQNVSHLILHMKQHILLLEIFVDVTSSVECLELRDTDLDTFHFS
ELSTGETNSLIKKFTFRNVKITDESLFQVMKLLNQISGLLELEFDDCTLNGVGNFRASDN
DRVIDPGKVETLTIRRLHIPRFYLFYDLSTLYSLTERVKRITVENSKVFLVPCLLSQHLK
SLEYLDLSENLMVEEYLKNSACEDAWPSLQTLILRQNHLASLEKTGETLLTLKNLTNIDI
SKNSFHSMPETCQWPEKMKYLNLSSTRIHSVTGCIPKTLEILDVSNNNLNLFSLNLPQLK
ELYISRNKLMTLPDASLLPMLLVLKISRNAITTFSKEQLDSFHTLKTLEAGGNNFICSCE
FLSFTQEQQALAKVLIDWPANYLCDSPSHVRGQQVQDVRLSVSECHRTALVSGMCCALFL
LILLTGVLCHRFHGLWYMKMMWAWLQAKRKPRKAPSRNICYDAFVSYSERDAYWVENLMV
QELENFNPPFKLCLHKRDFIPGKWIIDNIIDSIEKSHKTVFVLSENFVKSEWCKYELDFS
HFRLFDENNDAAILILLEPIEKKAIPQRFCKLRKIMNTKTYLEWPMDEAQREGFWVNLRA
AIKS
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Figure 9: Protein sequence of TLR2. The signal peptide is highlighted in green, while identified peptides after mass spectrometry are highlighted in yellow.

interacts with TLR2 and TLR4 in ligand recognition [3]. CD14 acts as a coreceptor for TLR2/TLR6 and for TLR2/TLR1 heterodimers in response to diacylated and triacylated lipopeptides, respectively [23], and belongs to the lipopolysaccharide (LPS) receptor, a multiprotein complex containing at least CD14, MD2, and TLR4 [24]. CD36 (cluster of differentiation 36) is a multifunctional glycoprotein involved in TLR pathways. It acts as a receptor for a broad range of ligands (proteins and lipids), interacting with multiple receptors and participating in signal transduction. CD36 is involved in TLR2 activation by microbial anionic ligands such as lipoteichoic acid and TLR4/TLR6 by endogenous ligands. A model proposed in [25] suggests an interaction between CD36 and CD14, where CD36 binds to anionic ligands and transfers them to CD14 which loads them into TLR2/TLR1 or TLR2/TLR6 heterodimers. In this work, we identified CD14 in the MFGM fraction only, while other studies report its presence in the skimmed fraction too [16]. On the contrary, CD36 was detected in both MFGM and skimmed fractions of milk.

Identified proteins in skimmed milk include zinc α2-glycoprotein and leucine-rich α-2-glycoprotein. Zinc α2-glycoprotein (ZAG) is a 40 kDa protein secreted in body fluids, which stimulates lipid degradation in adipocytes. The exact function of ZAG in physiological conditions is still unknown. ZAG shows high sequence homology with a lipid-mobilizing factor; thus, it is considered an adipokine. However, its structure is similar to that of MHC class I antigen-presenting molecule indicating that ZAG may have a role in the immune response [26]. Leucine-rich α2 glycoprotein (LRG1) is a 40 kDa serum protein and the founding member of the leucine-rich repeat (LRR) family of proteins. It is involved in neutrophil degranulation, positive regulation of angiogenesis, and proliferation of endothelial cells [27, 28]. LRG1 can bind to cytochrome c, a molecule that in the extracellular space is implicated in the initiation of arthritis via the NF-κB pathway, suggesting its role as a proinflammatory molecule [29]. LRG1 was found to mitigate apoptosis in lymphocytes treated with extracellular cytochrome c [30].

Leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1) is another LRR-containing protein found in both MFGM and skimmed fractions of milk. LRIG1 is a membrane protein with a series of LRRs, three immunoglobulin-like (Ig-like) domains, one transmembrane domain, and a cytosolic tail. It is a negative regulator of signaling by receptor tyrosine kinases. LRIG1 interacts with EGFR/ERBB1, ERBB2, ERBB3, and ERBB4 family receptors through the LRR and Ig-like extracellular domains [31]. However, its physiological role is still controversial, since LRIG1 may have low affinity for EGFR and their interaction could take place only with high quantity levels of both proteins [32].

Finally, tenascin, a glycoprotein of the extracellular matrix implicated in neuronal and axonal migration during development, was identified in the MFGM and skimmed fractions. It contains EGF-like and fibronectin type III domains and can exist in homoexameric or homotrimeric forms. Tenascin is involved in various physiological processes such as cell adhesion, cellular response to vitamin D and retinoic acid, extracellular matrix organization, and positive regulation of cell proliferation.

Its expression is induced by TGF-β1, and it is released after cell damage of infections. In fact, tenascin quickly reacts during acute inflammatory response to LPS, through the activation of TLR4 or integrins, promoting the innate immunity response with the synthesis of proinflammatory cytokines [33]. Although its role in the mammary gland and human breast milk is still unknown, tenascin may be involved in tissue reshaping associated with pregnancy. A different glycosylation pattern of tenascin has been reported in the milk of preterm with respect to term women. Since tenascin functions depend on adhesion ability with other MEC components and membrane receptors, a different glycosylation profile may change such properties [20]. We could identify tenascin in the skimmed milk, where probably it is more represented, as demonstrated by the higher number of peptides identified, but also in the MFGM fraction. Such observation is compatible with its role as an extracellular matrix protein, which could be secreted or associated with the membrane. The presence of tenascin in the MFGM of milk has previously been reported, and it shows higher expression levels in colostrum than in mature milk [34], whereas lower levels of tenascin were detected in the skimmed milk of preterm with respect to term women [16].

3.5. MS Analysis of Proteins after the Modified ELISA Test. Since TLR2 was detected only in MFGM, a further test on skimmed milk fractions was performed with the modified ELISA, in order to selectively detect proteins interacting with the anti-TLRs used in this study. We limited the assay to colostrum of preterm women, since after Western blotting analyses, we observed more intense immunoreactive profiles in these samples. After anti-TLR1 Ab incubation, we detected lactotransferrin and phosphatidylinositol 4,5-bisphosphate 5-phosphatase A, a protein with two isoforms of 100 and 70 kDa (in the WB, we observed a 75 kDa band). Anti-TLR2 incubation revealed again the presence of lactotransferrin and Rho GTPase-activating protein 7 (in the WB, a 75 kDa band was observed). Anti-TLR4 interacted with zinc α2-glycoprotein and lactotransferrin (in the WB, 25 kDa was observed) whereas anti-TLR6 Ab incubation with skimmed milk revealed the presence of lactotransferrin and β-casein (WB revealed a series of bands at 100-75, 50, 37, and 25 kDa). The MS of modified ELISA eluates did not reveal any TLR. Lactotransferrin was detected in all the samples: this highly abundant protein could have "obscured" other potential targets, but this event raises the question of Ab specificity.

4. Conclusions

MFGM fraction from preterm colostrum showed higher reactivity to anti-TLR1, anti-TLR2, and anti-TLR6 antibodies, compared to that from term samples. In the skimmed fraction, no immunoreactivity to anti-TLR2 was observed, whereas bands at low molecular weight appeared after incubation with anti-TLR4 and anti-TLR6 antibodies. LC-MS/MS analysis of immunoreactive bands confirmed the presence of the entire membrane receptor TLR2 in the MFGM of human milk, but not in the skimmed milk. With regard to TLR1, TLR4, and TLR6, despite the observation of immunoreactive bands, their presence was not confirmed after MS analysis. However, it is interesting to note that the MS analysis of these bands allowed the identification of proteins related to TLRs, because of the presence of LRR domains in their sequence or their direct interaction with TLRs. MS analysis of the ELISA fractions did not reveal any TLR but confirmed proteins identified in immunoreactive bands. The presence of soluble forms of TLRs in human milk remains an open question. It is possible that the soluble forms of TLRs in milk reported in literature, which were observed only by immunoblot assays, are related to cross-reactivity events with these proteins. Anyway, the lack of MS identification for TLR1, TLR4, and TLR6 cannot assure their total absence in milk, where their concentration may be inferior to the limit of detection of the instrument and the DDA method used in this study could have excluded them. It is clear that TLR2 is surely present in milk, and we suggest that soluble forms may be ascribed to other milk proteins sharing conformational domains capable of reacting with and modulating TLR signaling.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interest.

Acknowledgments

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Supplementary Materials

Table S1: list of proteins identified after mass spectrometry such as TLRs or TLR-related proteins. Figure S1: complete Western blot with anti-TLR2 for MFGM colostrum. Figure S2: complete Western blot with anti-TLR2 for MFGM mature milk. Figure S3: complete Western blot with anti-TLR4 for MFGM colostrum. Figure S4: complete Western blot with anti-TLR4 for MFGM mature milk. Figure S5: complete Western blot with anti-TLR2 for skimmed colostrum. Figure S6: complete Western blot with anti-TLR2 for skimmed mature milk. Figure S7: complete Western blot with anti-TLR4 for skimmed colostrum. Figure S8: complete Western blot with anti-TLR4 for skimmed mature milk. *(Supplementary Materials)*

References

[1] N. T. Cacho and R. M. Lawrence, "Innate immunity and breast milk," *Frontiers in Immunology*, vol. 8, p. 584, 2017.

[2] K. Hettinga, H. van Valenberg, S. de Vries et al., "The host defense proteome of human and bovine milk," *PLoS One*, vol. 6, no. 4, article e19433, 2011.

[3] M. J. J. Dalmaroni, M. E. Gerswhin, and I. E. Adamopoulos, "The critical role of toll-like receptors — From microbial recognition to autoimmunity: A comprehensive review," *Autoimmunity Reviews*, vol. 15, no. 1, pp. 1–8, 2016.

[4] J. Zhu, L. Garrigues, H. Van den Toorn, B. Stahl, and A. J. R. Heck, "Discovery and quantification of nonhuman proteins in human milk," *Journal of Proteome Research*, vol. 18, no. 1, pp. 225–238, 2019.

[5] E. LeBouder, J. E. Rey-Nores, N. K. Rushmere et al., "Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk," *Journal of Immunology*, vol. 171, no. 12, pp. 6680–6689, 2003.

[6] Y. Y. He, N. T. Lawlor, and D. S. Newburg, "Human milk components modulate Toll-like receptor-mediated inflammation," *Advances in Nutrition*, vol. 7, no. 1, pp. 102–111, 2016.

[7] M. Cavaletto, M. G. Giuffrida, D. Fortunato et al., "A proteomic approach to evaluate the butyrophilin gene family expression in human milk fat globule membrane," *Proteomics*, vol. 2, no. 7, pp. 850–856, 2002.

[8] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, pp. 248–254, 1976.

[9] G. Candiano, M. Bruschi, L. Musante et al., "Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis," *Electrophoresis*, vol. 25, no. 9, pp. 1327–1333, 2004.

[10] E. Bertino, G. M. Prandi, C. Fabris et al., "Human milk proteins may interfere in ELISA measurements of bovine beta-lactoglobulin in human milk," *Acta Paediatrica*, vol. 85, no. 5, pp. 543–549, 1996.

[11] C. Cattaneo, P. Cesaro, S. Spertino, S. Icardi, and M. Cavaletto, "Enhanced features of *Dictyoglomus turgidum* Cellulase A engineered with carbohydrate binding module 11 from *Clostridium thermocellum*," *Scientific Reports*, vol. 8, no. 1, p. 4402, 2018.

[12] F. Sievers, A. Wilm, D. G. Dineen et al., "Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega," *Molecular Systems Biology*, vol. 7, no. 1, p. 539, 2011.

[13] M. Cavaletto, A. Cantisani, L. Napolitano et al., "Comparative study of casein content in human colostrum and milk," *Milchwissenschaft*, vol. 49, pp. 303–305, 1994.

[14] D. Palmer, V. C. Kelly, A.-M. Smit, S. Kuy, C. G. Knight, and G. J. Cooper, "Human colostrum: identification of minor proteins in the aqueous phase by proteomics," *Proteomics*, vol. 6, no. 7, pp. 2208–2216, 2006.

[15] S. A. Atkinson, "Effects of gestational stage at delivery on human milk components," in *Handbook of Milk Composition*, R. G. Jensen, Ed., pp. 222–237, San Diego, Academic Press, 1995.

[16] C. E. Molinari, Y. S. Casadio, B. T. Hartmann et al., "Proteome mapping of human skim milk proteins in term and preterm milk," *Journal of Proteome Research*, vol. 11, no. 3, pp. 1696–1714, 2012.

[17] S. L. Zunt, L. V. Burton, L. I. Goldblatt, E. E. Dobbins, and M. Srinivasan, "Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumour necrosis factor-alpha secretion by macrophage-like cells," *Clinical and Experimental Immunology*, vol. 156, no. 2, pp. 285–293, 2009.

[18] A. T. Dulay, C. S. Buhimschi, G. Zhao et al., "Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection," *The Journal of Immunology*, vol. 182, no. 11, pp. 7244–7253, 2009.

[19] B. M. Henrick, K. Nag, X.-D. Yao, A. G. Drannik, G. M. Aldrovandi, and K. L. Rosenthal, "Milk matters: soluble Toll-like receptor 2 (sTLR2) in breast milk significantly inhibits HIV-1 infection and inflammation," *PLoS One*, vol. 7, no. 7, article e40138, 2012.

[20] C. E. Molinari, Y. S. Casadio, B. T. Hartmann, P. G. Arthur, and E. Peter, "Longitudinal analysis of protein glycosylation and β-casein phosphorylation in term and preterm human milk during the first 2 months of lactation," *British Journal of Nutrition*, vol. 110, no. 1, pp. 105–115, 2013.

[21] S. A. Armogida, N. M. Yannaras, A. L. Melton, and M. D. Srivastava, "Identification and quantification of innate immune system mediators in human breast milk," *Allergy and Asthma Proceedings*, vol. 25, no. 5, pp. 297–304, 2004.

[22] S. L. Kelley, T. Lukk, S. K. Nair, and R. I. Tapping, "The crystal structure of human soluble CD14 reveals a bent solenoid with a hydrophobic amino-terminal pocket," *Journal of Immunology*, vol. 190, no. 3, pp. 1304–1311, 2013.

[23] M. Triantafilou, F. G. Gamper, R. M. Haston et al., "Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting," *Journal of Biological Chemistry*, vol. 281, no. 41, pp. 31002–31011, 2006.

[24] J. da Silva Correia, K. Soldau, U. Christen, P. S. Tobias, and R. J. Ulevitch, "Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. Transfer from CD14 to TLR4 and MD-2," *Journal of Biological Chemistry*, vol. 276, no. 24, pp. 21129–21135, 2001.

[25] M. J. J. Dalmaroni, N. Xiao, A. L. Corper et al., "Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2," *PLoS One*, vol. 4, no. 10, article e7411, 2009.

[26] I. Hassan, A. Waheed, S. Yadav, T. P. Singh, and F. Ahmad, "Zinc 2-Glycoprotein: A Multidisciplinary Protein," *Molecular Cancer Research*, vol. 6, no. 6, pp. 892–906, 2008.

[27] L. J. Druhan, A. Lance, S. Li et al., "Leucine rich α-2 glycoprotein: a novel neutrophil granule protein and modulator of myelopoiesis," *PLoS One*, vol. 12, no. 1, article e0170261, 2017.

[28] X. Wang, S. Abraham, J. A. McKenzie et al., "LRG1 promotes angiogenesis by modulating endothelial TGF-β signalling," *Nature*, vol. 499, no. 7458, pp. 306–311, 2013.

[29] R. Pullerits, M. Bokarewa, I. M. Jonsson, M. Verdrengh, and A. Tarkowski, "Extracellular cytochrome c, a mitochondrial apoptosis-related protein, induces arthritis," *Rheumatology*, vol. 44, no. 1, pp. 32–39, 2005.

[30] R. Codina, A. Vanasse, A. Kelekar, V. Vezys, and R. Jemmerson, "Cytochrome c-induced lymphocyte death from the outside in: inhibition by serum leucine-rich alpha-2-glycoprotein-1," *Apoptosis*, vol. 15, no. 2, pp. 139–152, 2010.

[31] G. Gur, C. Rubin, M. Katz et al., "LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation," *EMBO Journal*, vol. 23, no. 16, pp. 3270–3281, 2004.

[32] Y. Xu, P. Soo, F. Walker et al., "LRIG1 extracellular domain: structure and function analysis," *Molecular Biology*, vol. 427, no. 10, pp. 1934–1948, 2015.

[33] A. M. Piccinini and K. S. Midwood, "Endogenous control of immunity against infection: tenascin-C regulates TLR4-mediated inflammation via microRNA-155," *Cell Reports*, vol. 2, no. 4, pp. 914–926, 2012.

[34] M. Yang, M. Cong, X. Peng et al., "Quantitative proteomic analysis of milk fat globule membrane (MFGM) proteins in human and bovine colostrum and mature milk samples through iTRAQ labeling," *Food and Function*, vol. 7, no. 5, pp. 2438–2450, 2016.

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Research Article

Functional Polymorphisms and Gene Expression of TLR9 Gene as Protective Factors for Nasopharyngeal Carcinoma Severity and Progression

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Nasopharyngeal carcinoma (NPC) is a disease that is closely associated with EBV infection. Toll-like receptor 9 is an important factor mediating the interaction between EBV and the host immune response. Any genetic (single nucleotide polymorphisms, SNPs) or expression variation in TLR9 gene may modify the ability of the receptor to respond correctly to viral infection as in NPC. This study is aimed at evaluating the effect of TLR9 functional polymorphisms (TLR9-1486 T/C and TLR9-1237 T/C) and TLR9 mRNA expression in NPC severity and progression at diagnosis and after treatment. This study included 322 patients with NPC. RFLP-PCR and real-time PCR were used to assess, respectively, the genotypes and the mRNA expression of TLR9 gene. The genotyping analysis showed that the presence of mutated allele -1237C (TLR9-1237 TC+CC) was associated with large tumor size ($p = 0.017$; OR (CI 95%) = 1.888 (1.11-3.19)) at diagnosis. After treatment, the -1237C allele was associated with a better chance of complete remission ($p = 0.031$, OR (CI 95%) = 0.486 (0.25-0.95)), a lower risk of distant metastasis ($p = 0.028$, OR (CI 95%) = 0.435 (0.18-1.02)), and a lower risk of death by NPC ($p = 0.003$, OR (CI 95%) = 0.20 (0.06-0.67)). Kaplan-Meier analysis showed that patients with -1237CC and -1237TC genotypes had a better overall survival (OVS) ($p < 0.01$) and distant metastasis-free survival (DMFS) ($p < 0.05$). A multivariate analysis revealed that TLR9-1237 T/C polymorphism was an independent prognostic factor in OVS ($p = 0.02$; HR = 0.244) and DMFS ($p = 0.048$; HR = 0.388). The transcriptomic analysis showed that the mRNA expression was reduced in patients with larger tumor size (T4) ($p = 0.013$) and advanced clinical stage (SIII-SIV) ($p = 0.037$). The TLR9 mRNA expression was inversely correlated with tumor size ($p = 0.014$; $r = -0.314$) at diagnosis. Our results indicated for the first time that the functional -1237 T/C polymorphism and mRNA expression of TLR9 gene may be considered as protective factors for NPC severity and progression.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a tumor derived from the epithelial cells and usually occurs in the fossa of Rosenmüller. NPC is a distinct entity compared to other epithelial cancers of the head and neck regions in its radiosensitivity, close association with EBV infection, and remarkable geographic distributions.

While it is rare in most parts of the world such as Europe and North America, this disease is the major cause of cancer death in China, particularly among people of Cantonese origin where the incidence exceeds 25 per 100000 (man/year)

(26.6/100000 in Guangdong, 15.7/100.000 in Maca, and 14.4/100000 in Hong Kong) [1]. Intermediate incidence rates were recorded in Southeast Asia and in North Africa, principally in Tunisia, Algeria, and Morocco, where the incidence rates reached 2-5 per 100000 inhabitants [2, 3]. The unequal geographic distribution in NPC incidence suggests that NPC is a multifactorial disease which results from the combined action of multiple etiological factors such as lifestyle, EBV infection, and genetic factors.

Various environmental factors such as salt-preserved food intake, tobacco consumption, and fume inhalation increase the risk of developing NPC [4, 5]. Likewise, exposure to Epstein-Barr virus has been consistently linked with the risk of developing NPC. In our previous study, we have demonstrated that in untreated NPC patients the EBV viral load was approximately 100 times higher compared with healthy controls [6]. More recently, it has been shown that high plasma EBV-DNA copies decrease the survival rates of patients with nasopharyngeal cancer [7]. During viral infection, the innate immune system is our first line of defense that organizes host responses to prevent the replication of the virus. The family of Toll-like receptors (TLRs) is an important factor mediating the interaction between viral agents and the host immune response. The TLRs are transmembrane pattern-recognition receptors (PRRs) that mediate innate immune responses when exposed to pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide, microbial RNA, or DNA [8]. So far, 13 TLRs have been identified in mammals and 10 in humans [9].

The human TLR9 is a DNA receptor that recognizes unmethylated nucleic acid containing Cytosine-phosphate-Guanine (CpG) motifs present in bacteria and viruses [10]. In the innate immune system, the single nucleotide polymorphisms (SNPs) within the TLR9 gene may alter the ability of the receptor to respond correctly to TLR9 ligands. They also participate in the physiopathology of many inflammatory and immune diseases, including cancer [11]. The SNP may influence gene expression, mRNA stability, protein function, and subcellular localization of mRNAs and/or proteins [12]. In the last years, several genetic association studies have demonstrated the impact of TLR9 polymorphisms on several cancers, including lymphoma, Hodgkin's lymphoma, endometrial cancer, gastric cancer, non-Hodgkin's lymphoma, cervical cancer, acute myeloid leukemia, breast cancer, and colorectal cancer [13–21].

Two functional SNPs (TLR9-1486 T/C (rs187084) and TLR9-1237 T/C (rs5743836)) are located in the promoter. They modify the transcriptional activity and the gene expression [22, 23]. The aim of the present study was to investigate firstly the effect of TLR9 promoter polymorphism on NPC pathogenesis at diagnosis and after treatment and their impact on survival rates. Secondly, the study was aimed at evaluating the expression levels of TLR9 mRNA in peripheral blood mononuclear cells (PBMCs) of NPC patients and their association with the NPC pathogenesis.

2. Materials and Methods

2.1. Study Population. This study included 322 patients with NPC. They were recruited from the Department of Radiation Oncology and Medical Oncology at Farhat Hached University Hospital. The study was approved by the National Ethical Committee, and an informed consent was obtained from all enrolled individuals prior to their participation. Age varied from 10 to 85 years, with a mean age of 42.62 ± 15.46 (Table 1). The distribution of age-specific rates for NPC patients showed a bimodal age distribution, with the first peak between 10 and 22 and the second one around the age of fifty. Respecting the distribution bimodal of age in intermediate risk regions, young patients were defined as those aged 35 years or under while adult patients were over 35. The sex ratio was 2.19 (219 men, 100 women). The diagnosis of NPC was confirmed by a histopathological analysis. In Tunisian NPC patients, the UCNT (undifferentiated carcinoma, type III, WHO classification) is the most predominant type in the histological repartition (89.6%) compared with differentiated types (only 5.3%) (type I, II, WHO classification) [24]. In accordance with the 1997 TNM staging of

Table 1: Demographic and clinical characteristics of the study population.

Characteristics	Patients ($n = 322$)	
	n	f (%)
Mean age (mean ± SD)	42.62 ± 15.46	
Age at diagnosis (years)[a]		
≤35	104	32.3
>35	215	66.8
Sex[a]		
Women	100	31.1
Men	219	68
Tumor size[a]		
T1-T2	141	43.8
T3-T4	178	55.3
Lymph node status[a]		
N0	69	21.5
N+	250	77.6
Metastasis[a]		
M0	285	88.5
M+	21	6.5
Clinical stage[a]		
SI-SII	24	7.5
SIII-SIV	294	91.3
State after treatment[a]		
Complete remission (CR)	215	66.9
Locoregional recurrence (LRR)	32	9.9
Distant metastasis (DM)	31	9.6
Locoregional recurrence and distant metastasis (LRR+DM)	13	4
Death after treatment and after recurrence[a]		
No	252	78.3
Yes	39	12.1

f: frequencies; SD: standard deviation. [a]The sum does not equal the total due to unavailable data.

malignant tumors, the NPC clinical stages ranged from I to IV [25]. In the present study, all NPC patients were diagnosed with UCNT with a lymph node extension N+ (77.6%), advanced clinical stage III, IV (91.3%) (Table 1).

At the time of this study, 215 patients were in complete remission and 79 patients relapsed after treatment. Among these, 32 patients developed locoregional recurrence, 31 patients developed distant metastasis, and 13 patients had both types of relapse after treatments. In our population, nearly half of the patients who relapsed early died of nasopharyngeal cancer (39 patients, 49.4%) (Table 1).

2.2. Genomic DNA Extraction and TLR9 Genotyping Analysis. Peripheral blood was collected in EDTA tubes. After centrifugation, genomic DNA was extracted from the blood sample by a salting out method [26, 27].

TLR9 promoter polymorphisms (TLR9-1486 T/C (rs187084) and TLR9-1237 T/C (rs5743836)) were genotyped using the restriction fragment length polymorphism of polymerase chain reaction (RFLP-PCR) analysis. All PCR reactions were performed in a 25 μl reaction volume containing 100 ng of genomic DNA, 200 μM dNTP, 1x Taq polymerase buffer with $MgCl_2$, unit of Taq DNA polymerase (Promega, Paris, France), and 0.3 μM of suitable primers described previously [28, 29]. For the genotyping of TLR9-1486 T/C polymorphism, 145 pb was amplified after 35 cycles of PCR with the following temperature program: denaturation at 95°C for 30 seconds, annealing primers at 62°C for 30 seconds, and primer extension at 72°C for 30 seconds. For TLR9-1237 T/C polymorphism, 154 pb was amplified using the following conditions, thermal cycler (Biometra, Göttingen, Germany): 95°C for 5 min followed by 30 cycles (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) and a final elongation step carried out at 72°C for 5 min.

After amplification, the PCR products were digested at 37°C overnight with 4 U of AflII restriction enzyme (Thermo Scientific) for the −1486 polymorphism and with 2 U of BstNI restriction enzyme (Thermo Scientific) for the −1237 polymorphism. The digestion products were then separated by a 3% agarose gel stained with ethidium bromide and visualized with ultraviolet light. After electrophoresis, for the -1486 T/C polymorphism, the -1486T allele was detected by the presence of 111 and 34 bp bands, whereas the -1486C allele was defined by the loss of the AflII site, yielding an undigested 145 bp fragment. For -1237 T/C, homozygous T alleles were represented by DNA bands with sizes at 129 and 25 bp. The presence of 81, 48, and 25 pb size fragments designated the homozygous C alleles while the heterozygous genotype displayed a combination of both alleles (129, 81, 48, and 25 bp). The band 25 pb indicated the digestion in the constant restriction site. All restricted fragments were analyzed by electrophoresis on a 3% agarose.

2.3. Quantitative Real-Time Reverse Transcription PCR (Real-Time RT-PCR) for TLR9 Gene Expression. Quantitative real-time PCR (qPCR) was used to evaluate TLR9 mRNA expression in the PBMCs. Peripheral blood samples were collected from patients in EDTA vacutainer tubes, and the PBMCs were isolated using the Ficoll density gradient technique. The total RNA was extracted from PBMC using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Using a NanoDrop 2000c Spectrophotometer, RNA concentration was assessed by measuring absorption at 260 nm and the purity was evaluated by measuring the 260/280 nm ratio. The integrity of the total RNA was then checked on 1% agarose gel by the presence of ribosomal RNA (rRNA) bands (28S and 18S). One hundred microgram of total RNA was reverse transcribed into cDNA by the use of the first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) which uses random Hexamer and Oligo (Dt) primers. For real-time PCR, 2 microliters of the cDNA was used and the reaction was carried out using iQ™ SYBR® Green Supermix (Bio-Rad, CA) in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The cycling conditions were initiated by a denaturation step at 95°C for 10 min which was followed by 40 cycles at 95°C for 15 s and 58.2°C for 1 min. For normalization, the housekeeping GAPDH gene was used for normalization as an internal control. The relative expression of TLR9 gene was determined using the $2^{-\Delta\Delta CT}$ method. The PCR primers used in TLR9 quantification were described previously [30, 31].

2.4. Statistical Analysis. The chi-square test (χ^2) was used to evaluate any significant association between clinicopathological features (age "≤35 years versus >35 years," sex "men versus women," primary tumor extension "T1-T2 versus T3-T4," regional lymph node extension "N0 versus N+," metastatic status "M0 versus M+," and clinical stages "I-II versus III-IV") and TLR9 polymorphisms. In the present study, we investigated the association between TLR9 promoter polymorphisms and responses after treatment: recurrence after treatment "No versus Yes," locoregional recurrence after treatment "No versus Yes," distant metastasis after treatment "No versus Yes," and death after treatment and after recurrence "No versus Yes." Fisher's exact test was appropriate when the sample sizes were small (sample is less than 5). Odds ratios (OR) and 95% confidence intervals (CI) were calculated to estimate the relative risk.

Nasopharyngeal carcinoma-specific overall survival (OVS) was defined as the time from the date of diagnosis to death if the patient died from nasopharyngeal carcinoma or to the last contact. Distant metastasis-free survival (DMFS) was defined as the time from the date of diagnosis to the first metastasis or to the last contact, and (LRRFS) locoregional relapse-free survival was defined as the time from the date of diagnosis to the first locoregional relapses or to the last contact. Survival rates were estimated using the Kaplan-Meier method for calculating survival curves. The differences between groups were calculated by the log-rank test. Univariate and multivariate analyses were carried out to identify the impact of clinicopathological variables and TLR9 promoter polymorphisms on survival rates. The hazard ratio (HR) and 95% CI were calculated using the Cox regression analysis. Variables with a p value less than 0.01 in the univariate model and TLR9 promoter polymorphisms were included as covariates in the multivariate model.

Table 2: Promoter TLR9 polymorphisms and their associations with the clinicopathological parameters at diagnosis.

Clinicopathological parameters	TLR9-1486		TLR9-1237	
	TT	TC+CC	TT	TC+CC
Age at diagnosis ($n = 319$)				
≤35	35 (0.34)	69 (0.66)	74 (0.71)	30 (0.29)
>35	71 (0.33)	144 (0.67)	164 (0.76)	51 (0.24)
p		0.910		0.324
OR (CI 95%)	1	1.028 (0.62-1.68)	1	0.767 (0.45-1.30)
Sex ($n = 319$)				
Women	35 (0.35)	64 (0.65)	68 (0.69)	31 (0.31)
Men	71 (0.32)	149 (0.68)	169 (0.77)	51 (0.23)
p		0.588		0.124
OR (CI 95%)	1	1.147 (0.70-1.89)	1	0.662 (0.39-1.12)
Tumor size ($n = 319$)				
T1–T2	40 (0.28)	101 (0.72)	114 (0.81)	27 (0.19)
T3–T4	65 (0.36)	113 (0.64)	123 (0.69)	55 (0.31)
p		0.124		**0.017**
OR (CI 95%)	1	0.688 (0.43-1.11)	1	**1.888 (1.11-3.19)**
Lymph node status ($n = 319$)				
N0	19 (0.28)	50 (0.72)	54 (0.78)	15 (0.22)
N+	86 (0.34)	164 (0.66)	183 (0.73)	67 (0.27)
p		0.283		0.394
OR (CI 95%)	1	0.725 (0.40-1.30)	1	1.318 (0.69-2.49)
Metastasis ($n = 306$)				
M0	90 (0.32)	195 (0.68)	214 (0.75)	71 (0.25)
M+	8 (0.38)	13 (0.62)	17 (0.81)	4 (0.19)
p		0.536		0.792[a]
OR (CI 95%)	1	0.750 (0.30-1.87)	1	0.709 (0.23-2.17)
Clinical stage ($n = 318$)				
SI-SII	7 (0.29)	17 (0.71)	21 (0.88)	3 (0.12)
SIII-SIV	98 (0.33)	196 (0.67)	215 (0.73)	79 (0.27)
p		0.676		0.149[a]
OR (CI 95%)		0.824 (0.33-2.05)	1	2.572 (0.75-8.86)

[a]Fisher's exact test; OR: odds ratio; CI: confidence interval.

To evaluate the role of TLR9 expression in NPC prognosis, the nonparametric Mann-Whitney U test wild-type was used. Spearman's rank correlation test was used to determine the direction of the relationship between the TLR9 mRNA expression levels and clinicopathological characteristics.

The data were analyzed using the Epi-Info statistical program (Version 7.1.3.10; Atlanta, USA) and a statistical software IBM SPSS for Windows (SPSS 20.0 SPSS Inc., Chicago, IL). For all experiments, a value of $p = 0.05$ was considered to indicate a statistically significant result.

3. Results

3.1. TLR9 Promoter Polymorphisms and Their Associations with NPC Clinicopathological Characteristics. The comparison of frequency distribution of TLR9 promoter genotypes with regard to demographic features of patients (sex and age) did not show any significant association (Table 2). According to the clinicopathological characteristics of NPC at diagnosis, we showed a significantly higher TLR9−1237 TC+CC distribution in the T3-T4 subgroup than in the T1-T2 subgroup. Therefore, patients with the TC+CC genotype had at the time of diagnosis a significantly larger tumor size compared to the individuals with the TT genotype ($p = 0.017$; OR (CI 95%) = 1.888 (1.11-3.19)). No significant associations were found between the other clinicopathological parameters and TLR9-1237 T/C polymorphism (Table 2). No significant associations were found between the clinicopathological parameters and TLR9-1486 T/C polymorphism either (Table 2).

In this study, we investigated the association between TLR9 promoter polymorphisms (TLR9-1486, TLR9-1237) and responses after treatment parameters including recurrence "No: complete remission versus Yes: relapse"; locoregional recurrence "No: negative locoregional recurrence versus Yes: positive locoregional recurrence"; distant

Table 3: Promoter TLR9 polymorphisms and their associations with the responses after treatment.

Responses after treatment parameters	TLR9-1486		TLR9-1237	
	TT	TC+CC	TT	TC+CC
Recurrence ($n = 291$)				
No	22 (0.29)	54 (0.71)	63 (0.83)	13 (0.17)
Yes	74 (0.34)	141 (0.66)	151 (0.70)	64 (0.30)
p		0.383		**0.031**
OR (CI 95%)	1	1.288 (0.73-2.28)	1	**0.486 (0.25-0.95)**
Locoregional recurrence ($n = 260$)				
No	74 (0.34)	141 (0.66)	151 (0.70)	64 (0.30)
Yes	13 (0.29)	32 (0.71)	38 (0.84)	7 (0.16)
p		0.474		**0.051**
OR (CI 95%)	1	1.292 (0.63-2.61)	1	**0.435 (0.18-1.02)**
Distant metastasis ($n = 259$)				
No	74 (34.4)	141 (65.6)	151 (70.2)	64 (29.8)
Yes	13 (29.6)	31 (70.5)	38 (86.4)	6 (13.6)
p		0.533		**0.028**
OR (CI 95%)	1	1.252 (0.62-2.53)	1	**0.373 (0.15-0.92)**
Death ($n = 291$)				
No	85 (0.34)	167 (0.66)	178 (0.71)	74 (0.29)
Yes	11 (0.23)	28 (0.78)	36 (0.92)	3 (0.08)
p		0.494		**0.003**[a]
OR (CI 95%)	1	1.29 (0.62-2.73)	1	**0.20 (0.06-0.67)**

[a]Fisher's exact test; OR: odds ratio; CI: confidence interval.

metastasis "No: negative distant metastasis versus Yes: positive distant metastasis"; and death after treatment and after recurrence "No: alive versus Yes: death."

Same significant associations were observed in the recurrence, distant metastasis, and death parameters (Table 3). Consequently, patients carrying the mutant allele -1237C (TC+CC) had a better chance of complete remission after treatment compared to those with the TT genotype ($p = 0.031$, OR (CI 95%) = 0.486 (0.25-0.95)). The same group of patients (TC+CC) was at low risk of developing distant metastasis ($p = 0.028$, OR (CI 95%) = 0.435 (0.18-1.02)) and at lowest risk of death ($p = 0.003$, OR (CI 95%) = 0.20 (0.06-0.67)) after treatment compared to patients with the TT genotype. The association was marginal between TLR9-1237 T/C and locoregional recurrence after treatment parameter ($p = 0.051$, OR (CI 95%) = 0.435 (0.18-1.02)). Unlike the TLR9-1237 T/C polymorphism findings, no significant associations were found between TLR9-1486 T/C and NPC responses after treatment (Table 3).

3.2. Survival Analysis and Prognostic Significance of TLR9 Promoter Polymorphisms. One of the main objectives of this study was to identify the relationship between the distribution of TLR9 promoter genotypes and the survival percentage (OVS, DMFS).

When we tested the relationship between TLR9-1237 genotypes in all the 292 patients and the survival (OVS, DMFS, and LRRFS), a significant difference was observed between the Kaplan-Meier OVS curves ($p < 0.01$) and DMFS curves ($p < 0.05$). Thus, 5 years after a cancer diagnosis, patients with CC and TC genotypes had a significantly better overall survival (100%) compared to patients with the wild-type genotype (TT) (78.2%) (Figure 1(a)). Therefore, the mean survival time was 65 and 44.2 months for patients with the variant genotypes CC and TC, respectively, and 43.5 months for those with the wild-type genotype TT (Figure 1(a)). The same significant difference was found in distant metastasis-free survival (DMFS) according to TLR9-1237 genotypes among the whole NPC patients. So, patients who were homozygous for the TLR9-1237 TT (79.4%) had a worse DMFS compared to those with other genotypes TC (92.2%) and CC (100%) ($p < 0.05$) (Figure 1(b)).

Although there was no statistical difference in LRRFS ($p > 0.05$), patients with CC and TC genotypes were at lower risk of LRRFS compared to patients carrying TT genotypes (Figure 1(c)).

Specific OVS rates were estimated and compared to previous clinicopathological parameters. Same significant associations with OVS were found for tumor size, lymph node extension, clinical stages, age subgroups, and sex (data not shown). Patients with TLR9-1237 variant genotypes (TC, CC) had a significantly better OVS compared to those with the wild-type genotype (TT). This was especially obvious in patients who had a large tumor size ($p < 0.02$), positive lymph node extension ($p < 0.01$), and advanced clinical stage ($p < 0.01$), were men ($p < 0.05$), and were older than 35 ($p < 0.02$) (data not shown). No significant differences were observed with different Kaplan-Meier survival curves (OVS, DMFS, and LRRFS) in the distribution of TLR9-1486

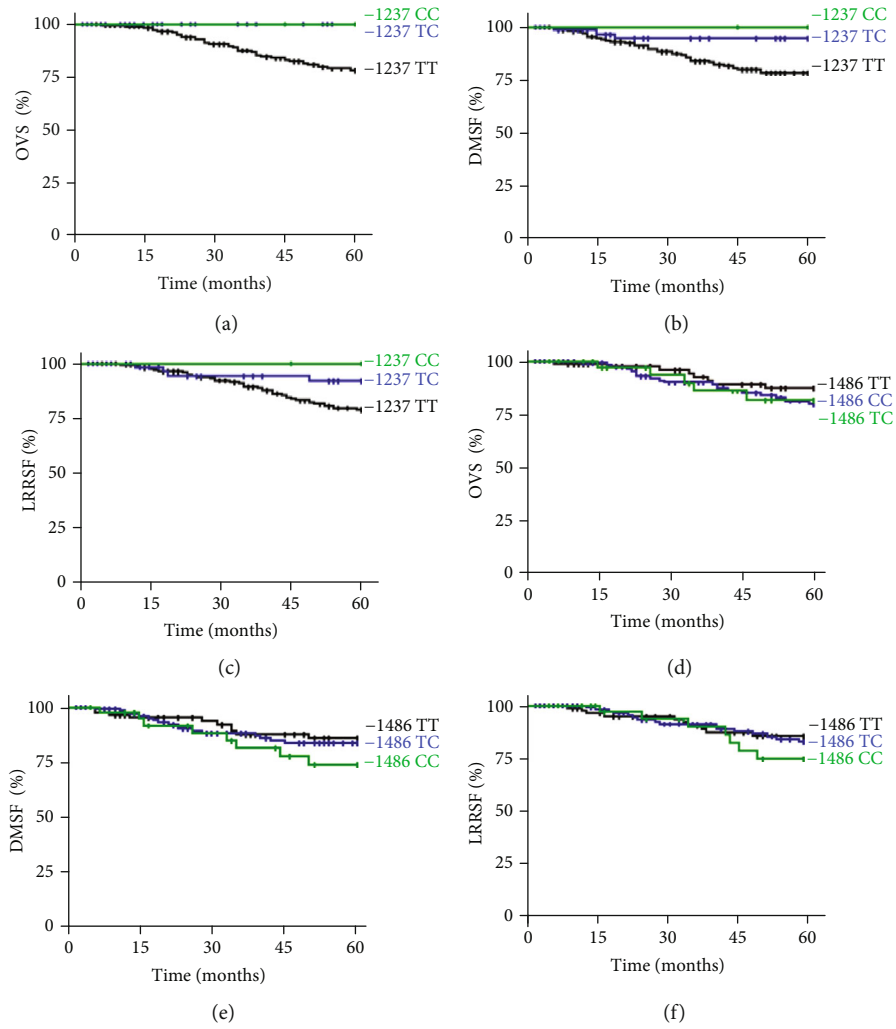

Figure 1: Survival analysis curves. (a, d) Overall survival ($p < 0.01$ and $p > 0.1$, respectively); (b, e) distant metastases-free survival ($p < 0.05$ and $p > 0.1$, respectively); (c, f) locoregional recurrence-free survival of NPC patients according to TLR9-1237 T/C and TLR9-1486 T/C genotypes ($p > 0.1$ and $p > 0.1$, respectively). p denotes the log-rank test value.

genotypes, among all NPC patients (Figures 1(d)–1(f)) and among the different clinicopathological parameters.

In this study, univariate and multivariate Cox regression analyses were performed to assess the effects of clinicopathological factors and the TLR9 promoter polymorphisms on OVS, DMFS, and LRRFS. The univariate analysis for OVS found prognosis to be significantly correlated to 3 clinicopathological factors at diagnosis. Therefore, we showed that age ($p = 0.001$, HR = 4.622), sex ($p = 0.034$, HR = 2.322), and metastases ($p = 0.007$, HR = 3.708) were significantly correlated with decreases of OVS. However, TLR9-1237 T/C was a significant prognostic factor for a better survival rate ($p = 0.010$, HR = 0.215) (Table 4). In a multivariate analysis, when age, metastases, and TLR9 promoter polymorphisms were included, we found that age, metastases, and TLR9-1237 T/C were independent prognostic factors (Table 4).

The multivariate analysis of DMFS showed that sex, metastasis at diagnosis, and TLR9-1237 T/C polymorphism were independent prognostic factors in DMFS (Table 4). For LRRFS, particular age was an independent prognostic factor (Table 4).

3.3. TLR9 Expression and Their Associations with Pathogenesis of NPC Cancer and with TLR9 Promoter Polymorphisms. In this study, we also analyzed the relation between TLR9 mRNA expression level in PBMCs and clinicopathological characteristics of NPC. As shown in Table 5, TLR9 mRNA expression was significantly reduced in NPC patients with advanced state of the disease (patients with larger tumor size (T4)) ($p = 0.013$) and advanced clinical stage (SIII-SIV) ($p = 0.0374$) (Table 5) compared to patients with early state of the disease (T1, T2) (SI-SII), respectively. The transcriptomic analysis also showed that the relative expression of TLR9 mRNA was inversely correlated with the tumor size at diagnosis ($p = 0.014$; $r = -0.314$) but not with clinical stage ($p = 0.117$; $r = -0.177$) (Figure 2(a)).

4. Discussion

TLR9 is a PRR that is involved in the detection of intracellular unmethylated Cytosine-phosphate-Guanine (CpG) motives in DNA pathogen. This receptor is implicated in the activation of both innate and adaptive immune responses

Table 4: Univariate and multivariate Cox proportional hazards model for prognostic significance of pathologic features and *TLR9* promoter polymorphisms on overall survival (OVS), distant metastases-free survival (DMFS), and locoregional recurrence-free survival (LRRFS).

	Overall survival (OVS)						Distant metastases-free survival (DMFS)						Locoregional recurrence-free survival (LRRFS)					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Age[a]	4.622	1.80-11.83	0.001	4.05	1.57-10.4	0.004	2.540	1.22-5.28	0.013	ni			2.816	1.35-5.84	0.005	2.716	1.30-5.65	0.008
Sex[b]	2.322	1.06-5.05	0.034	ni			3.134	1.39-7.02	0.006	2.855	1.19-6.84	0.019	2.496	1.20-5.17	0.014	ni		
Tumor size[c]	0.657	0.35-1.23	0.190	ni			0.739	0.41-1.32	0.310	ni			0.991	0.55-1.78	0.979	ni		
Lymph node[d]	0.965	0.48-1.94	0.920	ni			1.269	0.26-2.56	0.507	ni			1.378	0.68-2.77	0.370	ni		
Metastasis[e]	3.708	1.44-9.54	0.007	3.35	1.29-8.69	0.013	5.264	2.42-11.42	0.00002	5.016	1.19-6.84	0.00007	1.130	0.27-4.67	0.866	ni		
Clinical stage[f]	0.910	0.323-2.56	0.858	ni			1.376	0.426-4.44	0.593	ni			1.496	0.46-4.82	0.500	ni		
TLR9-1486 T/C[g]	1.416	0.70-2.84	0.328	ns			1.308	0.68-2.50	0.417	ns			1.365	0.71-2.60	0.344	ns		
TLR9-1237 T/C[h]	0.215	0.06-0.697	0.010	0.244	0.75-0.798	0.02	0.414	0.17-0.98	0.045	0.388	0.15-1.00	0.048	0.468	0.20-1.04	0.065	0.507	0.22-1.13	0.099

HR: hazard ratio; ni: not included in multivariate analysis; ns: nonsignificant; a: ≤35 versus >35 years; b: men versus women; c: T1-T2 versus T3-T4; d: N0 versus N+; e: M0 versus M+; f: I–II versus III-IV; g, h: TT versus TC+CC.

Table 5: Relative expression of *TLR9* mRNA according to NPC clinicopathological parameters at diagnosis.

NPC clinicopathological parameters (*n*)	Median of TLR9 expression (*E*-03)	*p* value[a]
Age		
Age ≤ 35 (17)	2.15	0.696
Age > 35 (33)	2.24	
Sex		
Women (14)	2.40	0.419
Men (36)	2.01	
Tumor size		
T1-T2-T3 (36)	2.52	**0.013**
T4 (13)	1.35	
Lymph node		
N0 (5)	3.13	0.469
N+ (44)	2.11	
Metastasis		
M0 (42)	2.20	0.653
M+ (4)	1.94	
Clinical stage		
SI-SII (6)	3.46	**0.0374**
SIII-SIV (41)	2.03	

a: Mann-Whitney's test.

through the stimulation of various cell types, especially B cells and pDC cells. The capacity to respond properly to the TLR9 ligand may be damaged by single nucleotide polymorphisms (SNPs) within the TLR9 gene. They alter susceptibility to inflammatory diseases, infectious diseases, and cancers. However, in this study, we tried to find out if TLR9 promoter polymorphisms (TLR9-1486 T/C, TLR9-1237 T/C) were implicated in NPC physiopathology.

In this study, we demonstrated that the genetic variation in TLR9 gene might influence NPC progression (prognostic). Therefore, some significant associations were reported in our study between TLR9-1237 T/C (rs5743836) and clinicopathological parameters at the time of diagnosis, after treatment and survival rate of NPC patients. Our findings indicate that the -1237C mutated allele was associated with large tumor sizes at the time of diagnosis. This result can be explained under hypotheses based on the role of viral infection in the NPC pathogenesis and its close relationship with the TLR9 receptor, one of the TLR family that recognizes EBV DNA [32]. The endemic nature as well as carcinogenesis of NPC is considered a consequence of Epstein-Barr virus (EBV) infection, which is one major NPC etiological factor. The presence of viral proteins in tumor cells and NPC biopsies highlights the role of EBV in NPC development [33, 34]. Other than TLR9 stimulation, EBV may use TLR9 in order to escape from the host's immune surveillance [35]. During infection, EBV expresses BGLF5, EBV lytic-phase protein, which contributes to downregulating TLR9 levels through RNA degradation [36]. Fathallah et al. showed that the oncoprotein latent membrane protein 1 (LMP1) is a strong inhibitor of TLR9 transcription. However, overexpression of LMP1 reduces TLR9 promoter activity, mRNA, and protein levels [37]. Moreover, it has been demonstrated that the involvement of TLR9-MyD88 signaling by EBV inhibited TLR9 expression [38]. Therefore, we propose the hypothesis that, in NPC tumor cells, the presence of the mutated allele is associated with higher TLR9 mRNA expression levels. Since the viral proteins are present in NPC tumor cells, they can inhibit and degrade the TLR9 mRNA. Thus, EBV can escape the immune system from their hosts. Such a mechanism can suppress cancer immunity and promote tumor growth. Independent of EBV infection, it has been demonstrated that BL cells expressing TLR9-1237C allele are more resistant to apoptosis compared to cells expressing TLR9-1237T allele [39]. On the basis of these results, we may suppose that in NPC tumor cells, the presence of the TLR9-1237C allele can help tumor cells to escape apoptosis and enhance tumor growth.

In the present study, we also showed that after treatment, the same mutated TLR9-1237C allele was associated with both a better chance of complete remission and a protective effect against locoregional recurrence and distant metastasis. We have also proved that the mutated TLR9-1237CC genotype is associated with a better overall survival (OVS), distant metastasis-free survival (DMFS), and locoregional recurrence-free survival (LRRFS), among all NPC patients. These results seem contradictory to those reported here which show that the -1237C mutated allele was associated with large tumor size. These results can be explained under several hypotheses including both the presence of the TLR9-1237C mutated allele and the EBV viral load after treatment, just like the immunogenic role of radiotherapy. Our patients were recruited from the Department of Radiation Oncology. Patients with NPC are typically treated with radiation therapy (RT) rather than surgery because of the NPC anatomical limitations as well as high radiosensitivity. In our previous study, Hassen et al. showed that after treatment, the EBV viral load level declined significantly compared to that before treatment [6]. Recently, it has been revealed that the intensity-modulated radiotherapy (IMRT) reduces the number of DNA-EBV copies [40]. Such results may illustrate that treatment reduces the presence of EBV in NPC patients. Consequently, the EBV inhibitor effect on TLR9 was decreased. Thereafter, the TLR9 receptor was expressed after treatment. TLR9 is highly expressed in patients with the mutated TLR9-1237CC genotype compared to the other patients (with TT and TC genotypes) knowing that the presence of the TLR9-1237C mutated allele appears to stimulate the host immune response copies [22, 41]. Carvalho et al. reported that the C allele of the TLR9-1237 T/C polymorphism generates an IL 6-responding element. In this study, it was shown that in peripheral blood mononuclear cells (PBMCs) carrying the TLR9-1237C variant allele (with TC genotype), IL 6 upregulates TLR9 expression, which exacerbates cellular responses to CpG, including IL 6 production and B cell proliferation [22].

Otherwise, radiotherapy destroys cancer by emitting high rays on the cancer cells. This treatment kills cells by damaging their DNA and molecules that make up cancer cells. We think that such a process may lead to the release of EBV DNA in the host body. Furthermore, the stimulation of the

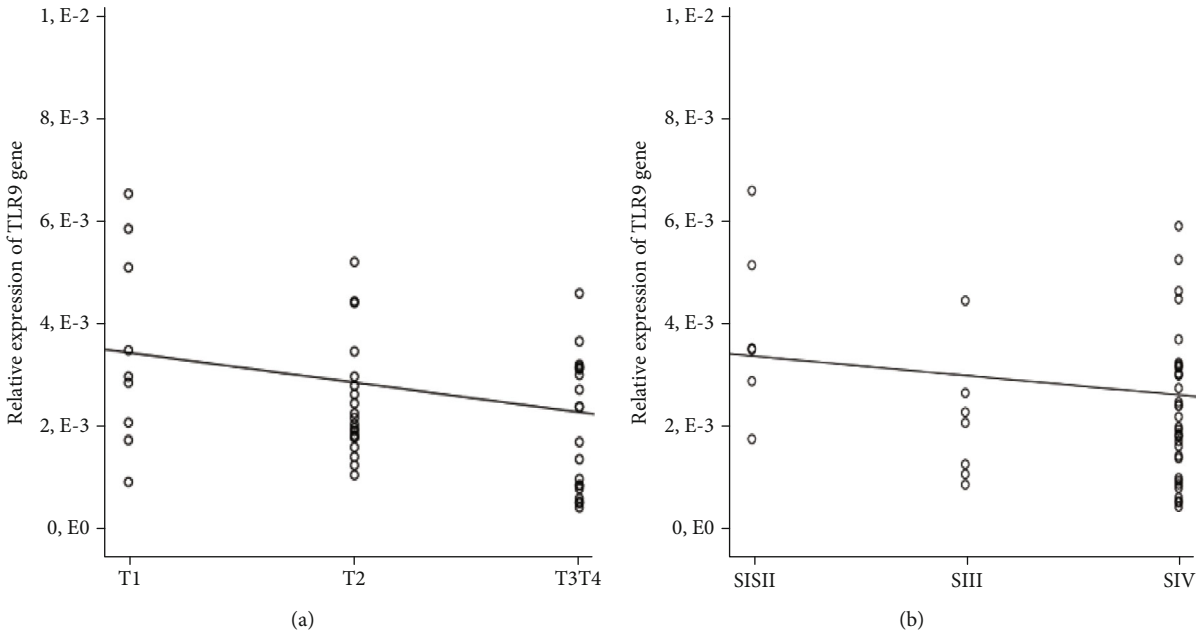

Figure 2: Relative expression of TLR9 mRNA according to (a) tumor size ($p = 0.014$, $r = -0.314$) and (b) clinical stage ($p = 0.017$, $r = -0.177$) (unilateral Spearman's correlation).

immune system would result from activation of the TLR9 signaling. Thus, it has been demonstrated that TLR9-mediated detection of EBV DNA by pDCs leads to type I IFN (IFN-I) production. Such a process is involved in the simulation of the innate immune cells and promotes adaptive immune responses [42]. We may, therefore, conclude that the findings reported in this study would be due to the combined immunostimulant effects of radiation therapy and the presence TLR9-1237C allele which in turn increases TLR9 expression.

Several studies are interested in examining the effect of combining RT with TLR9 stimulation on antitumoral immunity, primary tumor growth retardation. Therefore, it has been demonstrated that CpG ODN 107 (TLR9 agonist) combined with irradiation (5 Gy) significantly suppresses the growth of glioma cell line in vitro. In vivo, this same study showed that the survival rate of mice was significantly increased by treatment with TLR9 agonist in combination with radiotherapy (10 Gy) compared with local radiotherapy alone in an orthotropic implantation model of nude mice. Such a combination therapy significantly decreases the microvessel density (MVD), VEGF level, and HIF-1 α expression [43]. Indeed, it has been demonstrated that TLR9 stimulation combined with RT treatment leads to humoral antitumor immune responses, increases tumoral infiltration, reduces pulmonary metastases, and improves the survival in mice bearing a murine lung adenocarcinoma (Lewis lung adenocarcinoma) [44]. Another study proved, in the animal models, that tumor therapeutic vaccine compound of TLR9 agonists (CpG ODN) and irradiated tumor cell tracking of two other CpG ODNs injections lead to a long-term antitumor immune response against aggressive tumors. Repeated vaccination improves experimental animals' survival compared with a single vaccination [45]. A similar combination therapy with radiotherapy and TLR stimulation was applied in patients with low-grade B cell lymphoma which develop specific CD4+ T cell response against a tumor [46]. It has been demonstrated that in non-small-cell lung cancer (NSCLC) cells, radiotherapy combined with TLR9 stimulation by the CpG ODN 7909 was able to downregulate PD-L1 (programmed death-ligand 1) expression which plays an important role in tumor immune escapes [47, 48]. All these results show that CpG ODN TLR9 stimulation is the potent enhancer of tumor response and as such has a potential to improve clinical radiotherapy bearing in mind that standard CpG oligonucleotides have been shown to be highly active in murine models while showing limited activity in humans.

5. Conclusion

The associations observed in this study suggest for the first time the involvement of functional promoter polymorphisms and mRNA expression of TLR9 gene in the NPC pathogenesis at the time of diagnosis, after radiotherapy treatment, and in NPC survival. Therefore, our results allowed us not only to better understand the impact of the TLR9 gene in NPC severity at the time of diagnosis but also to predict the role of the TLR9 gene in the progression of the disease after treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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References

[1] D. Forman, F. Bray, D. H. Brewster et al., *Cancer Incidence in Five Continents, Vol. X (Electronic Version)*, International Agency for Research on Cancer, Lyon, 2013.

[2] M. Hamdi Cherif, D. Serraino, A. Mahnane et al., "Time trends of cancer incidence in Setif, Algeria, 1986–2010: an observational study," *BMC Cancer*, vol. 14, no. 1, 2014.

[3] B. A. H. Wided, B. Hamouda, H. Hamadi, and B. A. Mansour, "Nasopharyngeal carcinoma incidence in North Tunisia: negative trends in adults but not adolescents, 1994-2006," *Asian Pacific Journal of Cancer Prevention*, vol. 16, no. 7, pp. 2653–2657, 2015.

[4] B. J. Feng, M. Khyatti, W. Ben-Ayoub et al., "Cannabis, tobacco and domestic fumes intake are associated with nasopharyngeal carcinoma in North Africa," *British Journal of Cancer*, vol. 101, no. 7, pp. 1207–1212, 2009.

[5] L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal, "Global cancer statistics, 2012," *CA: A Cancer Journal for Clinicians*, vol. 65, no. 2, pp. 87–108, 2015.

[6] E. Hassen, K. Farhat, S. Gabbouj, N. Bouaouina, H. Abdelaziz, and L. Chouchane, "Epstein-Barr virus DNA quantification and follow-up in Tunisian nasopharyngeal carcinoma patients," *Biomarkers*, vol. 16, no. 3, pp. 274–280, 2011.

[7] M. Sengar, S. Chorghe, K. Jadhav et al., "Cell-free Epstein-Barr virus-DNA in patients with nasopharyngeal carcinoma: plasma versus urine," *Head & Neck*, vol. 38, no. S1, pp. E1666–E1673, 2016.

[8] M. Fukata and M. T. Abreu, "Pathogen recognition receptors, cancer and inflammation in the gut," *Current Opinion in Pharmacology*, vol. 9, no. 6, pp. 680–687, 2009.

[9] T. Kawai and S. Akira, "TLR signaling," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 816–825, 2006.

[10] S. Pandey and D. K. Agrawal, "Immunobiology of Toll-like receptors: emerging trends," *Immunology and Cell Biology*, vol. 84, no. 4, pp. 333–341, 2006.

[11] R. K. Mandal, G. P. George, and R. D. Mittal, "Association of Toll-like receptor (TLR) 2, 3 and 9 genes polymorphism with prostate cancer risk in North Indian population," *Molecular Biology Reports*, vol. 39, no. 7, pp. 7263–7269, 2012.

[12] B. S. Shastry, "SNPs: impact on gene function and phenotype," *Methods in Molecular Biology*, vol. 578, pp. 3–22, 2009.

[13] A. Nieters, L. Beckmann, E. Deeg, and N. Becker, "Gene polymorphisms in Toll-like receptors, interleukin-10, and interleukin-10 receptor alpha and lymphoma risk," *Genes and Immunity*, vol. 7, no. 8, pp. 615–624, 2006.

[14] V. Mollaki, T. Georgiadis, A. Tassidou et al., "Polymorphisms and haplotypes in *TLR9* and *MYD88* are associated with the development of Hodgkin's lymphoma: a candidate -gene association study," *Journal of Human Genetics*, vol. 54, no. 11, pp. 655–659, 2009.

[15] K. A. Ashton, A. Proietto, G. Otton et al., "Toll-like receptor (*TLR*) and nucleosome-binding oligomerization domain (*NOD*) gene polymorphisms and endometrial cancer risk," *BMC Cancer*, vol. 10, no. 1, 2010.

[16] H. M. Zeng, K. F. Pan, Y. Zhang et al., "The correlation between polymorphisms of Toll-like receptor 2 and Toll-like receptor 9 and susceptibility to gastric cancer," *Zhonghua Yu Fang Yi Xue Za Zhi [Chinese Journal of Preventive Medicine]*, vol. 45, no. 7, pp. 588–592, 2011.

[17] A. Carvalho, C. Cunha, A. J. Almeida et al., "The rs5743836 polymorphism in *TLR9* confers a population-based increased risk of non-Hodgkin lymphoma," *Genes & Immunity*, vol. 13, no. 2, pp. 197–201, 2012.

[18] S. Zidi, I. Sghaier, E. Gazouani, A. Mezlini, and B. Yacoubi-Loueslati, "Evaluation of toll-like receptors 2/3/4/9 gene polymorphisms in cervical cancer evolution," *Pathology Oncology Research*, vol. 22, no. 2, pp. 323–330, 2016.

[19] J. Rybka, K. Gębura, T. Wróbel et al., "Variations in genes involved in regulation of the nuclear factor - κB pathway and the risk of acute myeloid leukaemia," *International Journal of Immunogenetics*, vol. 43, no. 2, pp. 101–106, 2016.

[20] M. F. Al-Harras, M. E. Houssen, M. E. Shaker et al., "Polymorphisms of glutathione S-transferase π 1 and toll-like receptors 2 and 9: association with breast cancer susceptibility," *Oncology Letters*, vol. 11, no. 3, pp. 2182–2188, 2016.

[21] I. Messaritakis, M. Stogiannitsi, A. Koulouridi et al., "Evaluation of the detection of Toll-like receptors (TLRs) in cancer development and progression in patients with colorectal cancer," *PLoS One*, vol. 13, no. 6, p. e0197327, 2018.

[22] A. Carvalho, N. S. Osório, M. Saraiva et al., "The C allele of rs5743836 polymorphism in the human *TLR9* promoter links IL-6 and TLR9 up-regulation and confers increased B-cell proliferation," *PLoS One*, vol. 6, no. 11, p. e28256, 2011.

[23] D. Bharti, A. Kumar, R. S. Mahla et al., "The role of TLR9 polymorphism in susceptibility to pulmonary tuberculosis," *Immunogenetics*, vol. 66, no. 12, pp. 675–681, 2014.

[24] A. M. Ben, *Epidémiologie des Cancers en Tunisie: Registre de l'Institut Salah Azaiz (1994-1999)*, Association tunisienne de lutte contre le cancer, Tunis, 2004.

[25] L. H. Sobin and I. D. Fleming, "TNM classification of malignant tumors, fifth edition (1997)," *Cancer*, vol. 80, no. 9, pp. 1803-1804, 1997.

[26] O. Olerup and H. Zetterquist, "HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation," *Tissue Antigens*, vol. 39, no. 5, pp. 225–235, 1992.

[27] H. Memni, Y. Macherki, Z. Klayech et al., "E-cadherin genetic variants predict survival outcome in breast cancer patients," *Journal of Translational Medicine*, vol. 14, no. 1, p. 320, 2016.

[28] J. Hong, E. Leung, A. G. Fraser, T. R. Merriman, P. Vishnu, and G. W. Krissansen, "*TLR2, TLR4* and *TLR9* polymorphisms and Crohn's disease in a New Zealand Caucasian cohort," *Journal of Gastroenterology and Hepatology*, vol. 22, no. 11, pp. 1760–1766, 2007.

[29] R. Ramasawmy, E. Cunha-Neto, K. C. Fae et al., "Heterozygosity for the S180L variant of *MAL/TIRAP*, a gene expressing an adaptor protein in the Toll-like receptor pathway, is associated with lower risk of developing chronic Chagas cardiomyopathy," *The Journal of Infectious Diseases*, vol. 199, no. 12, pp. 1838–1845, 2009.

[30] W. Jiang, M. M. Lederman, R. J. Mohner et al., "Impaired naive and memory B-cell responsiveness to TLR9 stimulation in

human immunodeficiency virus infection," *Journal of Virology*, vol. 82, no. 16, pp. 7837–7845, 2008.

[31] C. A. Opitz, U. M. Litzenburger, U. Opitz et al., "The indoleamine-2,3-dioxygenase (IDO) inhibitor 1-methyl-D-tryptophan upregulates IDO1 in human cancer cells," *PLoS One*, vol. 6, no. 5, article e19823, 2011.

[32] S. Fiola, D. Gosselin, K. Takada, and J. Gosselin, "TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells," *Journal of Immunology*, vol. 185, no. 6, pp. 3620–3631, 2010.

[33] R. Fahraeus, H. L. Fu, I. Ernberg et al., "Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma," *International Journal of Cancer*, vol. 42, no. 3, pp. 329–338, 1988.

[34] L. Xiao, Z. Y. Hu, X. Dong et al., "Targeting Epstein-Barr virus oncoprotein LMP1-mediated glycolysis sensitizes nasopharyngeal carcinoma to radiation therapy," *Oncogene*, vol. 33, no. 37, pp. 4568–4578, 2014.

[35] L. Zauner and D. Nadal, "Understanding TLR9 action in Epstein-Barr virus infection," *Frontiers in Bioscience*, vol. 17, no. 1, pp. 1219–1231, 2012.

[36] M. van Gent, B. D. Griffin, E. G. Berkhoff et al., "EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection," *Journal of Immunology*, vol. 186, no. 3, pp. 1694–1702, 2011.

[37] I. Fathallah, P. Parroche, H. Gruffat et al., "EBV latent membrane protein 1 is a negative regulator of TLR9," *Journal of Immunology*, vol. 185, no. 11, pp. 6439–6447, 2010.

[38] C. Zannetti, P. Parroche, M. Panaye et al., "TLR9 transcriptional regulation in response to double-stranded DNA viruses," *Journal of Immunology*, vol. 193, no. 7, pp. 3398–3408, 2014.

[39] J. Noack, M. Jordi, L. Zauner et al., "TLR9 agonists induced cell death in Burkitt's lymphoma cells is variable and influenced by *TLR9* polymorphism," *Cell Death & Disease*, vol. 3, no. 6, p. e323, 2012.

[40] Q. Chen, W. Hu, H. Xiong et al., "Changes in plasma EBV-DNA and immune status in patients with nasopharyngeal carcinoma after treatment with intensity-modulated radiotherapy," *Diagnostic Pathology*, vol. 14, no. 1, p. 23, 2019.

[41] M. T. H. Ng, R. van't Hof, J. C. Crockett et al., "Increase in NF-B binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease," *Infection and Immunity*, vol. 78, no. 3, pp. 1345–1352, 2010.

[42] M. Severa, E. Giacomini, V. Gafa et al., "EBV stimulates TLR- and autophagy-dependent pathways and impairs maturation in plasmacytoid dendritic cells: implications for viral immune escape," *European Journal of Immunology*, vol. 43, no. 1, pp. 147–158, 2013.

[43] D. Liu, G. Cao, Y. Cen et al., "The radiosensitizing effect of CpG ODN107 on human glioma cells is tightly related to its antiangiogenic activity *via* suppression of HIF-1 α/VEGF pathway," *International Immunopharmacology*, vol. 17, no. 2, pp. 237–244, 2013.

[44] H. Zhang, L. Liu, D. Yu et al., "An in situ autologous tumor vaccination with combined radiation therapy and TLR9 agonist therapy," *PLoS One*, vol. 7, no. 5, p. e38111, 2012.

[45] P. Cerkovnik, B. J. Novakovic, V. Stegel, and S. Novakovic, "Tumor vaccine composed of C-class CpG oligodeoxynucleotides and irradiated tumor cells induces long-term antitumor immunity," *BMC Immunology*, vol. 11, no. 1, p. 45, 2010.

[46] J. D. Brody, W. Z. Ai, D. K. Czerwinski et al., "In situ vaccination with a TLR9 agonist induces systemic lymphoma regression: a phase I/II study," *Journal of Clinical Oncology*, vol. 28, no. 28, pp. 4324–4332, 2010.

[47] X. Chen, Q. Zhang, Y. Luo et al., "High-dose irradiation in combination with toll-like receptor 9 agonist CpG oligodeoxynucleotide 7909 downregulates PD-L1 expression via the NF-κB signaling pathway in non-small cell lung cancer cells," *OncoTargets and Therapy*, vol. Volume 9, pp. 6511–6518, 2016.

[48] M. Zhang, H. Sun, S. Zhao et al., "Expression of PD-L1 and prognosis in breast cancer: a meta-analysis," *Oncotarget*, vol. 8, no. 19, pp. 31347–31354, 2017.

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Research Article

43 kDa Glycoprotein (gp43) from *Paracoccidioides brasiliensis* Induced IL-17A and PGE2 Production by Human Polymorphonuclear Neutrophils: Involvement of TLR2 and TLR4

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The glycoprotein gp43 is the major antigenic/diagnostic component of *Paracoccidioides brasiliensis*, one of the etiologic agents of paracoccidioidomycosis (PCM). Gp43 has protective roles in mice, but due to adhesive properties, this glycoprotein has also been associated with immune evasion mechanisms. The present study evaluated gp43 interaction in vitro with Toll-like receptors 2 and 4 (TLR2 and TLR4) present in polymorphonuclear neutrophils (PMNs) from healthy human individuals and the consequent modulation of the immune response through the expression and release of cytokines and eicosanoids. PMNs were incubated in the absence or presence of monoclonal antibodies anti-TLR2 and anti-TLR4 (individually or in combination) before gp43 stimulation. Then, PMNs were analyzed for the expression of both surface receptors and the detection of intracytoplasmic IL-17A and IL-4 using flow cytometry, while the production of PGE2, LTB4, IL-6, IL-10, IL-12, IFN-γ, and TNF-α was evaluated in the supernatants by enzyme-linked immunosorbent assay (ELISA). Our results showed that gp43 increased TLR2 and TLR4 expression by PMNs and induced PGE2 and IL-17A via TLR4 and TLR2, respectively. Thus, our data suggest that gp43 from *P. brasiliensis* might modulate host susceptibility to the fungal infection by affecting PGE2 and IL-17A production.

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease prevalent in Latin America. The etiological agents belong to the *Paracoccidioides* gender of thermodimorphic fungi, among which *Paracoccidioides brasiliensis* is the most studied species [1–5]. PCM infection initiates through the inhalation of environmental mycelium propagules that reach

the pulmonary alveoli, where they can only survive upon transformation into yeasts, a process mediated by the body's temperature [6, 7]. Disease establishment, progression, and severity depend both on fungi virulence factors as well as the host's immunological response.

P. brasiliensis exhibits in the cytoplasm, and along the cell wall, a 43-kDa glycoprotein (gp43) that is considered the main fungal antigen. It is secreted by the fungus and frequently found in the serum of PCM patients [8–10]. Due to its adhesive properties, gp43 is associated with the fungal virulence factors, induction of cell apoptosis, and modulation of the local and systemic inflammatory response, which may contribute to fungal infection and dissemination to other tissues [11–17]. Inhibition of gp43 expression in genetically modified *P. brasiliensis* resulted in a less severe infection in experimentally infected mice due to diminishing adherence of the fungi to host cell proteins, increased yeast cell phagocytosis, and consequent production and action of proteases responsible for inhibiting fungal tissue diffusion [18]. The first cell types recruited to the infection sites are polymorphonuclear neutrophils (PMNs), which remain in the lesion to form a suppurative granuloma in the chronic phase of this mycosis [19, 20]. Effector and modulatory mechanisms of PMNs are dictated by the recognition of conserved structures presented by the microorganism denominated pathogen-associated molecular patterns (PAMPs), which are recognized by pattern-recognition receptors (PRRs) [21, 22]. Among the PRRs involved in fungal recognition, Toll-like receptors (TLRs) are a family of single-pass type I transmembrane-spanning proteins [23]. Following TLR binding to PAMPS, an intracellular signaling pathway is triggered, promoting the release of inflammatory mediators and modulation of innate and adaptive immunity [24–30]. PMNs can recognize *P. brasiliensis via* both TLR2 and TLR4, leading to the production of TNF-α, IL-8, IL-12, IL-10, prostaglandin E2 (PGE2), and leukotriene (LTB4) [24, 26, 27, 29–31]. Although most studies have evaluated PMN interactions with yeast or the mycelium of *P. brasiliensis*, the interactions and effects of gp43 in PMNs remain unknown. Therefore, the present study assessed whether gp43 is recognized by TLR2 and TLR4 on the surface of human PMNs, consequently modulating the production of immunomodulatory cytokines and eicosanoids.

2. Materials and Methods

2.1. Subjects. Heparinized venous blood was obtained from 7 voluntary healthy women, with age ranging from 19 to 37 years-old, after signing informed consent forms. This study was approved by the Research Ethics Committee from Botucatu Medical School, São Paulo State University (UNESP) (CAAE number: 80098817.8.0000.5411).

2.2. Isolation of Peripheral Blood Polymorphonuclear Neutrophils. Forty microliters of blood were layered above density gradients such as Histopaque 1119 g/ml, followed by Histopaque 1083 g/ml (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 405 g, for 30 min, at room temperature. The interface layers of polymorphonuclear neutrophils (PMNs) were collected; erythrocytes were lysed with 0.1% NaCl, and cell viability was assessed with trypan blue dye (95% viability). PMNs were then suspended in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 1% gentamicin (Cultilab, Campinas, São Paulo, Brazil; Sigma-Aldrich, St. Louis, MO, USA; and Schering-Plough, São Paulo, São Paulo, Brazil, respectively) and adjusted to 2×10^6 cells/ml in 96-well plates or 1×10^6 cells/ml in 48-well plates. Microcultures were intended to evaluate the expression of the receptors and the intracytoplasmic cytokines, whereas macrocultures were used in the cytokine and eicosanoid assays. All assays were performed in duplicate.

2.3. Purification of gp43. Gp43 was purified from *P. brasiliensis* (Pb339 strain) culture supernatants by affinity chromatography in Affi-Gel 10 columns (Bio-Rad) containing the anti-gp43 monoclonal antibody MAb17c [9], which recognizes all gp43 isoforms [32], as previously described [33].

2.4. TLR2 and TLR4 Expression and Blockage of the Receptor. The experimental protocol was conducted as described by Nakaira-Takahagi et al., with some adaptations [34]. PMNs were initially treated or not with monoclonal antibodies anti-human TLR2 (0.8 μg/10^5 cells) (BioLegend, San Diego, CA, USA) and mouse anti-human TLR4 (2 μg/10^5 cells) (BD Pharmingen™, Franklin Lakes, NJ, USA), individually or in combination, for 2 h in a 5% CO_2 atmosphere at 37°C. After the receptor blockade, cells were incubated in the presence of gp43 (20 ng/ml) or in its absence (control culture) for 4 hours at the same conditions described above. Supernatants were collected, centrifuged, and stored at -20°C for measuring IL-6, IL-10, IL-12, IFN-γ, PGE2, and LTB4 levels. PMNs were then incubated with PerCP anti-human CD16 antibody, FITC anti-human CD282 (TLR2) antibody (both antibodies from BioLegend, San Diego, CA, USA), and PE mouse anti-human TLR4 antibody (BD Pharmingen™, Franklin Lakes, NJ, USA) for 20 minutes at 4°C in the dark. Receptor expression was determined by FACSCanto II flow cytometry (BD, San Diego, CA, USA) using the FACSDiva software. The standard acquisition was set to 25,000 events, and cells were gated based on size (forward scattered (FSC)), granularity (side scattered (SSC)), and fluorescence parameters (PerCP-CD16+ and/or FITC-TLR2+ and/or PE-TLR4+). Data were analyzed with FLOWJo software.

2.5. Intracytoplasmic Detection of IL-4 and IL-17A by Flow Cytometry. After the receptor blockade, PMNs were incubated with gp43 (20 ng/ml) or LPS (1 μg/ml) as a positive control (data not shown) and, at the same time, with brefeldin A (5 mg/ml, BioLegend, San Diego, CA), for 4 h, to perform intracytoplasmic cytokine detection. After that, cells were incubated with PerCP anti-human CD16 antibody for 20 min at 4°C. Then, cells were centrifuged, the supernatant was carefully removed, and cell pellets were resuspended in 100 μl of reagent A from the FIX&PERM kit (Nordic MUbio, Susteren, The Netherlands) and incubated at 4°C for 15 min. The suspension was centrifuged, supernatants were removed, and cells were incubated with 100 μl of reagent B from the

FIX&PERM kit in the presence of PE mouse anti-human IL-4 or PE mouse anti-human IL-17A antibody (BD Pharmingen™, Franklin Lakes, NJ), individually, for 30 min at 4°C. Finally, cells were centrifuged to remove the supernatant and resuspended in 100 μl of fixing solution. All steps were performed in the dark to protect the cells from light exposure. The intracytoplasmic expression of IL-4 and IL-17A was determined by FACSCanto II flow cytometry, using FACSDiva software. The standard acquisition was set to 30,000 events, and cells were gated based on size (forward scattered (FSC)), granularity (side scattered (SSC)), and fluorescence parameters (PerCP-CD16+ and PE-IL-4+ or PE-IL-17A+). Data were analyzed with FLOWJo software.

2.6. Determination of Cytokines and Eicosanoids in Cultures Supernatant. Concentrations of IL-6, IL-10, IL-12, IFN-γ, and TNF-α were detected using Human IL-6, IL-10, and IL-12 TNF-α ELISA Sets (BD OptEIA™, BD, San Diego, CA, USA) and IFN-γ by DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). PGE2 and LTB4 levels were measured using a competitive ELISA kit from Cayman Chemical Company (Ann Arbor, MI, USA). All assay types were conducted according to the manufacturer's protocol.

2.7. Statistical Analysis. Results were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), and the significance level was set at $p < 0.05$. Nonparametric data were presented as median and analyzed using the Friedman test, followed by the posttest of the multiple Dunn comparisons. Parametric data were expressed as mean ± SD and analyzed by Analysis of One-Way Variance (ANOVA) and Tukey's multiple comparison posttest. The Shapiro-Wilk was the normality test used.

3. Results

3.1. Expression of TLR2 and TLR4 following PMN Stimulation with gp43. To test the hypothesis that gp43 interacts with either TLR2 or TLR4 from PMNs, we initially incubated human PMN cells with 20 ng of gp43 for 4 hours and analyzed the expression of these two receptors by flow cytometry. Figure 1 shows a significant increase in TLR2+ (Figure 1(a)) and TLR4+ (Figure 1(b)) cells after stimulation with gp43 alone. We then blocked TLR2 or TLR4 using a monoclonal antibody (anti-TLR2 or anti-TLR4) prior gp43 stimulation, and we observed that the percentage of TLR2+ and TLR4+ cells remained similar to nonstimulated control cells (Figure 1(a) and 1(b)). TLR2 and TLR4 expression also remained identical to the control group when both receptors were blocked simultaneously and then incubated with glycoprotein (Figure 1(a)). These data indicate that gp43 is sufficient to induce TLR2 and TLR4 expression by human PMN cells.

3.2. Role of TLR2 and TLR4 in PGE2 and LTB4 Production by PMNs Incubated with gp43. To investigate whether TLR2 or TLR4 are involved in the production of PGE2 by PMNs during the recognition of gp43, we then measured PGE2 in the supernatant of cells stimulated or not with gp43 and incubated or not with anti-TLR2 and anti-TLR4 monoclonal antibodies. Our results showed that TLR4 is the main receptor involved in the production of PGE2 by PMNs. PMNs incubated with gp43 showed a slightly increased production of PGE2 than unstimulated cells. Blocking TLR2 alone or simultaneously TLR2 and TLR4 resulted in a low production of PGE2 by gp43-stimulated PMNs when compared to control unstimulated cells (Figure 2(a)). However, after TLR4 blockage, PGE2 levels substantially declined in the presence of gp43.

To determine whether other eicosanoids besides PGE2 are induced during gp43 recognition, we evaluated the levels of leukotriene B4 (LTB4) in the supernatant of PMN-stimulated cells. However, gp43 did not influence LTB4 production by PMNs, and contrary to PGE2, no difference was observed in LTB4 levels after TLR2 and TLR4 blockage (Figure 2(b)).

3.3. Role of TLR2 and TLR4 in IL-17A and IL-4 Intracytoplasmic Detection in PMNs Stimulated with gp43. Since IL-17A has an important role in protecting against fungal infections and given that PMNs are capable of producing IL-17 during infections, we investigated whether this cytokine is produced following gp43 stimulation. Thus, IL-17A was measured intracellularly in PMNs (CD16+ cells) coincubated or not with gp43, and our data demonstrated that IL-17A production involved the participation of TLR2 (Figure 3(a)). Gp43 alone resulted in a slight increase in the percentage of IL-17-producing cells when compared to nonstimulated PMNs. Moreover, blockage of TLR2 or TLR4 alone did not alter the percentage of IL-17+ cells (Figure 3(a)). However, PMNs cultured with anti-TLR4 and stimulated with gp43 showed an increased percentage of IL-17-producing PMNs when compared with unstimulated control cells, suggesting the participation of TLR2 in the production of IL-17A by gp43-stimulated neutrophils. To confirm this hypothesis, we blocked both TLR4 and TLR2 before gp43 stimulation and we observed that the percentage of IL-17A+ cells was similar to control unstimulated cells, confirming that TLR2 and TLR4 might compete for gp43 binding, and by blocking TLR4 responses, we can favor TLR2 actions in IL-17A production.

We next measured the capacity of PMNs to produce IL-4 following gp43 stimulation, a cytokine involved with high susceptibility to PCM infection. However, we did not observe differences in IL-4-producing PMNs independently of whether the cells were stimulated with gp43 or whether TLR2 or TLR4 were blocked during the culture conditions (Figure 3(b)).

3.4. Role of TLR2 and TLR4 in IL-6, IL-10, IL-12, IFN-γ, and TNF-α Production by PMNs Incubated with gp43. Since the production of inflammatory cytokines frequently accompanies PCM infection, we next evaluated the effects of gp43 on the release of IL-6, IL-10, IL-12, IFN-γ, and TNF-α by human PMNs. Our results demonstrated that gp43 did not induce any of the cytokines evaluated (IL-6 (Figure 4(a)), IL-10 (Figure 4(b)), IL-12 (Figure 4(c)), IFN-γ (Figure 4(d)), and TNF-α (Figure 4(e))) after 4 hours of incubation with this glycoprotein. The data regarding IL-6 and

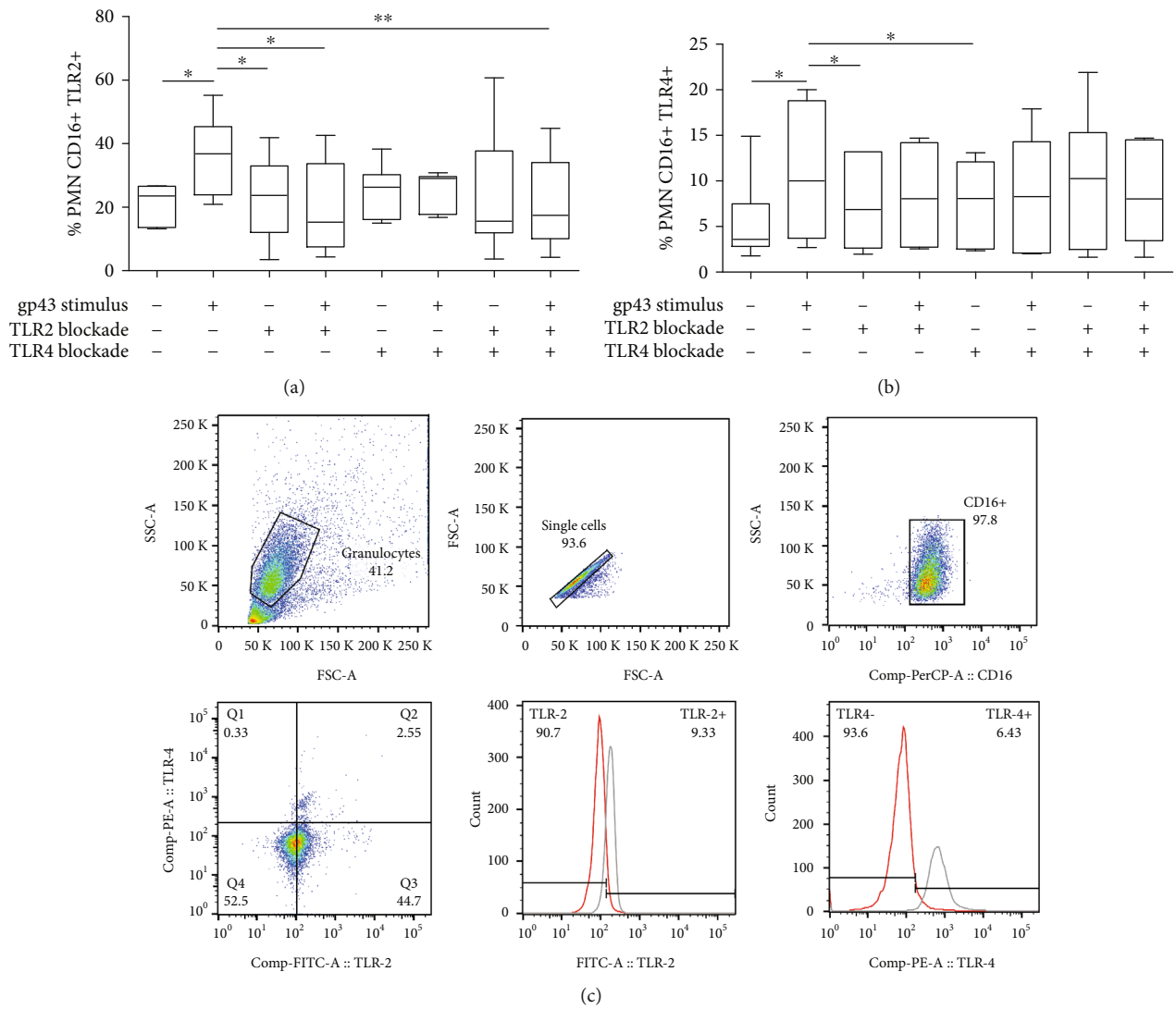

Figure 1: Percentage of TLR2 (a) and TLR4 (b) expression on the surface of CD16+ human polymorphonuclear neutrophils (PMNs). PMNs were incubated in the absence (control) or presence of gp43 (20 ng/ml) for 4 h, and PMNs were analyzed using flow cytometry. Results are presented as percentage of PMNs expressing TLR2 and TLR4 using box-and-whiskers (min to max) graphs with data from 7 women tested, considering $p < 0.05$. Statistically significant differences between groups are indicated as follows: $^{*}p < 0.05$ and $^{**}p < 0.01$. (c) Representative experiment showing the expression of PMN (control culture) CD16 PerCP+, TLR2 FITC+, and TLR4 PE+, and histograms display the percentage of TLR2 and TLR4 blockade (red line) and the remaining positive receptor expression (gray lines).

TNF-α productions showed considerable variability between individuals, while IL-10, IL-12, and IFN-γ productions were not detected regardless of the presence or absence of gp43 and receptor blockage (Figure 4).

4. Discussion

Studies evaluating the role of PMNs in PCM have increased in the last few years due to the high number of PMNs in the initial phase of this fungal infection. To date, the studies have focused on modulatory functions of PMNs using whole yeast or the mycelium of *P. brasiliensis*. Here, we assessed the interaction of PMNs with gp43, the main immunodominant antigen of *P. brasiliensis*. In this context, we evaluated gp43 ability to modulate the expression of TLR2 and TLR4 by PMNs obtained from healthy donors and the subsequent release of inflammatory cytokines and lipid mediators.

Our data showed that PMNs exposed to gp43 upregulated both TLR2 and TLR4 expression. According to previous literature, the recognition of *P. brasiliensis* yeasts by PMNs involves different cell surface receptors, including dectin-1, mannose receptor (MR), and TLR2 or TLR4, which act collaboratively to provide mechanisms of resistance or susceptibility to fungal infection [24, 26, 27, 30]. Although we have not evaluated the involvement of other receptors besides TLRs, our data indicate that TLR2 and TLR4 might play essential roles in the modulation of PMN functions by gp43 during an active infection.

In this study, we also observed that PMNs produced both PGE2 and LTB4. However, while LTB4 was produced by

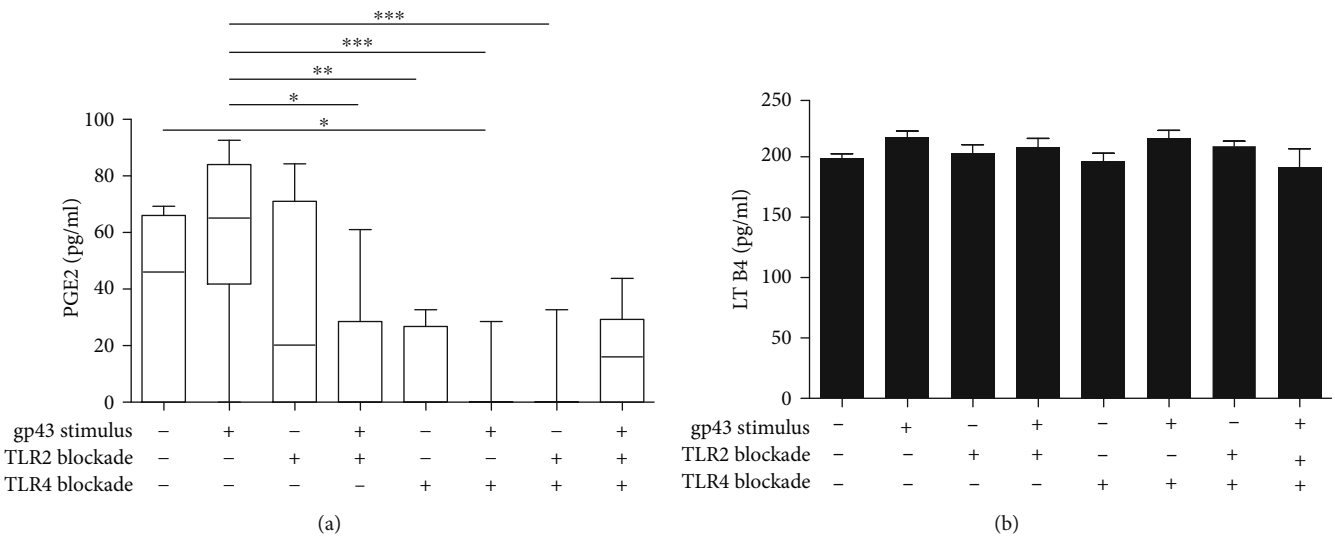

Figure 2: Involvement of TLR2 and TLR4 in PGE2 (a) and LTB4 (b) production by human polymorphonuclear neutrophils (PMNs) stimulated with gp43 from *P. brasiliensis*. Cells were incubated in the absence or presence of anti-TLR2 and anti-TLR4 antibodies, individually or in combination for 2 h. PMNs were then stimulated or not with gp43 (20 ng/ml) during 4 h, and eicosanoid concentrations (pg/ml) were measured by competitive ELISA in the culture supernatants. Results are expressed using a box-and-whiskers (min to max) graph with data from 7 women tested, considering $p < 0.05$ (a) or mean ± SD (b), considering $p < 0.05$. Statistically significant differences between groups are indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

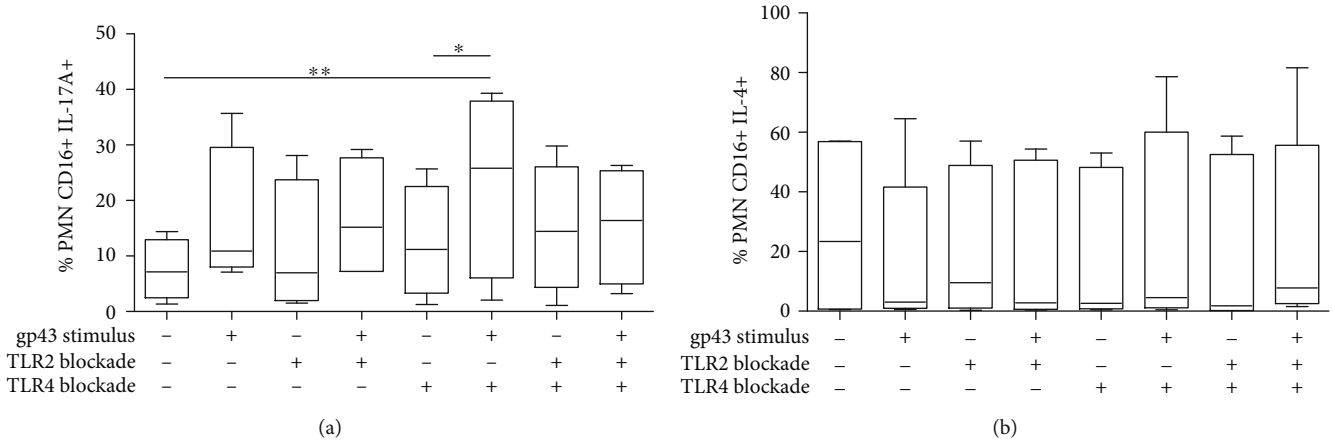

Figure 3: Percentage of human polymorphonuclear neutrophils (PMNs) producing intracytoplasmic IL-17A (a) and IL-4 (b) after stimulation or not with gp43 from *P. brasiliensis* and the involvement of TLR2 and TLR4. Cells were incubated in the absence or presence of anti-TLR2 and anti-TLR4 antibodies, individually or in combination, for 2 h. PMNs were then stimulated or not with gp43 (20 ng/ml) during 4 h and intracellular IL-4 and IL-17 were analyzed by flow cytometry. Results are presented as the percentage of PMNs expressing IL-17A (a) and IL-4 (b) using box-and-whiskers (min to max) graphs with data from 7 women tested, considering $p < 0.05$. (a) Statistically significant differences between groups are indicated as follows: $^{*}p < 0.05$ and $^{**}p < 0.01$.

gp43-stimulated or not stimulated PMNs, PGE2 appears to be induced in a TLR2- and TLR4-dependent manner. Balderramas et al. showed that PMNs release LTB4 when cells are incubated with *P. brasiliensis* yeasts, but neither TLR2 nor TLR4 were involved in LTB4 production [30]. Contrariwise, our data showed that gp43-induced PGE2 production required both TLR2 and TLR4, as evidenced by a substantial decrease in this eicosanoid production following blockage of these receptors. As seen in other studies, PGE2 production by PMNs in response to yeasts of *P. brasiliensis* involved not only TLR2 but also MR and dectin-1 receptors [26, 30]. Balderramas et al. [30] found spontaneous PGE2 levels similar to those that are present in this study, but increased levels of PGE2 were observed after challenge with two different fungal strains, a phenomenon mediated by simultaneous activation of different receptors. PGE2 production seemed to have a harmful role for hosts infected with *P. brasiliensis*. In murine PCM, PGE2 had an

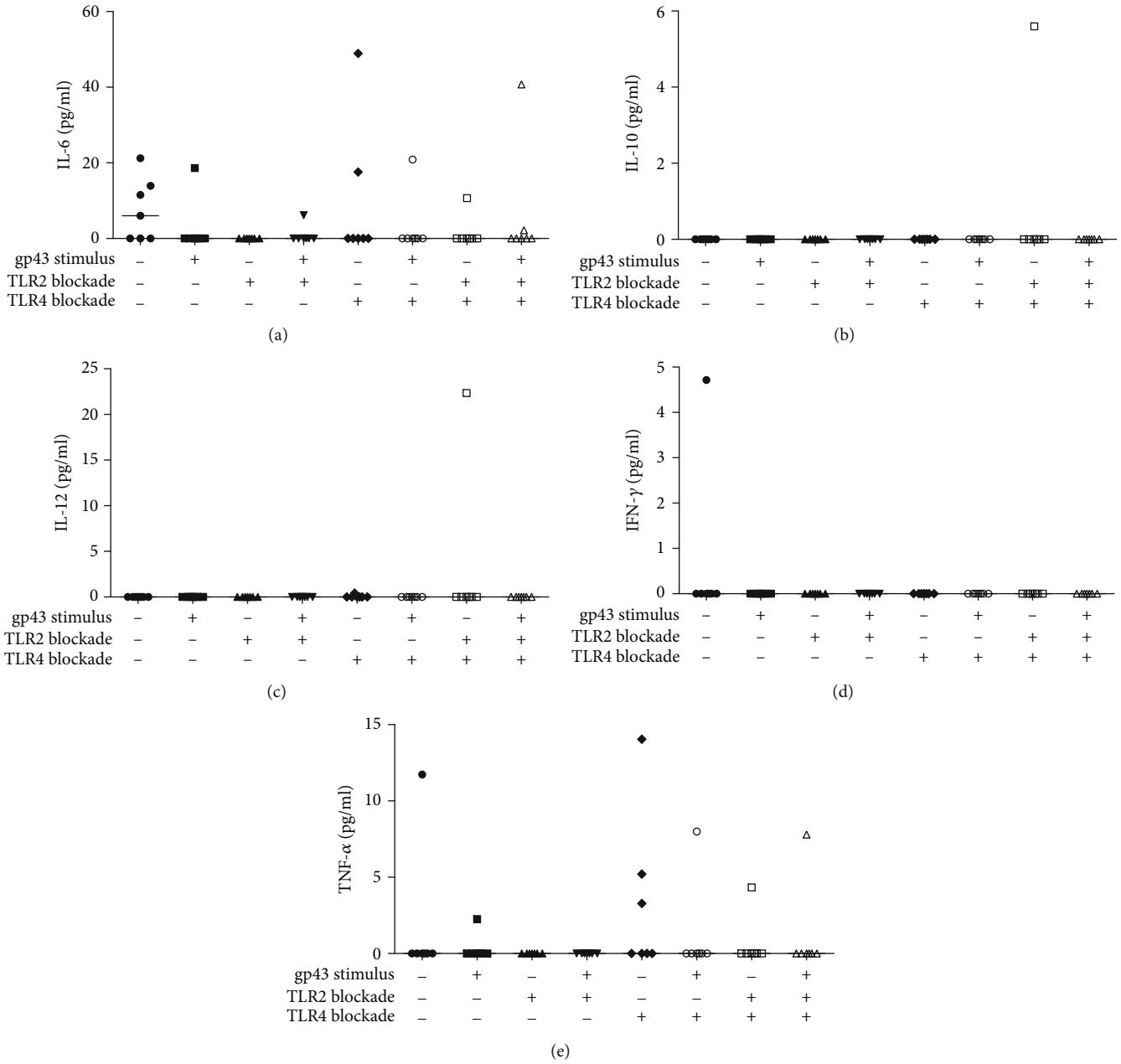

Figure 4: Involvement of TLR2 and TLR4 in the production of IL-6 (a), IL-10 (b), IL-12 (c), IFN-γ (d), and TNF-α (e) by human polymorphonuclear neutrophils (PMN) stimulated or not with gp43 from *P. brasiliensis*. Cells were incubated in the absence or presence of anti-TLR2 and anti-TLR4 antibodies, individually or in combination, for 2 h. PMNs were then stimulated or not with gp43 (20 ng/ml) during 4 h, and cytokine concentrations (pg/ml) were measured in the supernatant culture. Results are expressed as median using data from 7 women tested, considering $p < 0.05$.

immunosuppressive effect at early steps of infection, by a mechanism dependent on IL-10 and IL-4 [35].

Moreover, PGE2 inhibition using indomethacin resulted in the production of hydrogen peroxide and TNF-α by monocytes and improved fungicidal activity [36, 37]. Additionally, studies showed that *P. brasiliensis* yeasts synthesize prostaglandins, being this lipid mediator required for fungal survival [38, 39]. Conversely, *P. brasiliensis* can affect dendritic cell maturation by inhibiting PGE2 production and causing inadequate production of IL-12p70 and TNF-α [40]. Thus, together, these findings suggest that *P. brasiliensis* might take advantage of PGE2 production for its survival as well as for the modulation of the immune response. We showed here that gp43 could be considered an essential component of *Paracoccidioides* responsible for the release of this lipid mediator mainly via TLR4. Per our findings, other studies have shown that *P. brasiliensis* utilizes TLR4 and TLR2 as a mechanism to infect human neutrophils and murine

macrophages, taking advantage of the release of IL-10 and IL-8 following the activation of these receptors for increasing their multiplication inside these cells [25, 27]. Importantly, *P. brasiliensis* recognition via TLR4 in macrophages is associated with a more severe PCM in an experimental disease model [24].

We also observed a robust decrease in PGE2 production by gp43-activated PMNs after TLR4 blockage, indicating critical crosstalk between PGE2 and TLR4 during gp43 recognition. Other studies have shown the involvement of TLRs and PGE2 production. During gram-negative *Escherichia coli* infections, COX-2 and PGE2 are synthesized by vascular smooth muscle cells via TLR4 stimulation [41]. Also, it was identified that the stimulation of TLR7/8 by the synthetic agonist R-848 increases COX-2 expression in neutrophils [42]. However, a recent study showed that PGE2 restricts TLR4 signaling, indicating that more studies are warranted to determine the contribution of TLR4 to the control of PCM [43].

In this study, we also observed that gp43 results in IL-17A production by PMNs, most likely via TLR2, but not TLR4 activation. We showed that during gp43 stimulation, TLR4 blockage using a monoclonal antibody resulted in an increased percentage of IL-17A+ PMNs when compared to nonstimulated control cells. We then hypothesized that TLR2 and TLR4 compete for binding to gp43, and once the environment favors TLR2 binding to gp43, i.e., in the absence of TLR4 availability, the production of IL-17A is then significantly increased. Simultaneous blockade of TLR2 and TLR4 using monoclonal antibodies resulted in impaired augmentation of IL-17+ cells (different from blocking TLR4 alone), indicating a possible interaction between the two receptors in the modulation of IL-17A production by gp43. We have previously shown that monocytes from healthy individuals produced IL-17A after incubation with *P. brasiliensis* yeasts via activation of the Dectin-1 receptor [44]. However, until this moment, IL-17A production by PMNs incubated with yeasts or other components of *P. brasiliensis* had not been reported. Despite the controversial ability of neutrophils to produce this cytokine, our study detected intracellular IL-17A by PMNs stimulated or not with gp43. Thus, it is possible that TLR4 binding to gp43 drives both PGE2 production and inhibition of IL-17A production by PMNs, a mechanism that could lead to poor control of the fungal infection. However, the most severe form of PCM is characterized by a predominant Th17/Th22 response, along with the substantial participation of Th1 cells [45]. Thus, although high levels of IL-12 and IL-17 contribute to partial resistance to fungal infection, an exacerbated inflammatory response can be detrimental due to the induction of tissue damage and fibrosis [45–47].

Given that PMNs are observed in all stages of PCM lesions, the production of IL-17A by gp43-stimulated cells could intensify the inflammatory response and contribute to tissue damage. Loures et al. showed that TLR2 is a negative regulator of the pathogenic Th17 immunity, shifting the T cell responses to a balanced Th1/Th2 response modulated by Tregs [25]. In our study, gp43 stimulated PMNs to produce IL-17A only after blocking TLR4, suggesting that gp43 might take advantage of the TLR4/TLR2 interactions during IL-17 production to control inflammatory reactions during an active infection.

Although we have observed IL-17 production by gp43-stimulated human PMNs, we did not detect substantial levels of other inflammatory cytokines, including IL-4, IL-6, IL-10, IL-12, IFN-γ, and TNF-α following gp43 stimulation. Balderramas et al. reported the production of IL-12p40 and IL-10 after 4 hours of incubation with *P. brasiliensis* yeasts [30], indicating the participation of other fungal components in PMN activation. *P. brasiliensis* induces the production of all of these cytokines *in vitro* and *in vivo* modulating different arms of the immune system [27, 30, 31, 45]; but according to our data, gp43 seems not to participate in this process at least for a short period of incubation. This is a new and important study about PCM, demonstrating the recognition of gp43 by PMNs and its participation in PGE2 and IL-17A production, highlighting the use of the glycoprotein by *P. brasiliensis* to escape from host defense mechanisms.

5. Conclusion

Taken together, our data reinforce that gp43 from *P. brasiliensis* can function as an escape mechanism modulating the release of PGE2 and IL-17A by PMNs via TLR2/TLR4 interaction.

Data Availability

All relevant data are within the paper and its supporting information files.

Conflicts of Interest

The authors declare that they have no conflict of interests.

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References

[1] A. Restrepo and A. M. Tobón, "Paracoccidioides brasiliensis," in *Principles and Practice of Infectious Diseases*, G. L. Mandell, J. E. Bennett, and R. Dollin, Eds., pp. 3062–3068, ESA. Elsevier, Philadelphia, 2005.

[2] D. R. Matute, J. G. McEwen, R. Puccia et al., "Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies," *Molecular Biology and Evolution*, vol. 23, no. 1, pp. 65–73, 2006.

[3] M. M. Teixeira, R. C. Theodoro, M. J. de Carvalho et al., "Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus," *Molecular Phylogenetics and Evolution*, vol. 52, no. 2, pp. 273–283, 2009.

[4] S. A. Marques, "Paracoccidioidomycosis: epidemiological, clinical, diagnostic and treatment up-dating," *Anais Brasileiros de Dermatologia*, vol. 88, no. 5, pp. 700–711, 2013.

[5] R. Martinez, "Epidemiology of paracoccidioidomycosis," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 57, suppl 19, pp. 11–20, 2015.

[6] M. Franco, "Host-parasite relationships in paracoccidioidomycosis," *Journal of Medical and Veterinary Mycology*, vol. 25, no. 1, pp. 5–18, 1987.

[7] B. R. da Silva Neto, P. F. Z. Carvalho, A. M. Bailão, W. S. Martins, C. M. de Almeida Soares, and M. Pereira, "Transcriptional profile of *Paracoccidioides* spp. in response to itraconazole," *BMC Genomics*, vol. 15, no. 1, 2014.

[8] R. Puccia, S. Schenkman, P. A. Gorin, and L. R. Travassos, "Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen," *Infection and Immunity*, vol. 53, no. 1, pp. 199–206, 1986.

[9] R. Puccia and L. R. Travassos, "43-kilodalton glycoprotein from *Paracoccidioides brasiliensis*: immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease," *Journal of Clinical Microbiology*, vol. 29, no. 8, pp. 1610–1615, 1991.

[10] A. H. Straus, E. Freymüller, L. R. Travassos, and H. K. Takahashi, "Immunochemical and subcellular localization of the 43 kDa glycoprotein antigen of *Paracoccidioides brasiliensis* with monoclonal antibodies," *Journal of Medical and Veterinary Mycology*, vol. 34, no. 3, pp. 181–186, 1996.

[11] A. P. Vicentini, J. L. Gesztesi, M. F. Franco et al., "Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis," *Infection and Immunology*, vol. 62, no. 4, pp. 1465–1469, 1994.

[12] J. L. Gesztesi, R. Puccia, L. R. Travassos et al., "Monoclonal antibodies against the 43,000 Da glycoprotein from *Paracoccidioides brasiliensis* modulate laminin-mediated fungal adhesion to epithelial cells and pathogenesis," *Hybridoma*, vol. 15, no. 6, pp. 415–422, 1996.

[13] C. P. Taborda, M. A. Juliano, R. Puccia, M. Franco, and L. R. Travassos, "Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice," *Infection and Immunity*, vol. 66, no. 2, pp. 786–793, 1998.

[14] F. A. F. Popi, J. D. Lopes, and M. Mariano, "GP43 from *Paracoccidioides brasiliensis* inhibits macrophage functions. An evasion mechanism of the fungus," *Cellular Immunology*, vol. 218, no. 1-2, pp. 87–94, 2002.

[15] F. T. Konno, J. Maricato, A. Y. Konno et al., "*Paracoccidioides brasiliensis* GP43-derived peptides are potent modulators of local and systemic inflammatory response," *Microbes and Infection*, vol. 14, no. 6, pp. 517–527, 2012.

[16] H. C. de Oliveira, J. . F. Ã.¡. da Silva, L. Scorzoni et al., "Importance of adhesins in virulence of *Paracoccidioides* spp.," *Frontiers in Microbiology*, vol. 6, no. 303, 2015.

[17] J. de Fátima da Silva, J. Vicentim, H. C. de Oliveira et al., "Influence of the *Paracoccidioides brasiliensis* 14-3-3 and gp43 proteins on the induction of apoptosis in A549 epithelial cells," *Memórias do Instituto Oswaldo Cruz*, vol. 110, no. 4, pp. 476–484, 2015.

[18] I. Torres, O. Hernandez, D. Tamayo et al., "Inhibition of PbGP43 expression may suggest that gp43 is a virulence factor in *Paracoccidioides brasiliensis*," *PLoS One*, vol. 8, no. 7, p. e68434, 2013.

[19] C. L. Silva, L. M. Alves, and F. Figueiredo, "Involvement of cell wall glucans in the genesis and persistence of the inflammatory reaction caused by the fungus *Paracoccidioides brasiliensis*," *Microbiology*, vol. 140, no. 5, pp. 1189–1194, 1994.

[20] A. M. Della Coletta, T. F. Bachiega, J. C. de Quaglia e Silva et al., "Neutrophil extracellular traps identification in tegumentary lesions of patients with paracoccidioidomycosis and different patterns of NETs generation in vitro," *PLOS Neglected Tropical Diseases*, vol. 9, no. 9, article e0004037, 2015.

[21] R. P. Gazendam, A. van de Geer, D. Roos, T. K. van den Berg, and T. W. Kuijpers, "How neutrophils kill fungi," *Immunological Reviews*, vol. 273, no. 1, pp. 299–311, 2016.

[22] L. Chen and L. A. DiPietro, "Toll-like receptor function in acute wounds," *Advances in Wound Care*, vol. 6, no. 10, pp. 344–355, 2017.

[23] E. M. Moresco, D. LaVine, and B. Beutler, "Toll-like receptors," *Current Biology*, vol. 21, no. 13, pp. R488–R493, 2011.

[24] V. L. G. Calich, T. A. da Costa, M. Felonato et al., "Innate immunity to *Paracoccidioides brasiliensis* infection," *Mycopathologia*, vol. 165, no. 4-5, pp. 223–236, 2008.

[25] F. V. Loures, A. Pina, M. Felonato, and V. L. Calich, "TLR2 is a negative regulator of Th17 cells and tissue pathology in a pulmonary model of fungal infection," *Journal of Immunology*, vol. 183, no. 2, pp. 1279–1290, 2009.

[26] C. V. Bonfim, R. L. Mamoni, and M. H. Blotta, "TLR-2, TLR-4 and dectin-1 expression in human monocytes and neutrophils stimulated by *Paracoccidioides brasiliensis*," *Medical Mycology*, vol. 47, no. 7, pp. 722–733, 2009.

[27] M. J. Acorci-Valério, A. P. Bordon-Graciani, L. A. Dias-Melicio, M. de Assis Golim, E. Nakaira-Takahagi, and A. M. de Campos Soares, "Role of TLR2 and TLR4 in human neutrophil functions against *Paracoccidioides brasiliensis*," *Scandinavian Journal of Immunology*, vol. 71, no. 2, pp. 99–108, 2010.

[28] F. S. M. Tristão, F. A. Rocha, A. P. Moreira, F. Q. Cunha, M. A. Rossi, and J. S. Silva, "5-Lipoxygenase activity increases susceptibility to experimental *Paracoccidioides brasiliensis* infection," *Infection and Immunity*, vol. 81, no. 4, pp. 1256–1266, 2013.

[29] H. A. Balderramas, O. G. Ribeiro, A. M. Soares, and S. L. Oliveira, "The role of leukotriene B4 in early stages of experimental paracoccidioidomycosis induced in phenotypically selected mouse strains," *Medical Mycology*, vol. 51, no. 6, pp. 625–634, 2013.

[30] H. A. Balderramas, M. Penitenti, D. R. Rodrigues et al., "Human neutrophils produce IL-12, IL-10, PGE2 and LTB4 in response to *Paracoccidioides brasiliensis*. Involvement of TLR2, mannose receptor and dectin-1," *Cytokine*, vol. 67, no. 1, pp. 36–43, 2014.

[31] D. R. Rodrigues, R. K. Fernandes, H. . A. Balderramas et al., "Interferon-gamma production by human neutrophils upon stimulation by IL-12, IL-15 and IL-18 and challenge with *Paracoccidioides brasiliensis*," *Cytokine*, vol. 69, no. 1, pp. 102–109, 2014.

[32] Z. P. D. Camargo, J. L. Gesztesi, E. C. Saraiva, C. P. Taborda, A. P. Vicentini, and J. D. Lopes, "Monoclonal antibody capture enzyme immunoassay for detection of *Paracoccidioides brasiliensis* antibodies in paracoccidioidomycosis," *Journal of Clinical Microbiology*, vol. 32, no. 10, 1994.

[33] R. Puccia, L. R. Travassos, E. G. Rodrigues, A. K. Carmona, M. C. F. Oliveira, and L. Juliano, "Purification of the specific exocellular antigen Gp43 from paracoccidioides brasiliensis: immunological and proteolytic activities," in *Molecular Biology of Pathogenic Fungi: A Laboratory Manual*, B. Maresca and G. S. Kobayashi, Eds., pp. 507–515, Telos Press, New York, 1994.

[34] E. Nakaira-Takahagi, M. A. Golim, C. F. Bannwart, R. Puccia, and M. T. Peraçoli, "Interactions between TLR2, TLR4, and mannose receptors with gp43 from *Paracoccidioides brasiliensis* induce cytokine production by human monocytes," *Medical Mycology*, vol. 49, no. 7, pp. 694–703, 2011.

[35] M. A. Michelin, F. Figueiredo, and F. Q. Cunha, "Involvement of prostaglandins in the immunosuppression occurring during experimental infection by *Paracoccidioides brasiliensis*," *Experimental Parasitology*, vol. 102, no. 3-4, pp. 170–177, 2002.

[36] A. M. Soares, S. A. Calvi, M. T. Peraçoli, A. C. Fernandez, L. A. Dias, and A. R. Dos Anjos, "Modulatory effect of prostaglandins on human monocyte activation for killing of high- and low-virulence strains of *Paracoccidioides brasiliensis*," *Immunology*, vol. 102, no. 4, pp. 480–485, 2001.

[37] A. P. Bordon, L. A. Dias-Melicio, M. J. Acorci, S. A. Calvi, M. T. Serrão Peraçoli, and A. M. Victoriano de Campos Soares, "Prostaglandin E_2 inhibits *Paracoccidioides brasiliensis* killing by human monocytes," *Microbes and Infection*, vol. 9, no. 6, pp. 744–747, 2007.

[38] A. P. Bordon, L. A. Dias-Melicio, M. J. Acorci et al., "Prostaglandin E(2) production by high and low virulent strains of *Paracoccidioides brasiliensis*," *Mycopathologia*, vol. 163, no. 3, pp. 129–135, 2007.

[39] G. A. Biondo, L. A. Dias-Melicio, A. P. Bordon-Graciani, M. J. Acorci-Valério, and A. M. Soares, "*Paracoccidioides brasiliensis* uses endogenous and exogenous arachidonic acid for PGE x production," *Mycopathologia*, vol. 170, no. 2, pp. 123–130, 2010.

[40] R. K. Fernandes, T. F. Bachiega, D. R. Rodrigues et al., "Correction: *Paracoccidioides brasiliensis* interferes on dendritic cells maturation by inhibiting PGE2 production," *PLoS One*, vol. 10, no. 6, p. e0131380, 2015.

[41] R. Jimenez, E. Belcher, S. Sriskandan et al., "Role of Toll-like receptors 2 and 4 in the induction of cyclooxygenase-2 in vascular smooth muscle," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 12, pp. 4637–4642, 2005.

[42] K. Hattermann, S. Picard, M. Borgeat, P. Leclerc, M. Pouliot, and P. Borgeat, "The toll-like receptor 7/8-ligand resiquimod (R-848) primes human neutrophils for leukotriene B4, prostaglandin E2 and platelet-activating factor biosynthesis," *The FASEB Journal*, vol. 21, no. 7, pp. 1575–1585, 2007.

[43] D. J. Perkins, K. Richard, A. M. Hansen et al., "Autocrine-paracrine prostaglandin E_2 signaling restricts TLR4 internalization and TRIF signaling," *Nature Immunology*, vol. 19, no. 12, pp. 1309–1318, 2018.

[44] A. G. Romagnolo, J. C. de Quaglia e Silva, A. M. D. Coletta et al., "Role of Dectin-1 receptor on cytokine production by human monocytes challenged with *Paracoccidioides brasiliensis*," *Mycoses*, vol. 61, no. 4, pp. 222–230, 2018.

[45] L. F. de Castro, M. C. Ferreira, R. M. da Silva, M. H. Blotta, L. N. Longhi, and R. L. Mamoni, "Characterization of the immune response in human paracoccidioidomycosis," *The Journal of Infection*, vol. 67, no. 5, pp. 470–485, 2013.

[46] M. Franco, M. T. Peracoli, A. Soares, R. Montenegro, R. P. Mendes, and D. A. Meira, "Host-parasite relationship in paracoccidioidomycosis," *Current Topics in Medical Mycology*, vol. 5, pp. 115–149, 1993.

[47] S. J. Oliveira, R. L. Mamoni, C. C. Musatti, P. M. Papaiordanou, and M. H. Blotta, "Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidioidomycosis: comparison with infected and non-infected controls," *Microbes and Infection*, vol. 4, no. 2, pp. 139–144, 2002.

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Research Article

The Nod2 Agonist Muramyl Dipeptide Cooperates with the TLR4 Agonist Lipopolysaccharide to Enhance IgG2b Production in Mouse B Cells

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Many studies have shown that Toll-like receptors (TLRs) and Nod-like receptors (NLRs) were expressed in B cells and their signaling affects B cell functions. Nonetheless, the roles played by these receptors in B cell antibody (Ab) production have not been completely elucidated. In the present study, we examined the effect of the Nod2 agonist muramyl dipeptide (MDP) in combination with the TLR4 agonist lipopolysaccharide (LPS), a well-known B cell mitogen, on B cell viability, proliferation, and activation, and Ab production by in vitro culture of purified mouse spleen resting B cells. MDP combined with LPS to reinforce B cell viability, proliferation, and activation. Moreover, MDP enhanced LPS-induced IgG2b production, germline γ2b transcript (GLTγ2b) expression, and surface IgG2b expression. In an experiment with Nod2- and TLR4-deficient mouse B cells, we observed that the combined effect of MDP and LPS is dependent on Nod2 and TLR4 receptors. Furthermore, the combined effect on B cell viability and IgG2b switching was not observed in Rip2-deficient mouse cells. Collectively, this study suggests that Nod2 signaling enhances TLR4-activated B cell proliferation, IgG2b switching, and IgG2b production.

1. Introduction

Pattern recognition receptors such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs) recognize specific conserved bacterial structures (pathogen-associated molecular patterns). TLR signaling can directly affect B cell functions, even without the support of T cells [1–4]. We recently reported that TLR1/2 agonist Pam3CSK4 and TLR7 agonist imiquimod directly inhibit IgG1 and IgE class switching, respectively, in activated mouse B cells [5, 6]. In addition, we found that Dectin-1 (a type of CLR) agonist selectively induced IgG1 class switching by TLR4 agonist lipopolysaccharide (LPS)-activated mouse B cells [7, 8]. Many studies have shown that TLR signaling interplays with other receptor signaling such as other TLRs, B cell receptor (BCR), and CD40 signaling in B cells [9–14]. For instance, TLR2 stimulation arrests TLR4 agonist LPS-promoted B cell maturation [15]; BCR signaling synergizes with TLR signaling for activation-induced cytidine deaminase (AID) expression and Ig class switch recombination (CSR) by B cells [16]. Thus, TLRs play various roles in B cell activation, differentiation, and function.

However, the roles played by NLRs (Nod1, Nod2, NLRC4, NLRP3, etc.) in B cells remain to be elucidated. The effects of Nod1 and Nod2 stimulation on B cell activation have been investigated in only a few studies: Cohen and Parant reported that Nod2 agonist muramyl dipeptide (MDP) increases surface Ig (membrane κ-light chain) expression and enhances the response to LPS in the mouse pre-B cell line 70Z/3 [17]; Petterson et al. reported that Nod1 or Nod2 stimulation augments BCR- or TLR-induced human B cell activation (proliferation, viability,

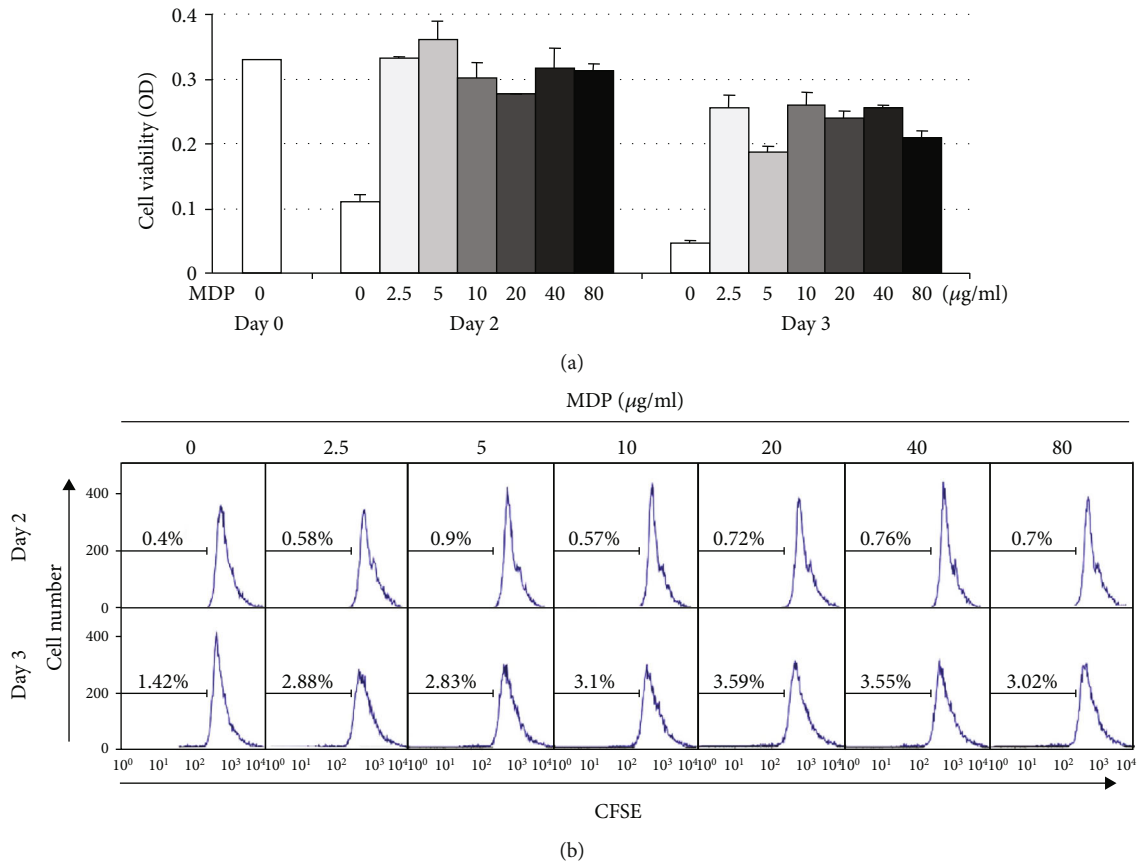


Figure 1: MDP sustains B cell viability in vitro, but MDP itself hardly induces B cell proliferation. Resting B cells were stimulated with the indicated MDP concentrations. After 2 and 3 days of culture, cell viability was measured by (a) EZ-Cytox assay and (b) cell proliferation was measured by CFSE assay. Data shown are representative of two independent experiments.

and expression of cell surface markers) independently of physical T cell assistance [18, 19].

In the present study, to further elucidate the role of Nod2 in B cell response, we directly stimulated mouse resting B cells with MDP in the absence and presence of LPS in vitro and analyzed B cell viability, proliferation, activation, antibody (Ab) production, and Ig class switching.

2. Materials and Methods

2.1. Animals. Wild-type (WT) C57BL/6 mice were purchased from Damool Science (Daejeon, Korea). TLR4-deficient (*Tlr4*$^{-/-}$), Nod2-deficient (*Nod2*$^{-/-}$), and Rip2-deficient (*Rip2*$^{-/-}$) mice with a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained on an 8 : 16 h light:dark cycle in an animal environmental control chamber. Eight- to twelve-week-old mice were used, and animal care was provided in accordance with the guidelines of the Institutional Animal Care and Use Committee of Konyang University.

2.2. Cell Culture and Reagents. The mouse B cell lines L10A6.2 (surface μ^+, mature B cell line) and A20.3 (surface γ2a$^+$) were provided by Dr. J. Stavnezer (University of Massachusetts Medical School, Worcester, MA, USA). The mouse B lymphoma cell line CH12F3-2A (surface μ^+) was provided by Dr. T. Honjo (Kyoto University, Kyoto, Japan). Mouse spleen resting B cells were obtained by depletion of CD43$^+$ cells using anti-CD43 microbeads and high-gradient magnetic cell separation according to the manufacturer's instruction (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described [5]. The purity of resting B cells (CD43$^-$B220$^+$) was assessed using FACSCalibur (BD Biosciences, San Jose, CA, USA) following staining of the cells with anti-CD43 FITC (eBioscience, San Diego, CA, USA) and anti-B220 PE (BD Biosciences) (Supplementary Figure 1(a)). The cells were cultured at 37°C in a humidified CO_2 incubator (Forma Scientific, Marietta, OH, USA) in RPMI-1640 medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (PAA Laboratories, Etobicoke, ON, Canada). The cells were stimulated with LPS (ultrapure LPS, *E. coli* 0111:B4; InvivoGen, San Diego, CA, USA), MDP (InvivoGen), and iE-DAP (InvivoGen). The mouse macrophage cell line RAW264.7 was cultured in DMEM (Welgene) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum in a humidified CO_2 incubator. Anti-mouse IgG2b-

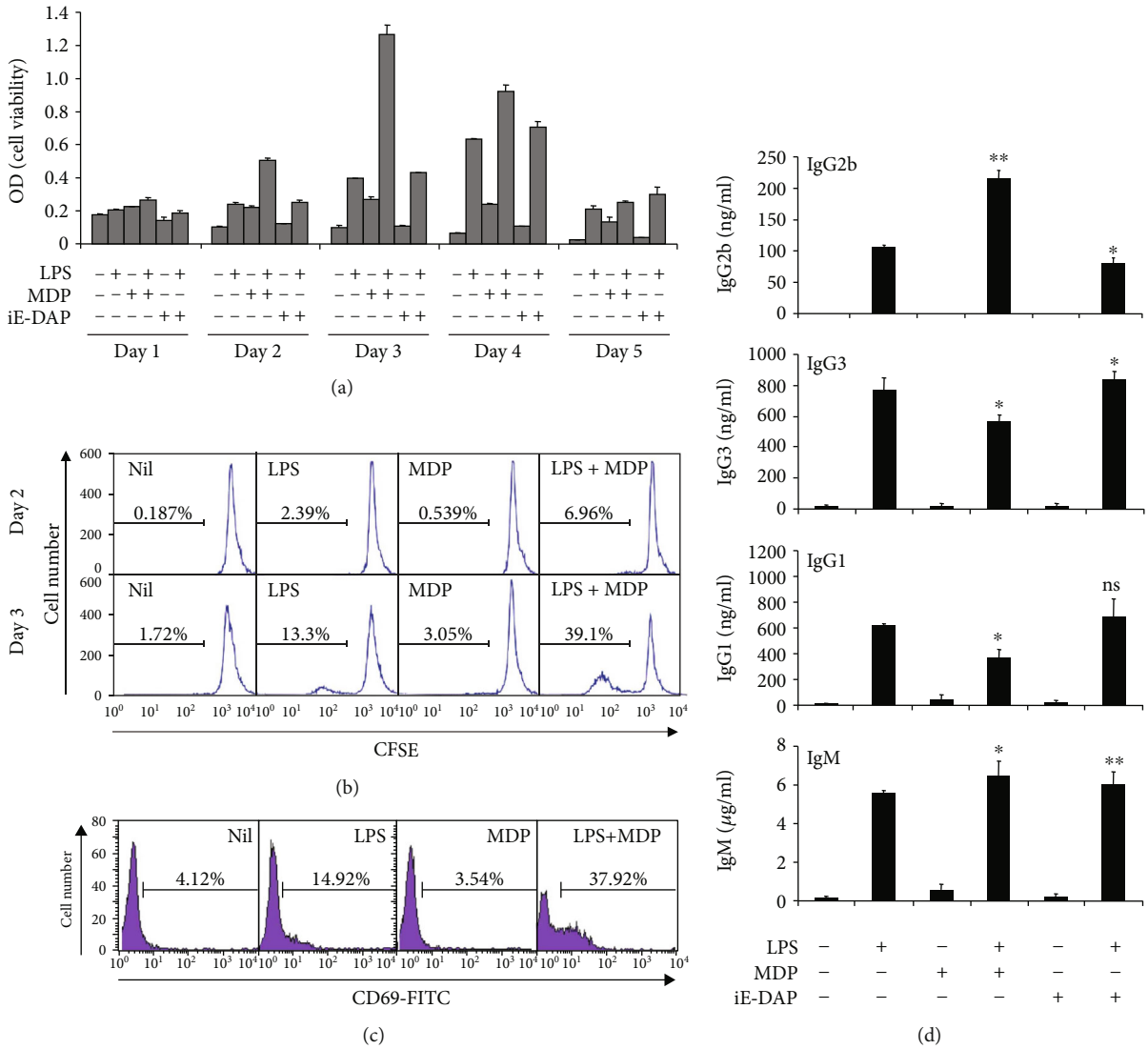


Figure 2: Combined effects of LPS and MDP on B cell viability, proliferation, activation, and Ab production. Resting B cells were stimulated with MDP (10 μg/mL) or iE-DAP (10 μg/mL) in the presence or absence of LPS (1 μg/mL). (a) Cell viability was measured by EZ-Cytox assay at the indicated time points (days). Data presented are means of duplicate samples with ranges (bars). (b) After 2 and 3 days of culture, cell proliferation was measured using CFSE assay. (c) After 2 days of culture, B cell activation was determined by surface CD69 expression. (d) After 7 days of culture, supernatants were harvested, and the levels of Ab production were measured using isotype-specific ELISA. Data presented are the means ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, SEM: standard error of the mean; ns: not significant.

PE and anti-mouse IgG3-PE were purchased from Southern Biotech (Birmingham, AL, USA). Anti-mouse IgM-PE was obtained from eBioscience.

2.3. Cell Viability, Proliferation, and Activation Assays. Cell viability was determined using the EZ-Cytox cell viability assay (DaeilLab Service Co., Ltd., Seoul, Korea) according to the manufacturer's instructions [8]. For the cell proliferation assay, purified mouse resting B cells were labeled with CFSE (eBioscience) and then supplemented with MDP, iE-DAP, and LPS. CFSE dilution was measured by counting 10,000 cells with the FACSCalibur. For the cell activation assay, cultured cells were stained with anti-CD69-FITC (BD Biosciences), and the expression levels were analyzed by flow cytometry (FACSCalibur).

2.4. Isotype-Specific ELISAs. Antibodies produced in B cell cultures were detected using isotype-specific ELISAs as previously described [8].

2.5. RNA Isolation and RT-PCR. RNA isolation and RT-PCR were performed as previously described [6]. The PCR primers (Supplementary Table 1) were synthesized by Bioneer (Daejeon, Korea). PCR for β-actin was performed in parallel to normalize for cDNA concentrations within each set of samples. PCR products were resolved by electrophoresis on 2% agarose gels. Semiquantitative RT-PCR analysis was performed using cDNA dilutions.

2.6. Flow Cytometric Analysis. Surface staining was performed with anti-mouse IgG2b-PE, anti-mouse IgG3-PE,

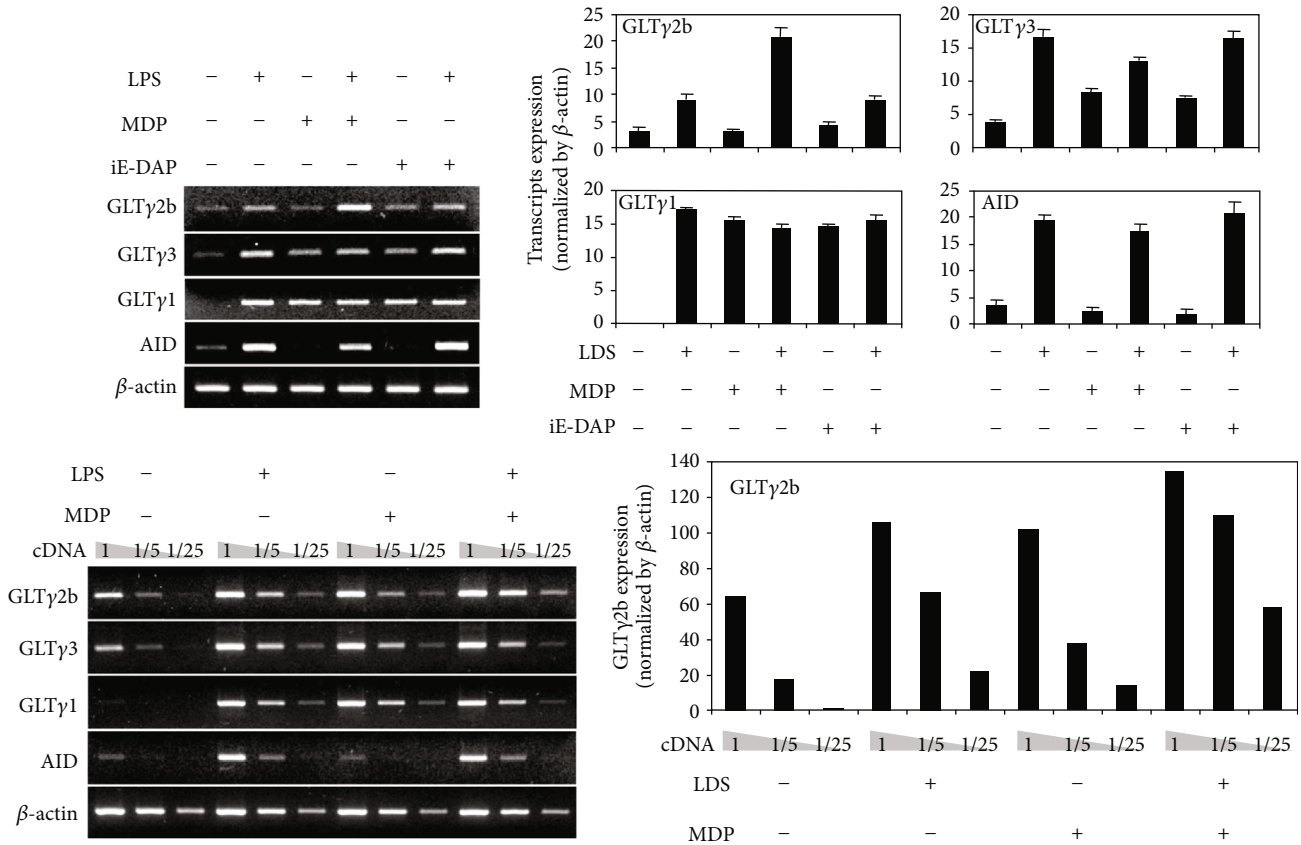


Figure 3: Combined effects of LPS and MDP on the expression of germline transcripts and AID mRNA. Resting B cells were stimulated with MDP (10 μg/mL) and iE-DAP (10 μg/mL) in the presence or absence of LPS (1 μg/mL). After 2.5 days of culture, RNAs were isolated, and the levels of germline transcripts and AID mRNA were measured by RT-PCR. The levels of germline transcripts and AID mRNA were measured by semiquantitative RT-PCR with 1/5 and 1/25 diluted cDNA (lower panel). The graphs show relative transcript levels normalized to the expression of β-actin cDNA by ImageJ (NIH, Bethesda, MD, USA) analysis. Densitometric data are averages of two independent experiments with ranges (bars).

or anti-mouse IgM-PE in the dark for 30 min at 4°C, and surface Ig-expressing B cells were analyzed by flow cytometry (FACSCalibur). Dead cells were excluded from analysis using Zombie Red™ Fixable Viability Kit according to the manufacturer's instruction (BioLegend, San Diego, CA).

2.7. Statistical Analysis. Statistical differences between experimental groups were determined by analysis of variances. All p values were calculated using unpaired 2-tailed Student's t-tests to assess statistical significance.

3. Results and Discussion

3.1. Dosage Effect of Nod2 Agonist MDP on B Cell Viability and Proliferation. First, to determine the direct effect of the Nod2 agonist MDP on B cell viability and proliferation, we purified resting B cells from mouse spleen (Supplementary Figure 1(a)) and treated them with MDP. The resting B cells expressed Nod1 and Nod2 as well as TLR4 (Supplementary Figure 1(b)). The RAW264.7 mouse macrophage cell line was used as a positive control for TLR and NLR expression. Resting B cells could not survive in the absence of stimuli in vitro and died (Figure 1(a), white bars). MDP treatment sustained B cell viability but did not increase it in a dose-dependent manner. MDP very slightly induced B cell proliferation (Figure 1(b)). However, MDP alone did not induce any Ab production (data not shown). These results suggest that MDP sustains B cell viability, but MDP itself hardly induces B cell proliferation and plasma cell differentiation.

3.2. Nod2 Agonist MDP but Not Nod1 Agonist iE-DAP Combines with TLR4 Agonist LPS to Induce B Cell Viability and Proliferation and IgG2b Production. New functions in innate immune cells have been reported for the crosstalk between TLRs and NLRs [20–22]. There is a synergistic stimulation of human monocytes and dendritic cells by TLR4 and Nod1- and Nod2-activating agonists [23]. Furthermore, Nod2 is involved in TLR4-mediated signaling of inflammation regulation [24, 25]. LPS stimulates TLR4 and is a well-known mitogen for mouse B cells [26, 27]. TLR4 on B cells recognizes LPS and stimulates B cell proliferation, differentiation, and Ig CSR. LPS in vitro stimulation

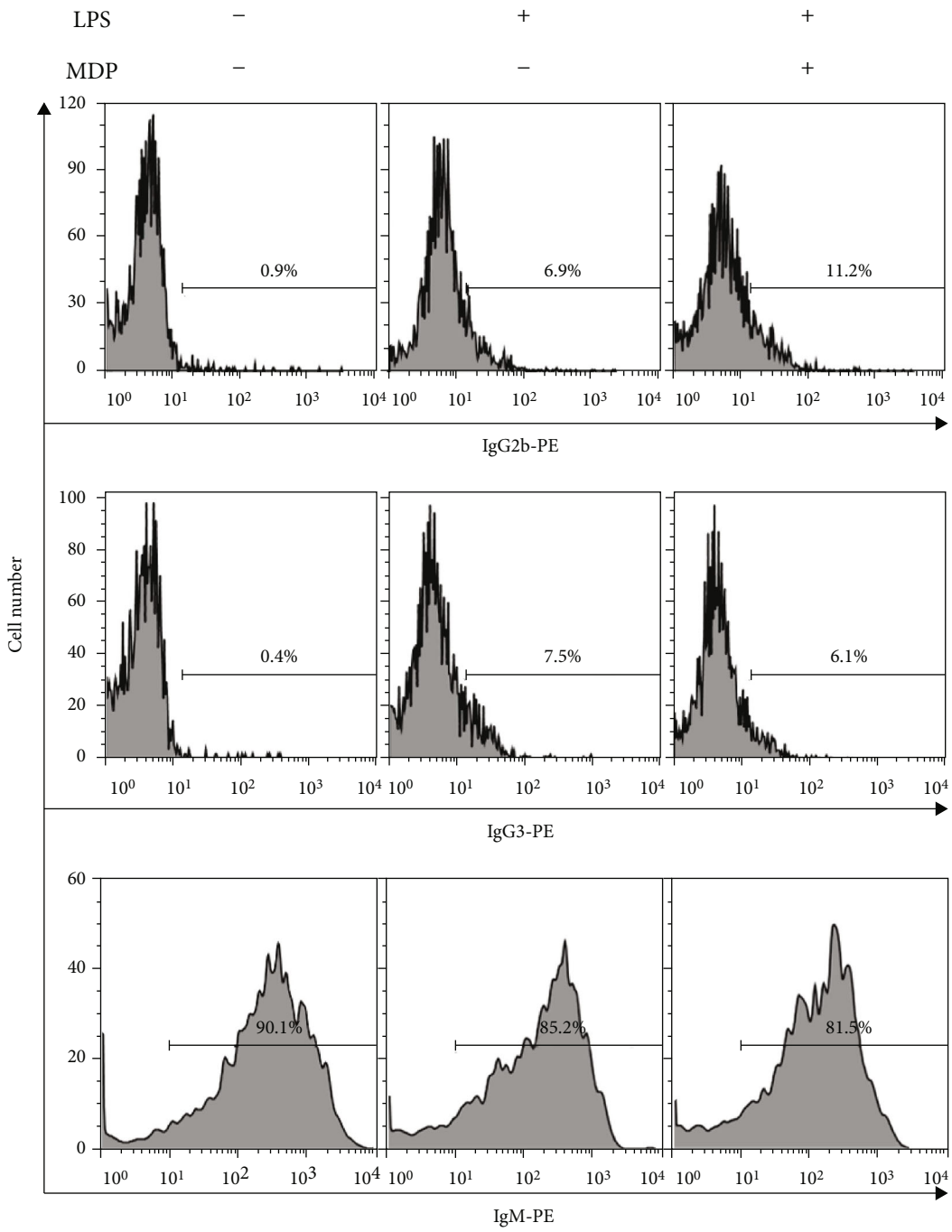
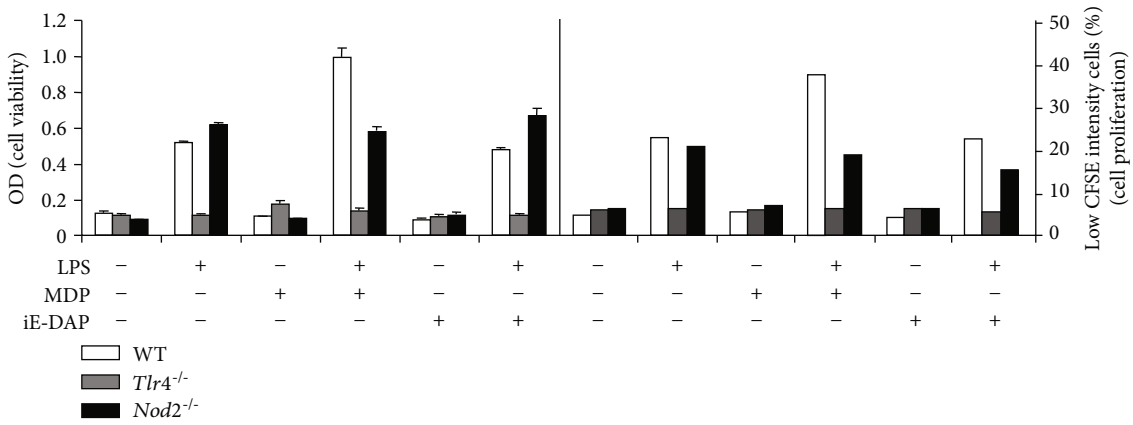
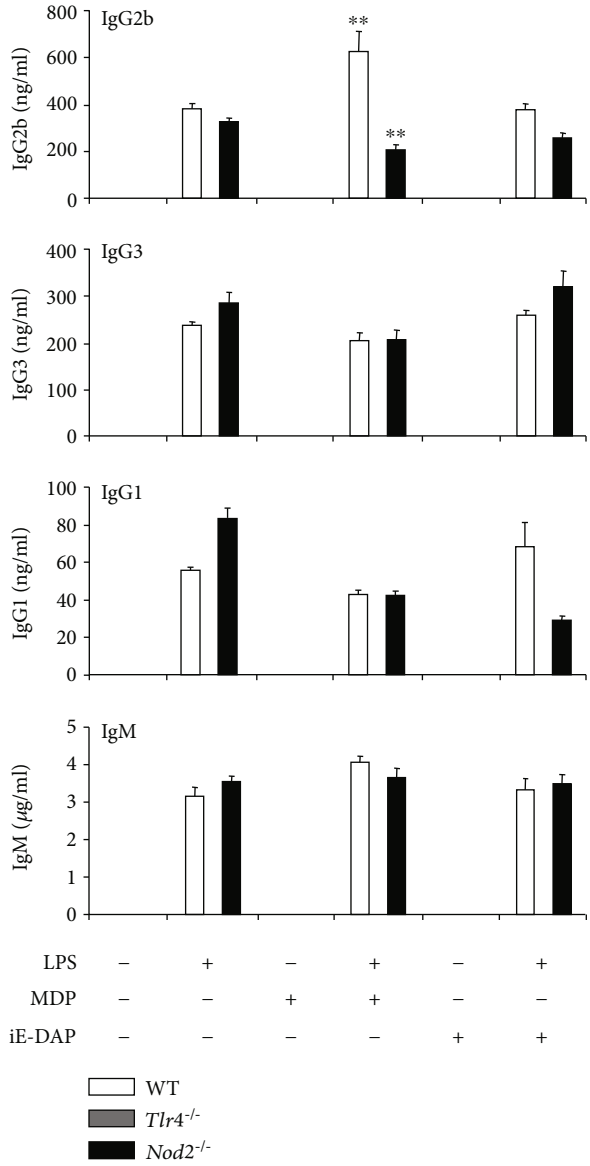


Figure 4: Combined effects of LPS and MDP on surface Ig expression. Resting B cells were stimulated with LPS (1 μg/mL) and MDP (10 μg/mL). After 4 days of culture, cells were stained with anti-IgG2b-PE, anti-IgG3-PE, or anti-IgM-PE, and surface Ig expression was analyzed by flow cytometry.

increases IgG2b and IgG3 production through IgG2b and IgG3 class switching, respectively, by mouse B cells [4, 28–33]. Therefore, we investigated the combined effect of TLR4 agonist LPS and Nod2 agonist MDP or Nod1 agonist iE-DAP on B cell viability, proliferation, activation, and Ab production. Resting B cells were stimulated with MDP or iE-DAP in the presence or absence of LPS. After 2 and 3 days of culture, LPS-induced cell viability was significantly enhanced by MDP but not by iE-DAP (Figure 2(a)). In addition, MDP reinforced LPS-induced cell proliferation (Figure 2(b)). These results indicate that MDP combined with LPS to induce B cell viability and proliferation, while iE-DAP does not. Further, MDP enhanced LPS-induced expression of CD69, which is an activation marker (Figure 2(c)). Next, we examined the effect of MDP on LPS-induced Ab production, particularly IgG2b and IgG3 production. MDP increased LPS-induced IgG2b production but decreased LPS-induced IgG3 and IgG1 production



(a)



(b)

Figure 5: Continued.

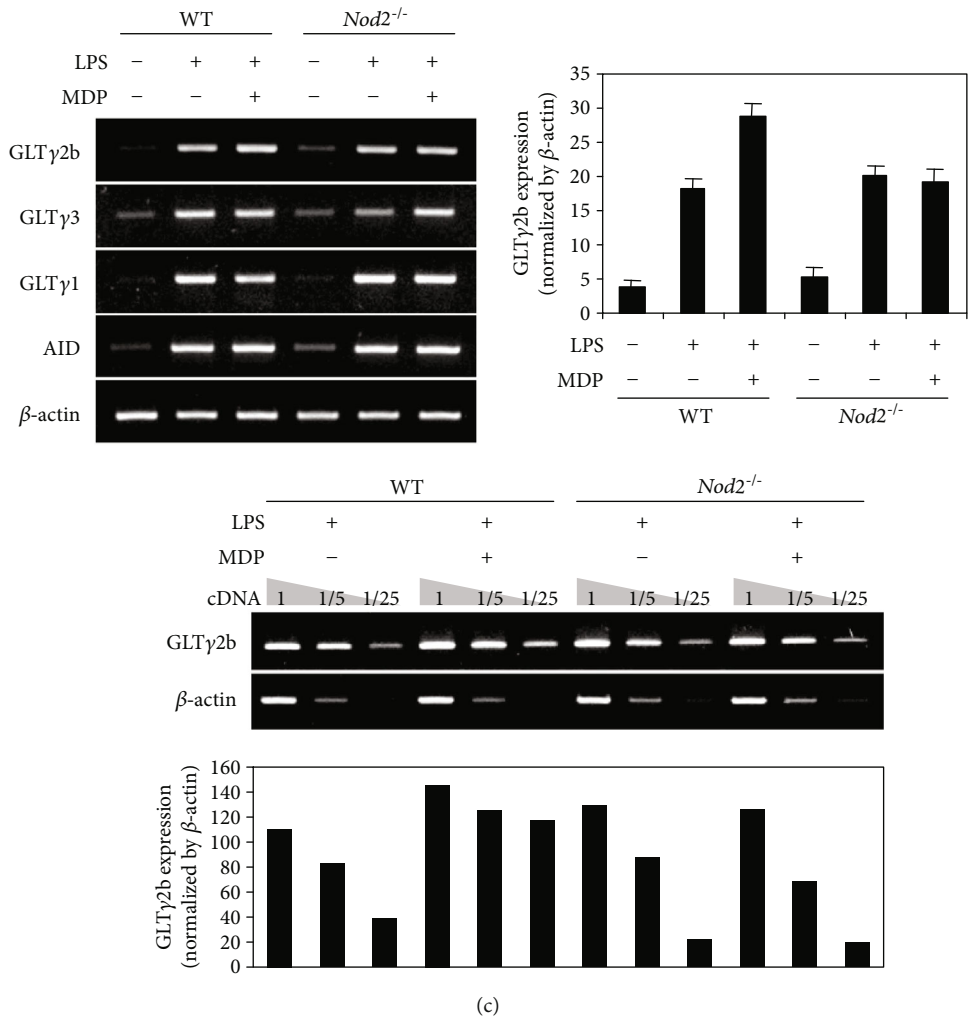


Figure 5: Effects of LPS and MDP on cell viability, proliferation, and Ab production and germline transcripts expression in TLR4- and Nod2-deficient B cells. Resting B cells were purified from wild-type (WT), TLR4-deficient (*Tlr4*$^{-/-}$), and Nod2-deficient (*Nod2*$^{-/-}$) B cells and stimulated with MDP (10 μg/mL) and iE-DAP (10 μg/mL) in the presence or absence of LPS (1 μg/mL). (a) After 2 and 3 days of culture, cell viability (OD) was measured by EZ-Cytox assay kit, and cell proliferation was measured by CFSE assay. Low CFSE intensity cell (%) means the proportion of proliferating cells. (b) After 7 days of culture, supernatants were harvested, and the levels of Ab production were measured by isotype-specific ELISA. Data shown are averages of triplicate cultures with SEM error bars. SEM: standard error of the mean. $^{**}p < 0.01$. (c) After 2.5 days of culture, RNAs were isolated and the levels of germline transcripts and AID mRNA were measured by RT-PCR. The levels of germline transcripts and AID mRNA were measured by semiquantitative RT-PCR with 1/5 and 1/25 diluted cDNA (c, lower panel). The graphs show relative GLTγ2b level normalized to β-actin cDNA expression using ImageJ, and data are averages of two independent experiments with ranges (bars).

(Figure 2(d)). iE-DAP had no significant effect on LPS-induced Ab production. Instead, iE-DAP decreased LPS-induced IgG2b production. These results indicate that MDP combines with LPS to selectively induce IgG2b production.

3.3. MDP Combines with LPS to Induce Germline γ2b Transcripts and Surface IgG2b Expression. The transcription of germline transcripts (GLT) is a prerequisite for subsequent Ig CSR [34–36]. Therefore, GLT expression can serve as a marker of Ig class switching. LPS induces the expression of GLTγ2b as well as that of GLTγ3 [34, 37, 38]. To evaluate the effect of MDP on LPS-induced IgG2b class switching, we examined whether LPS and MDP together induce the expression of germline γ2b transcripts (GLTγ2b) and surface IgG2b. Resting B cells were stimulated with MDP and iE-DAP in the presence or absence of LPS, and GLT expression were measured by RT-PCR (Figure 3). LPS-induced GLTγ2b expression was enhanced by MDP, whereas MDP did not affect LPS-induced GLTγ3 and GLTγ1 expression (Figure 3). In contrast, iE-DAP neither had any effect on LPS-induced GLTγ2b expression nor on GLTγ3 and GLTγ1 expression. Because AID is an essential enzyme for class switching [39], we assessed its expression. MDP did not affect LPS-induced AID mRNA expression (Figure 3). In addition, MDP selectively enhanced LPS-induced surface IgG2b expression (Figure 4). MDP alone did not induce surface IgG2b expression (data not shown). Collectively, these

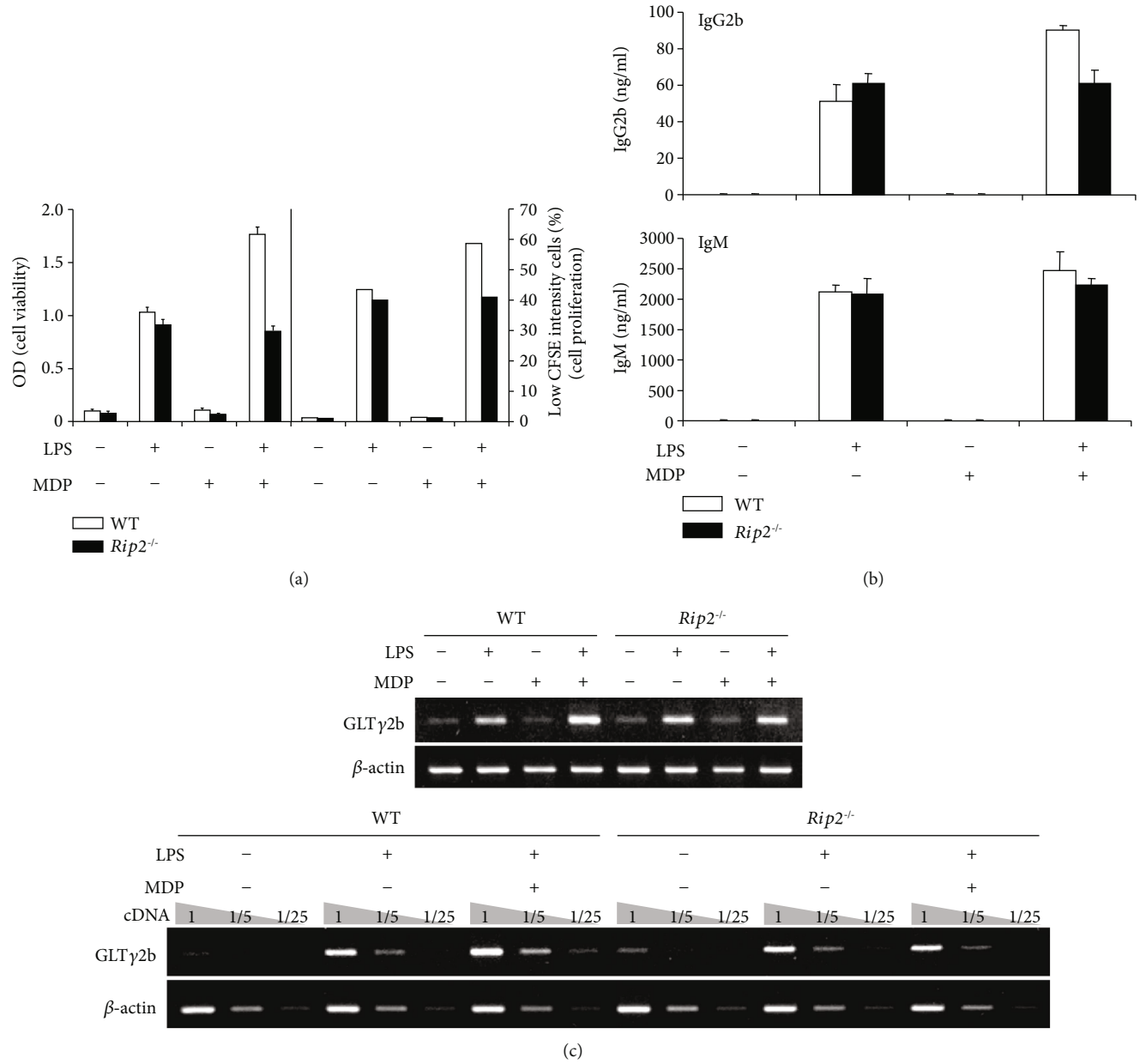


Figure 6: Effects of LPS and MDP on cell viability, cell proliferation, IgG2b production, and germline γ2b transcripts expression in Rip2-deficient B cells. Resting B cells were purified from WT and Rip2-deficient (*Rip2*$^{-/-}$) B cells and stimulated with MDP (10 μg/mL) and LPS (1 μg/mL). (a) After 2 and 3 days of culture, cell viability (OD) and proliferation were measured by EZ-Cytox assay and CFSE assay, respectively. Low CFSE intensity cell (%) means the proportion of proliferating cells. (b) After 7 days of culture, supernatants were harvested and the levels of Ab production were measured using isotype-specific ELISA. Data shown are averages of triplicate cultures with SEM error bars. SEM: standard error of the mean. (c) After 2.5 days of culture, RNAs were isolated and the levels of germline γ2b transcripts were measured by RT-PCR. The levels of germline γ2b transcripts were measured by semiquantitative RT-PCR with 1/5 and 1/25 diluted cDNA (c, lower panel).

results indicate that LPS and MDP together induce IgG2b production through increasing IgG2b class switching.

3.4. Combination Effect of LPS and MDP Is Abrogated in TLR4- and Nod2-Deficient B Cells. LPS and MDP are specific agonists for TLR4 and Nod2, respectively. Therefore, we examined whether the effects of LPS and MDP on B cell responses are dependent on their specific receptors by comparing B cells from WT and TLR4-deficient (*Tlr4*$^{-/-}$) or Nod2-deficient (*Nod2*$^{-/-}$) mice. WT, TLR4-, and Nod2-deficient B cells were stimulated with MDP or iE-DAP in the presence or absence of LPS, and cell viability, cell proliferation, and Ab production were measured (Figure 5). In TLR4-deficient B cells, LPS did not induce cell viability and proliferation (Figure 5(a), gray bars). This finding confirms that B cell proliferation by LPS is dependent on TLR4. In Nod2-deficient B cells, MDP did not enhance LPS-induced cell viability and proliferation (Figure 5(a), black bars). This

indicates that MDP can enhance LPS-induced B cell proliferation through Nod2. Next, we examined the effects of LPS and MDP on Ab production in TLR4- and Nod2-deficient B cells. TLR4-deficient B cells did not produce all Abs production upon stimulation of LPS (Figure 5(b)). In Nod2-deficient B cells, MDP did not increase LPS-induced IgG2b production. Furthermore, MDP did not increase LPS-induced GLTγ2b expression in Nod2-deficient B cells (Figure 5(c)). These results suggest that the combined effect of LPS and MDP on B cell proliferation and IgG2b production is dependent on their receptors, TLR4 and Nod2. In addition, we investigated the effects of LPS and MDP on B cell responses in receptor-interacting protein 2 (Rip2)-deficient (*Rip2*$^{-/-}$) B cells (Figure 6), because Rip2 is a critical mediator of Nod2 signaling in innate and adaptive immune responses [40–43]. MDP neither reinforced LPS-induced cell viability nor increased cell proliferation (Figure 6(a)), IgG2b production (Figure 6(b)), or GLTγ2b expression (Figure 6(c)) in Rip2-deficient B cells. Thus, Nod2-Rip2-mediated signaling could cooperatively play a critical role in LPS-induced B cell responses. However, the underlying molecular mechanisms remain to be determined.

4. Conclusions

Our present observations demonstrate that direct stimulation of Nod2 selectively enhances TLR4 agonist LPS-induced IgG2b production by enhancing IgG2b class switching in mouse B cells. IgG2b is particularly important early in the immune response, when T cell support may be limited (i.e., T-independent response), and provides early FcγR-mediated effector functions and efficient complement activation through binding on C1q [31, 44–46]. Consequently, Nod2 agonist MDP can be used as B cell adjuvant to protect from fast-replicating bacterial infection through enhancing direct B cell activation and IgG2b production independent of T cells and BCR stimulation.

Abbreviations

TLR: Toll-like receptor
NLR: Nod-like receptor
LPS: Lipopolysaccharide
MDP: Muramyl dipeptide
Ab: Antibody
GLT: Germline transcripts
CSR: Class switch recombination.

Data Availability

All data supporting the findings of this study, including its supplementary information files, are available from the corresponding author upon reasonable request.

Disclosure

The preliminary results of the current work have been presented as poster presentation on the 15th International Congress of Immunology 2013 (Milan, Italy; Abstract no.: P3.07.27). Lee Sang-Hoon's present address is the Curocell Inc., Daejeon, Republic of Korea.

Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table 1: RT-PCR primers. Supplementary Figure 1: Purity of resting B cells and expression of TLR4, Nod1, and Nod2 in the resting B cells. (a) Purity of isolated mouse spleen resting B cells (CD43^{-}B220^{+}) was measured using flow cytometric analysis. (b) Total RNA was isolated from the resting B cells and the indicated cell lines. The levels of TLR4, Nod1, and Nod2 mRNA were measured by RT-PCR. *(Supplementary Materials)*

References

[1] N. Liu, N. Ohnishi, L. Ni, S. Akira, and K. B. Bacon, "CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells," *Nature Immunology*, vol. 4, no. 7, pp. 687–693, 2003.

[2] T. Kusunoki, M. Sugai, H. Gonda et al., "CpG inhibits IgE class switch recombination through suppression of NFκB activity, but not through Id2 or Bcl6," *Biochemical and Biophysical Research Communications*, vol. 328, no. 2, pp. 499–506, 2005.

[3] E. Shen, L. Lu, and C. Wu, "TLR7/8 ligand, R-848, inhibits IgE synthesis by acting directly on B lymphocytes," *Scandinavian Journal of Immunology*, vol. 67, no. 6, pp. 560–568, 2008.

[4] I. Bekeredjian-Ding and G. Jego, "Toll-like receptors – sentries in the B-cell response," *Immunology*, vol. 128, no. 3, pp. 311–323, 2009.

[5] S. H. Lee and S. R. Park, "Toll-like receptor 1/2 agonist Pam3CSK4 suppresses lipopolysaccharide-driven IgG1 production while enhancing IgG2a production by B cells," *Immune Network*, vol. 18, no. 1, article e10, 2018.

[6] H. K. Yoon, Y. S. Shim, P. H. Kim, and S. R. Park, "The TLR7 agonist imiquimod selectively inhibits IL-4-induced IgE production by suppressing IgG1/IgE class switching and germline ε transcription through the induction of BCL6 expression in B cells," *Cellular Immunology*, vol. 338, pp. 1–8, 2019.

[7] B. S. Seo, S. H. Lee, J. E. Lee, Y. C. Yoo, J. Lee, and S. R. Park, "Dectin-1 stimulation selectively reinforces LPS-driven IgG1 production by mouse B cells," *Immune Network*, vol. 13, no. 5, pp. 205–212, 2013.

[8] B. S. Seo, H. Y. Park, H. K. Yoon, Y. C. Yoo, J. Lee, and S. R. Park, "Dectin-1 agonist selectively induces IgG1 class switching by LPS-activated mouse B cells," *Immunology Letters*, vol. 178, pp. 114–121, 2016.

[9] S. Minguet, E. P. Dopfer, C. Pollmer et al., "Enhanced B-cell activation mediated by TLR4 and BCR crosstalk," *European Journal of Immunology*, vol. 38, no. 9, pp. 2475–2487, 2008.

[10] E. J. Pone, H. Zan, J. Zhang, A. Al-Qahtani, Z. Xu, and P. Casali, "Toll-like receptors and B-cell receptors synergize to induce immunoglobulin class-switch DNA recombination: relevance to microbial antibody responses," *Critical Reviews in Immunology*, vol. 30, no. 1, pp. 1–29, 2010.

[11] E. Boeglin, C. R. Smulski, S. Brun, S. Milosevic, P. Schneider, and S. Fournel, "Toll-like receptor agonists synergize with CD40L to induce either proliferation or plasma cell differentiation of mouse B cells," *PLoS One*, vol. 6, no. 10, article e25542, 2011.

[12] S. Jain, S. B. Chodisetti, and J. N. Agrewala, "CD40 signaling synergizes with TLR-2 in the BCR independent activation of resting B cells," *PLoS One*, vol. 6, no. 6, article e20651, 2011.

[13] E. J. Pone, Z. Xu, C. A. White, H. Zan, and P. Casali, "B cell TLRs and induction of immunoglobulin class-switch DNA recombination," *Frontiers in Bioscience*, vol. 17, no. 7, pp. 2594–2615, 2012.

[14] E. J. Pone, Z. Lou, T. Lam et al., "B cell TLR1/2, TLR4, TLR7 and TLR9 interact in induction of class switch DNA recombination: modulation by BCR and CD40, and relevance to T-independent antibody responses," *Autoimmunity*, vol. 48, no. 1, pp. 1–12, 2015.

[15] E. A. Hayashi, S. Akira, and A. Nobrega, "Role of TLR in B cell development: signaling through TLR4 promotes B cell maturation and is inhibited by TLR2," *Journal of Immunology*, vol. 174, no. 11, pp. 6639–6647, 2005.

[16] E. J. Pone, J. Zhang, T. Mai et al., "BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-κB pathway," *Nature Communications*, vol. 3, no. 1, p. 767, 2012.

[17] L. Y. Cohen and M. A. Parant, "Differential regulation of surface immunoglobulin expression by various muramyl dipeptides in a murine pre-B cell line," *Immunology Letters*, vol. 45, no. 3, pp. 210–214, 1995.

[18] T. Petterson, J. Jendholm, A. Mansson, A. Bjartell, K. Riesbeck, and L. O. Cardell, "Effects of NOD-like receptors in human B lymphocytes and crosstalk between NOD1/NOD2 and toll-like receptors," *Journal of Leukocyte Biology*, vol. 89, no. 2, pp. 177–187, 2011.

[19] L. A. J. O'Neill, "Editorial: Synergism between NOD-like receptors and Toll-like receptors in human B lymphocytes," *Journal of Leukocyte Biology*, vol. 89, no. 2, pp. 173–175, 2011.

[20] M. S. Lee and Y. J. Kim, "Signaling pathways downstream of pattern-recognition receptors and their cross talk," *Annual Review of Biochemistry*, vol. 76, pp. 447–480, 2007.

[21] D. M. Underhill, "Collaboration between the innate immune receptors dectin-1, TLRs, and Nods," *Immunological Reviews*, vol. 219, pp. 75–87, 2007.

[22] K. Dolasia, M. K. Bisht, G. Pradhan, A. Udgata, and S. Mukhopadhyay, "TLRs/NLRs: shaping the landscape of host immunity," *International Reviews of Immunology*, vol. 37, no. 1, pp. 3–19, 2018.

[23] J. H. Fritz, S. E. Girardin, C. Fitting et al., "Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists," *European Journal of Immunology*, vol. 35, no. 8, pp. 2459–2470, 2005.

[24] W. H. Tsai, D. Y. Huang, Y. H. Yu, C. Y. Chen, and W. W. Lin, "Dual roles of NOD2 in TLR4-mediated signal transduction and –induced inflammatory gene expression in macrophages," *Cellular Microbiology*, vol. 13, no. 5, pp. 717–730, 2011.

[25] H. Kim, Q. Zhao, H. Zheng, X. Li, T. Zhang, and X. Ma, "A novel crosstalk between TLR4- and NOD2-mediated signaling in the regulation of intestinal inflammation," *Scientific Reports*, vol. 5, no. 1, article 12018, 2015.

[26] G. Janossy, J. Snajdr, and M. Simak-Ellis, "Patterns of B-lymphocyte gene expression elicited by lipopolysaccharide mitogen," *Immunology*, vol. 30, no. 6, pp. 799–810, 1976.

[27] A. Poltorak, X. He, I. Smirnova et al., "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene," *Science*, vol. 282, no. 5396, pp. 2085–2088, 1998.

[28] D. Yuan and E. S. Vitetta, "Structural studies of cell surface and secreted IgG in LPS-stimulated murine B cells," *Molecular Immunology*, vol. 20, no. 4, pp. 367–375, 1983.

[29] C. Esser and A. Radbruch, "Immunoglobulin class switching: molecular and cellular analysis," *Annual Review of Immunology*, vol. 8, pp. 717–735, 1990.

[30] C. M. Snapper and J. J. Mond, "Towards a comprehensive view of immunoglobulin class switching," *Immunology Today*, vol. 14, no. 1, pp. 15–17, 1993.

[31] E. K. Deenick, J. Hasbold, and P. D. Hodgkin, "Switching to IgG3, IgG2b, and IgA is division linked and independent, revealing a stochastic framework for describing differentiation," *The Journal of Immunology*, vol. 163, no. 9, pp. 4707–4714, 1999.

[32] Q. Pan-Hammarstrom, Y. Zhao, and L. Hammarstrom, "Class switch recombination: a comparison between mouse and human," *Advances in Immunology*, vol. 93, pp. 1–61, 2007.

[33] F. J. Quintana, A. Solomon, I. R. Cohen, and G. Nussbaum, "Induction of IgG3 to LPS via toll-like receptor 4 co-stimulation," *PLoS One*, vol. 3, no. 10, article e3509, 2008.

[34] E. Severinson, C. Fernandez, and J. Stavnezer, "Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination," *European Journal of Immunology*, vol. 20, no. 5, pp. 1079–1084, 1990.

[35] J. Stavnezer, "Immunoglobulin class switching," *Current Opinion in Immunology*, vol. 8, no. 2, pp. 199–205, 1996.

[36] J. Stavnezer, "Molecular processes that regulate class switching," *Current Topics in Microbiology and Immunology*, vol. 245, no. 2, pp. 127–168, 2000.

[37] S. Lutzker, P. Rothman, R. Pollock, R. Coffman, and F. W. Alt, "Mitogen- and IL-4-regulated expression of germ-line Ig γ2b transcripts: Evidence for directed heavy chain class switching," *Cell*, vol. 53, no. 2, pp. 177–184, 1988.

[38] P. Rothman, S. Lutzker, B. Gorham, V. Stewart, R. Coffman, and F. W. Alt, "Structure and expression of germline immunoglobulin γ^3 heavy chain gene transcripts: implications for mitogen and lymphokine directed class-switching," *International Immunology*, vol. 2, no. 7, pp. 621–627, 1990.

[39] M. Muramatsu, K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo, "Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme," *Cell*, vol. 102, no. 5, pp. 553–563, 2000.

[40] A. I. Chin, P. W. Dempsey, K. Bruhn, J. F. Miller, Y. Xu, and G. Cheng, "Involvement of receptor-interacting protein 2 in

innate and adaptive immune responses," *Nature*, vol. 416, no. 6877, pp. 190–194, 2002.

[41] K. Kobayashi, N. Inohara, L. D. Hernandez et al., "RICK/-Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems," *Nature*, vol. 416, no. 6877, pp. 194–199, 2002.

[42] J. G. Magalhaes, J. Lee, K. Geddes, S. Rubino, D. J. Philpott, and S. E. Girardin, "Essential role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands," *European Journal of Immunology*, vol. 41, no. 5, pp. 1445–1455, 2011.

[43] Q. Gong, Z. Long, F. L. Zhong et al., "Structural basis of RIP2 activation and signaling," *Nature Communications*, vol. 9, no. 1, article 4993, 2018.

[44] R. R. McKendall and W. Woo, "Murine IgG subclass responses to herpes simplex virus type 1 and polypeptides," *The Journal of General Virology*, vol. 69, no. 4, pp. 847–857, 1988.

[45] S. A. da Silveira, S. Kikuchi, L. Fossati-Jimack et al., "Complement activation selectively potentiates the pathogenicity of the IgG2b and IgG3 isotypes of a high affinity anti-erythrocyte autoantibody," *The Journal of Experimental Medicine*, vol. 195, no. 6, pp. 665–672, 2002.

[46] A. M. Collins, "IgG subclass co-expression brings harmony to the quartet model of murine IgG function," *Immunology and Cell Biology*, vol. 94, no. 10, pp. 949–954, 2016.

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Review Article

The Role of Toll-Like Receptors in Skin Host Defense, Psoriasis, and Atopic Dermatitis

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As the key defense molecules originally identified in Drosophila, Toll-like receptor (TLR) superfamily members play a fundamental role in detecting invading pathogens or damage and initiating the innate immune system of mammalian cells. The skin, the largest organ of the human body, protects the human body by providing a critical physical and immunological active multilayered barrier against invading pathogens and environmental factors. At the first line of defense, the skin is constantly exposed to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and TLRs, expressed in a cell type-specific manner by various skin cells, serve as key molecules to recognize PAMPs and DAMPs and to initiate downstream innate immune host responses. While TLR-initiated inflammatory responses are necessary for pathogen clearance and tissue repair, aberrant activation of TLRs will exaggerate T cell-mediated autoimmune activation, leading to unwanted inflammation, and the development of several skin diseases, including psoriasis, atopic dermatitis, systemic lupus erythematosus, diabetic foot ulcers, fibrotic skin diseases, and skin cancers. Together, TLRs are at the interface between innate immunity and adaptive immunity. In this review, we will describe current understanding of the role of TLRs in skin defense and in the pathogenesis of psoriasis and atopic dermatitis, and we will also discuss the development and therapeutic effect of TLR-targeted therapies.

1. Introduction

The skin, poised at the interface between the host body and the environment, is constantly exposed to pathogens and environmental insults and therefore has evolved to provide rapid and specific immune responses to these stimuli. Precise and situation-specific innate immune responses of skin cells to insults lead to rapid induction of host defense molecules including antimicrobial peptides (AMPs) and proinflammatory cytokines that shapes the adaptive immune responses, leading to immediate as well as long-term protection against pathogens or physical dangers.

Pattern recognition receptors (PRRs) are the vast array of germline-encoded surveillance receptors responsible for recognizing pathogens, activating the innate immune system, and priming antigen-specific adaptive immunity [1]. Upon infection or injury, pathogen-associated molecular patterns (PAMPs) released by a pathogen or damage-associated molecular patterns (DAMPs) by damaged cells are taken up by affected cells to activate membrane and/or cytosolic PRRs. PAMPs or DAMPs, such as pathogenic or host nucleic acid (DNA or RNA), protein, lipid, or lipoprotein, can be detected by unique PRR and initiate differential downstream signaling cascades, leading to situation-specific host immune responses after bacterial, viral, and parasitic infection and skin injury [2, 3].

Mammals have several highly conserved and distinct classes of PPRs including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type lectin, and intracellular DNA sensors such as cGAS-STING. Among different classes of PRRs, TLRs were the first to be characterized and are the most extensively studied innate immune receptors in both vertebrates and invertebrates [4]. Mammalian TLRs were first identified based on their sequence homology with the Drosophila Toll gene, which was originally discovered by Dr. Jules Hoffmann as the crucial receptor detecting microorganisms and activating the fly's innate immune defense

response against bacterial infection [5, 6]. The discovery of Toll-mediated innate immunity in Drosophila soon led to the discovery of mammalian TLRs by providing evidence that resistance to infection is mediated by inducible antimicrobial genes secondary to activation of the TLR signaling pathway. Dr. Hoffmann was therefore awarded one half of the 2011 Nobel Prize in Physiology or Medicine to acknowledge his contribution to innate immunity.

The unique multilayered structure of the skin enables an effective barrier against a relentless barrage of pathogens and insults. Anatomically, the skin comprises three consecutive layers, including the stratified epidermis, the fibroblast-rich dermis, and the dermal fat (also known as dermal white adipose tissue (dWAT)) [7–9]. Keratinocytes (KCs) are the main epidermal cell type (~95%), and the remaining epidermal cells include Langerhans cells, melanocytes, Merkel cells, and infiltrated immune cells. Dermal fibroblasts (dFBs), the major resident cell types in the dermis, are highly heterogeneous [9, 10]. While dFBs located in the upper (papilla) dermis support epidermal growth and regulate hair cycling, dFBs located in the lower (reticular) dermis have the potential to commit to preadipocytes (pAd) and differentiate to adipocytes, forming the last and deepest barrier of the skin, dWAT [9, 10]. PRR-mediated innate immune activation of these skin resident cells by PAMPs or DAMPs leads to the production of antimicrobial peptides as well as proinflammatory cytokines that recruit and activate myeloid and lymphatic immune cells, such as neutrophils, monocytes, dendritic cells, macrophages, and T lymphocytes. A proper interplay between innate and adaptive immune cells confers immediate and long-term immune protection against pathogens and insults.

While PRR activation is essential for inflammatory responses that initiate skin's host defense against invasive pathogens, overactivation of PRRs often leads to uncontrolled inflammation and the subsequent development of autoimmunity and/or inflammatory skin diseases, such as psoriasis, atopic dermatitis, systemic lupus erythematosus, and diabetes-induced impaired wound healing [11–13]. Here, we will review current literatures on the role of TLRs in host defense and how aberrant activation of TLRs leads to the development of psoriasis and atopic dermatitis and recent advances in therapeutic targeting of TLR to treat these skin diseases.

2. TLR Signaling

2.1. The Structure of TLRs and Their Cognate Ligands. TLRs are type I transmembrane proteins consisting of three domains including an extracellular domain, a single transmembrane domain, and an intracellular tail [14]. The extracellular domain (ectodomains) contains tandem copies of leucine-rich repeats (LRR) that recognize specific PAMPs or DAMPs as a homo- or heterodimer along with a coreceptor molecule. The intracellular tail of TLRs is homologous to that of IL1 receptor, called the Toll/IL1R domain (TIR), and it is required for downstream signaling transduction. Upon PAMP or DAMP recognition, the TIR domain recruits adaptor proteins, such as MyD88 (myeloid differentiation primary response gene 88) or TRIF (TIR domain-containing adaptor inducing IFNβ), which initiate signaling cascades that activate NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), MAPKs, or TBK1 (TANK-binding kinase 1) signaling cascades to regulate the expression of cytokines, chemokines, and antimicrobial peptides that ultimately provide host defense against danger signals [15, 16].

To date, 13 mammalian TLRs have been identified and characterized, namely, TLR1 to TLR13, including TLR1-TLR11 in human. Each TLR can interact with specific PAMPs or DAMPs including lipopeptides for TLR1, TLR2, and TLR6, lipopolysaccharide for TLR4, bacterial flagellin for TLR5, dsRNA for TLR3, ssRNA for TLR7 and TLR8, and DNA for TLR9 to initiate various intracellular signaling events triggering innate immune responses (Figure 1). TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) are localized at the cell plasma membrane to sense various cell wall components from gram-positive bacteria or mycoplasma, or protein derivatives from damaged host cells. TLR4, together with its extracellular partner CD14, recognizes lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. TLR5 recognizes flagellins from either gram-positive or gram-negative bacteria. TLR2 and/or TLR4 can also be activated by endogenous ligands or DAMPs, such as biglycans, hyaluronic acid, heat shock proteins, oxidized lipid, or lipoproteins [17, 18]. In contrast, TLR3, TLR7, TLR8, and TLR9 are localized in the endosome to detect nucleic acids derived from viruses, bacteria, or damaged cells [19]. TLR3 recognizes viral double-stranded RNA (dsRNA), TLR7 and TLR8 recognize single-stranded RNA (ssRNA) found during viral replication, and TLR9 detects unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) DNA motifs commonly found in bacterial and viral genomes. Studies have shown that guanosine is also a TLR7 agonist, and binding of guanosine and ssRNA to two distinct sites on TLR7 leads to synergistic activation of TLR7 [20, 21]. Under homeostatic conditions, endogenous nucleic acids are usually not recognized by these endosomal TLRs, but increasing evidences have shown that TLR activation by endogenous RNA or DNA is often associated with the development of autoimmunity and inflammatory diseases [22]. The ligands for TLR10 or TLR11 remain unclear. Evidences have suggested that TLR10 can form a heterodimer with TLR1, TLR2, or TLR6. TLR11 may also play an important role in host defense against certain infection as mice lacking TLR11 were highly susceptible to uropathogenic bacterial infection in the kidney. However, TLR11 may not be functional in human due to the presence of stop codons in the open reading frame of human TLR11 DNA which may represent a form of genetic polymorphism and may lead to failure of the translation of a full-length TLR11 protein [15, 23].

2.2. Signaling Pathway of TLRs. Activation of TLR signaling requires homodimerization or heterodimerization of TLRs or with coreceptors, which form an "m"-shaped dimer sandwiching the ligand molecule structure to facilitate dimerization of the intracellular TIR domains and to trigger a downstream signaling cascade [24]. TLR2 is known to form

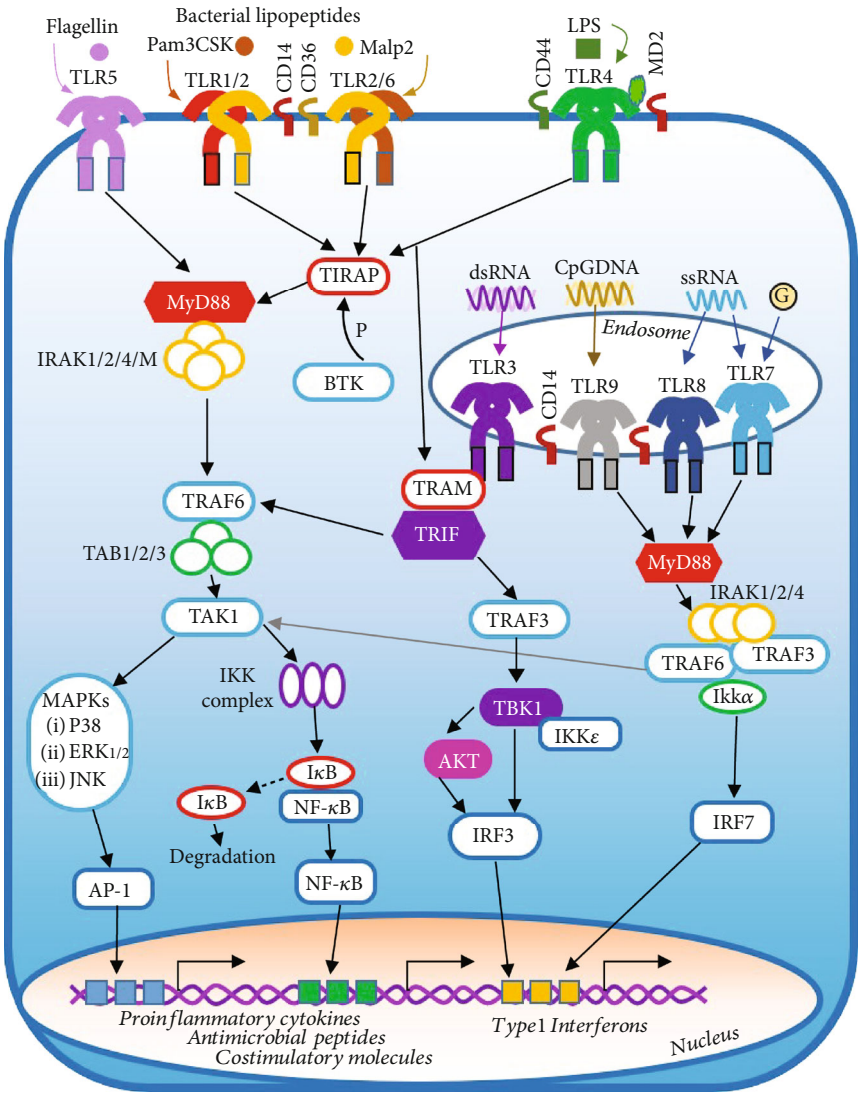

Figure 1: MyD88-dependent and TRIF-dependent TLR signaling pathways. Ligand binding of TLRs by their respective ligands induces dimerization of TLRs and initiates MyD88-dependent or TRIF-dependent signaling cascades. The presence of coreceptors, such as CD14 for TLR2, TLR3, TLR4, TLR7, and TLR9, CD36 for TLR2 and TLR6, and CD44 for TLR4, promotes ligand binding efficiency to TLRs. MD2 is a receptor component associated with TLR4 and enables TLR4 to respond to LPS. Activation of TLR1-TLR2 by the lipopeptide Pam3CSK, TLR2-TLR6 by the lipopeptide Malp2, TLR5 by flagellin, or TLR4 by LPS recruits MyD88 through the adaptor molecule TIRAP. MyD88 then recruits and activates the IRAK complex, which in turn activates TRAF6, which serves as a platform to recruit and activate TAK1 in cooperation with TAB1-3. Once activated, TAK1 activates the IKK-NFκB pathway and the MAPK- (including P38, ERK1/2, and JNK) AP1 pathway. Activated NFκB or AP1 translocates to the nucleus, driving the transcription of genes encoding proinflammatory cytokines, antimicrobial peptides, and costimulatory molecules. Activation of endosomal TLR7 by ssRNA or guanosine, TLR8 by ssRNA, or TLR9 by CpG-DNA not only initiates the MyD88-TRAF6-dependent activation of AP1 and NFκB but also triggers the IRAK-, TRAF6-, TRAF3-, and IKKα-dependent activation of IRF7, translocation of which induces the transcription of type1 interferon genes including IFNα and IFNβ. In contrast, activation of TLR3 by dsRNA initiates the TRIF-dependent pathway, whereas TLR4 activation induces both MyD88- and TRIF-dependent pathways. Once recruited to the intracellular domain of TLRs by TRAM, TRIF initiates a TRAF3-dependent activation of the TBK1-type 1 IFN pathway and/or a TRAF6-dependent activation of the TAK1-proinflammatory cytokine pathway. The TRAF3-dependent activation of TBK1 and IKKε and TBK1-mediated activation of AKT result in the coordinate activation of the transcription factor IRF3, which translocates to the nucleus and induces the transcription of type 1 interferon genes upon activation. Pam3CSK4: tripalmitoyl-S-glycero-Cys-(Lys)4; Malp2: macrophage-activating lipopeptide-2; LPS: lipopolysaccharide; dsRNA: double-stranded RNA; ssRNA: single-stranded RNA; CpG: deoxycytidyl-phosphate-deoxyguanosine; MyD88: myeloid differentiation primary response gene 88; TIRAP: TIR domain-containing adaptor protein; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adaptor inducing IFNβ; TRAF: TNFR-associated factor; IRAK: IL1R-associated kinase; TAK: transforming growth factor beta-activated kinase 1; TAB: TAK1-binding protein; IKK: inhibitor of nuclear factor kappa-B kinase; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; IκB: inhibitor of NFκB; TBK1: TANK binding kinase 1; AMPs: antimicrobial peptides.

heterodimers with TLR1 or TLR6 to recognize distinct peptidoglycan (PGN) or lipopeptides from gram-positive bacteria or mycoplasma. For example, TLR2-TLR1 recognize the bacterial lipopeptide Pam3CSK4 (tripalmitoyl-S-glycero-Cys-(Lys)4), whereas TLR2-TLR6 recognize bacterial PGN, lipoteichoic acid (LTA), and diacylated lipopeptides such as Malp2 (macrophage-activating lipopeptide-2) [25, 26] (Figure 1). The presence of coreceptors can promote the ligand binding efficiency for several TLRs, such as CD14 for TLR2, TLR4, TLR3, TLR7, and TLR9, CD36 for TLR2 and TLR6, and CD44 for TLR4 [15, 27]. MD2 is a receptor component associated with TLR4 and enables TLR4 to respond to LPS or lipid A [16].

As shown in Figure 1, following ligand-induced dimerization of the ectodomains of TLRs, the intracellular TIR domains of TLRs dimerize and recruit TIR domain-containing adapter proteins, such as MyD88, TIRAP (TIR domain-containing adaptor protein), TRIF, and TRAM (TRIF-related adaptor molecule). Depending on the adapter usage, TLR signaling is generally divided into the MyD88-dependent and TRIF-dependent pathways. All TLRs, except TLR3, use the MyD88-dependent pathway to initiate signaling, and TLR4 uniquely utilizes both MyD88 and TRIF pathways. After TLR engagement, TIRAP mediates recruitment of MyD88, which then forms a complex with IRAK (IL1R-associated kinase) family kinases, including IRAK1, IRAK2, IRAK4, and IRAK-M, to induce TRAF6 (TNFR-associated factor 6) activation. TRAF6, as an E3 ubiquitin ligase, activates TAK1 (transforming growth factor beta-activated kinase 1) through the cooperation with TAB1/2/3 (TAK1-binding protein). Activated TAK1 then phosphorylates the IKK complex (inhibitor of nuclear factor kappa-B kinase), which promotes the degradation of IκB (inhibitor of NFκB), and the dissociated NFκB then translocates to the nucleus for the induction of targeted genes. On the other hand, TAK1 can activate MAPK (mitogen-activated protein kinase) family kinases, including stress-activated protein kinase p38, Jun N-terminal kinase JNK, and signal-regulated kinase ERK1/2; activation of these MAPKs leads to the activation of the heterodimer of ATF2 and c-Jun, called AP-1. AP-1 translocates to the nucleus where it coordinates with NFκB to initiate transcription of various inflammatory cytokines, chemokines, and costimulatory factors [15].

Activation of TLR3 by dsRNA or TLR4 by LPS mediates type 1 interferon (IFN) production via the TRIF-dependent pathway. TRIF is first recruited to the TIR domain of TLRs by TRAM, and TRIF recruits TRAF6 and/or TRAF3. TRAF6 recruits RIP1 (receptor-interacting protein 1) kinase, which activates TAK1 and the subsequent NFκB and MAPK pathways. In contrast, TRAF3 recruits TBK1 (TANK-binding kinase 1) and IKKε (inhibitor of κB kinase ε), which in turn lead to the phosphorylation and nuclear translocation of IRF3, an important transcription factor regulating IFNβ production [28]. Ligand binding of TLR3 also activates the AKT in a TBK1-dependent manner, and AKT contributes to IRF3 phosphorylation by interacting with TBK1 [29, 30]. In contrast, IFNα production upon activation of TLR7/8 by ssRNA or TLR9 by CpG-DNA in plasmacytoid dendritic cells (pDCs) is mediated by the MyD88 pathway [31]. A signaling cascade involving MyD88, IRAKs, TRAF6, and IRF7 leads to the activation and nuclear translocation of NFκB and IRF7, which mediate the transcription of IFNα in activated pDCs.

3. TLRs in Skin Inflammatory Diseases

TLR signaling plays an essential role in host defense against danger signals by producing a diverse range of cytokines, chemokines, antimicrobial peptides, and costimulatory factors, and it is also required for adaptive immunity activation for long-term protection. However, aberrant activation of TLRs may disturb the homeostatic balance of the immune system and may trigger the development of systemic autoimmune diseases. For example, type 1 interferons, which are the key antiviral cytokines induced during viral infection, are potential triggers of several autoimmune diseases such as systemic lupus erythematosus (SLE), psoriasis, rheumatoid arthritis, diabetes mellitus, Sjogren's syndrome, dermatomyositis (DM), and systemic sclerosis [31]. In addition to psoriasis and SLE, unbalanced activation of TLRs may lead to other skin diseases, such as atopic dermatitis, impaired wound closure, diabetic foot ulcers, and skin cancer. Progression of these localized skin diseases may lead to systemic diseases, posing a serious threat to human health and life [12, 32].

3.1. Expression and Function of TLRs in Skin Cells. TLRs are expressed by various skin cell types in a cell-specific manner [11, 13]. Keratinocytes, localized at the surface of the skin, is the major epidermal cell type and are the first responders to external pathogens or injury. TLR2 and TLR3 are the most studied TLRs in keratinocytes, whereas the expression levels of other TLRs, such as TLR4, TLR7, TLR8, and TLR9, are much higher in myeloid immune cells compared to keratinocytes [22, 33–39]. Bacterial lipopeptide-mediated TLR2 activation in keratinocytes not only triggers the production of proinflammatory cytokines such as TNFα and IL6 but also enhances the tight junction barrier function of the epidermis upon pathogen invasion [37, 39]. In contrast, dsRNA-mediated TLR3 activation is required for normal inflammatory response during viral infection, skin injury, or UV irradiation [33–35]. TLR3 is also required for normal skin barrier repair following tissue damage, and activation of TLR3 induces the expression and function of tight junction components and markedly enhances reepithelialization, granulation, and neovascularization required for wound healing [36, 40].

While the innate immune function of keratinocytes has been extensively studied, the immune functions of dFBs and adipocytes in host defense and tissue repair have only been unrevealed and recognized recently [9, 22, 41, 42]. dFBs express functional TLR2 and TLR4 [43, 44]. TLR2-mediated recognition of bacterial lipopeptides or fungal pathogen *Candida albicans* and TLR4-mediated recognition of LPS stimulate the production of proinflammatory cytokines such as IL6 to promote both innate and adaptive immunity against pathogen invasion [43, 44]. However, excessive activation of TLR2 or TLR4 in dFBs by endogenous DAMPs such as TLR4 ligands hyaluronan, fibrinogen, and other ECM proteins or TLR2 ligand serum amyloid A (SAA) may lead to

the pathogenesis of fibrotic skin disorders, such as hypertrophic scarring and systemic sclerosis (SSc) [43–45]. dFBs have the potential to commit to preadipocytes (pAds) which can differentiate into adipocytes upon stimulation. Our group has shown that dermal infection with the gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) triggers a dermal reactive adipogenesis response, characterized by dFB commitment to pAd in response to infection, and then pAd proliferation followed by adipocyte differentiation, and during this process, the antimicrobial peptide cathelicidin (CAMP) is abundantly secreted by differentiating adipocytes, conferring host resistance to the invasive *S. aureus* infection [9, 41, 42]. In vitro, *S. aureus* or TLR2 ligand treatments enhance the adipogenic potential of primary mouse dFBs [42], suggesting that TLR2 activation may drive the commitment of dFB to adipocyte lineage and enable dFB's ability to produce antimicrobial peptide during the subsequent differentiation step. Together, with emerging roles for dFBs in host defense being unrevealed, studies are urgently needed to define the role of TLRs in activating dFBs.

Dendritic cells (DCs), known as the professional antigen-presenting cells (APCs), function as immune sentinels and play a pivotal role in bridging innate and adaptive immunity in the skin [46]. DCs uptake and process antigens and become functional mature antigen-presenting cells followed by migration to lymph nodes, where they prime T cell differentiation and activation to induce adaptive immune responses to microbials, vaccines, and self-antigens. TLRs are critical molecules for antigen presentation and induction of cytokines, chemokines, and costimulated molecules in DCs. Langerhans cells (LCs), a unique subset of APCs located in the epidermis between keratinocytes, rapidly sense PAMPs, DAMPs, or antigens and migrate to lymph nodes to prime T cells to elicit appropriate cutaneous immune responses for host defense [47, 48]. TLR2 is the most prominent TLR expressed in LCs, and LCs also express TLR8, TLR4, and TLR3 [49]. Activation of TLR2 or TLR7/8 in LCs leads to the production of proinflammatory cytokines such as IL12, CCL3, and IL8, whereas TLR3 stimulation in LCs induces the expression of chemokines (CXCL9, CXCL11, and CXCL10) and IFNβ [47]. DCs in the dermis can be subdivided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [50]. While cDCs normally reside in the skin under homeostatic condition, pDCs are not present in healthy skin but rapidly infiltrate the skin dermis upon injury [50, 51]. We and others have shown that cDCs express most TLR family genes at moderate levels whereas TLR7 and TLR9 and their downstream signaling molecule IRF7 are preferentially expressed at high levels in pDCs [22, 52]. This unique TLR expression signature enables pDCs to rapidly respond to ssRNA or DNA and produce high levels of type 1 IFNs, especially IFNα family genes, to promote autoimmune activation [22].

TLR-mediated innate immune activation of skin-resident keratinocytes, fibroblasts, and dendritic cells promotes the activation or recruitment of myeloid-derived immune cells such as neutrophils and macrophages or adaptive immune cells such as T cells, leading to immediate and long-term immunity against danger signal. During wound healing, cessation of the initial defensive/inflammatory phase is required for the subsequent proliferative and remodeling phases to complete the healing process and return to homeostatic condition. Therefore, unresolved or excessive inflammation not only can lead to the development of autoimmune skin diseases such as psoriasis, atopic dermatitis, rosacea, lupus, and systemic sclerosis but also can lead to defective or aberrant wound healing as seen in wound ulcers, diabetic foot ulcers, keloid, or hypertrophic scars [12, 53, 54] (Figure 2). We will next focus on reviewing the roles of TLRs in the pathogenesis of the two most common inflammatory skin diseases: psoriasis and atopic dermatitis.

3.2. Innate Immune Activation of TLRs and Psoriasis Initiation. Psoriasis is a chronic, recurrent, genetic autoimmune skin disorder featured by well-demarcated, raised areas of erythematous plaques, often covered by silvery scaling [55]. It is estimated that ~1.7% of the world population is affected by psoriasis, including ~3% of the US and European populations and ~0.5% of the Chinese or Asians [56]. Principal histological features of psoriasis are hyperplastic epidermis, increased vascularity in the dermis, and dermal infiltration with inflammatory leukocytes. There is no cure for psoriasis, and the recurrence of psoriasis can be triggered by several factors such as skin injury, infection, stress, and drugs such as β blockers, lithium, type 1 interferons, and imiquimod [57].

Psoriasis is considered a T cell-mediated disease, because T cell-derived cytokines such as IL17A and IL22 are responsible for the hyperproliferation and aberrant differentiation of keratinocytes that ultimately leads to psoriatic plaque formation. However, PRR-mediated recognitions of DAMPs or PAMPs and the resultant innate immune responses in keratinocytes or pDCs are believed to be the early initiating events in psoriasis that drive the subsequent adaptive immunity and autoimmunity development. Upon DAMP or PAMP stimulation, keratinocytes are capable of producing an array of proinflammatory cytokines, such as IFNβ, IL1β, IL36, TNF, IL6, IL8, IL25, and CXCL10, to initiate the inflammatory T cell phenotype in psoriasis [22, 58–60].

Skin injury even superficial tattoos can trigger psoriasis, and this is known as the "Koebner phenomenon." We have recently shown that PRR-mediated activation of the innate immune responses in keratinocytes plays a role in triggering psoriasis upon skin injury [22]. During skin injury, damaged cells release DAMPs such as dsRNA, ssRNA, and DNA, and we have found that the antimicrobial peptide LL37 which is upregulated during wounding enables dsRNA recognition in keratinocytes through the TLR3 and mitochondrial MAVS (mitochondrial antiviral signaling protein) signaling pathway, leading to IFNβ production from KC or pDC, respectively [22, 61]. Dr. Gilliet's group has also shown that LL37 can also enable ssRNA or DNA recognition by TLR7 or TLR9 in pDCs, which then produce a large quantity of IFNα [51, 61]. The self-ssRNA-LL37 complexes also activate cDC through TLR8, leading to the production of TNFα and IL6 and cDC maturation [62]. Direct comparison of the transcript levels of PRRs in KC, pDC, and cDC reveals that while

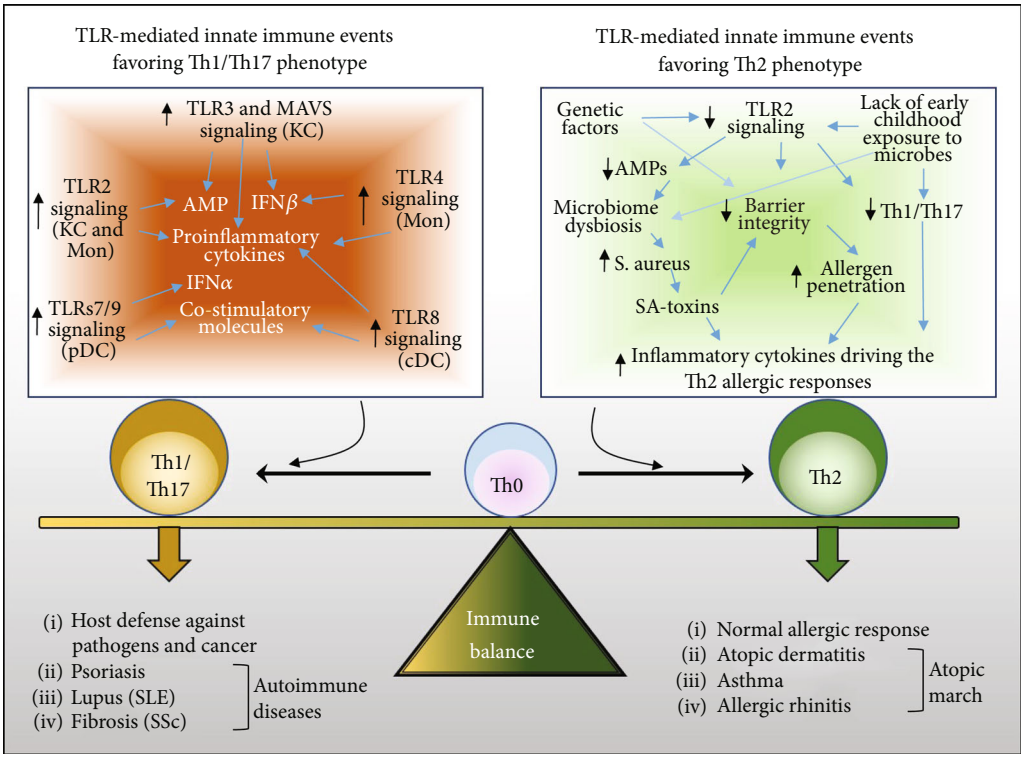

Figure 2: Proposed model for the role of TLR-mediated innate immune events in regulating the Th1/Th17 and Th2 immune balance. The development of Th1/Th17 T cells can be initiated upon innate immune activation of several TLRs, including TLR3 and MAVS (mitochondrial antiviral signaling protein) in keratinocytes (KCs), TLR7 and TLR9 in pDCs, TLR8 in cDCs, TLR2 in KCs and monocytes (Mon), and TLR4 in monocytes. Activation of these TLR-mediated signaling events leads to elevated expression of proinflammatory cytokines, type 1 interferons (including IFNβ from KCs and IFNα from pDCs), antimicrobial peptides (AMP), and costimulatory molecules (on cDCs and pDCs), which ultimately promote the differentiation of T cells from the Th0 to Th1/Th17 phenotype. In contrast, impaired TLR2 may play a role in the development of Th2 immune response. Genetic factors (such as TLR2 polymorphisms) or lack of early childhood exposure to microbes impairs TLR2 expression, and the resultant defective TLR2 signaling leads to decreased expression of antimicrobial peptides (AMPs), compromised epithelial barrier integrity, and decreased expression of Th1/Th17 cytokines. Impaired barrier integrity plays a central role in driving the allergic Th2 immune response by allowing allergens to penetrate through the skin surface. In addition, lack of AMP expression in the skin epidermis promotes dysbiosis of the skin microbiome and overgrowth of *S. aureus*, which releases several virulent toxins that exacerbate the disruption of barrier integrity and the expression of inflammatory Th2 cytokines. Activation of the Th1/Th17 immune system is necessary to promote autoimmunity and host defense against pathogens and cancer cells, but overstimulation of the Th1/Th17 pathway drives the development of several autoimmune diseases, including psoriasis, systemic lupus erythematosus (SLE), and fibrotic skin diseases (e.g., hypertrophic scarring and systemic sclerosis (SSc)). On the other hand, activation of the Th2 immune system is necessary to elicit normal allergic immune responses to allergens or pathogens, but overstimulation of Th2 immune response early in life initiates the progression of allergic diseases including atopic dermatitis, asthma, and allergic rhinitis, a pathological process known as "atopic march."

TLR3 is expressed at similar levels in all cells, MAVS is preferentially in KCs, TLR4 and TLR8 are expressed at higher levels in cDCs, and TLR7-TLR9 are preferentially expressed by pDCs [22]. These cell type-specific expression patterns of PRRs can explain the cell type-dependent responsiveness to various DAMPs or PAMPs during skin injury. Type 1 IFNs, including IFNβ from KCs and IFNα from pDCs, serve as early cytokines released upon injury to promote cDC activation and maturation with consequent Th17 T cell development and the beginning of the autoimmune self-amplification loop that drives pathogenic hyperproliferation of KCs and manifestations of psoriasis.

The roles of TLR2 or TLR4 in psoriasis still remain unclear. The expression of TLR2 and TLR4 on peripheral blood mononuclear cells and keratinocytes is elevated in patients with psoriasis [63, 64]. There is also an association between polymorphisms within TLR4 with chronic plaque type psoriasis and psoriatic arthritis [65]. A recent study has shown that epidermal infiltration of neutrophils drives inflammatory responses in the skin through activation of the epidermal TLR4-IL36R crosstalk in the imiquimod-(IMQ-) induced psoriasis-like mouse model [66]. Additionally, heat shock proteins (HSPs), such as HSP27, HSP60, HSP70, and HSP90, are overexpressed in KCs of psoriasis patients, and these HSPs can function as autoantigens to activate antigen-presenting cells (APC) through TLR4 to promote APC maturation and secretion of TNFα and IL12 [67–69].

In summary, psoriasis is a complicated autoimmune disease mediated by the dynamic interplay between the innate and the adaptive immune cells. TLR-mediated activation of keratinocytes, pDCs, and/or cDC initiates early innate

immune events that link to T cell activation and the development of autoimmunity in psoriasis. Current psoriasis therapies targeting T cell activation are effective in clinical trials [57], but potential problems including lack of long-term efficacy and rapid relapse of the disease upon drug removal [70–72] suggest that targeting the T cell alone is not enough. Targeting PRR-mediated innate immune activation of KCs or pDCs in the combination of T cell therapies may result in more sustainable effect to treat psoriasis.

3.3. Dysbiosis of Skin Microbiome, Impaired TLR2 Function, and Atopic Dermatitis. Atopic dermatitis (AD), a chronic, inflammatory skin disease characterized by an eczema-like lesion and intense pruritus and high serum immunoglobulin E (IgE), is a major health problem worldwide affecting 15~20% of children and 2~3% of adults [73–76]. AD often begins early in infancy around 3 months of age, and about 80% children have a spontaneous remission of the disease before adolescence, whereas the remaining 20% continue to have eczema into adulthood. Children with persistent AD symptoms often develop asthma and/or allergic rhinitis from 3 years of age, a process known as "atopic march" [75, 77]. Studies suggest that environmental factors may be critical in disease progression of AD. First, the prevalence of symptoms of AD is about 5~10 times higher in developed countries such as the United Kingdom, Japan, Australia, and the USA compared to developing countries such as Iran and China [75, 76]. Furthermore, the development of AD is inversely associated with early childhood exposure to infections or microbe-rich environment such as living with older siblings or pets or on a farm [78] (Figure 2). A hygiene hypothesis has therefore been proposed to describe the protective influence of microbial exposure to early life on the development of AD [78].

Recent studies have shown that dysbiosis of skin microbial community (microbiome) may promote disease progression of AD (Figure 2). The lesional skin of AD patients is often colonized with *S. aureus*, and skin *S. aureus* colonization not only positively correlates with disease severity but also precedes the clinical diagnosis of AD, suggesting that *S. aureus* may actively contribute to AD pathogenesis [79–81]. High-throughput DNA sequencing of the bacterial 16S rRNA has revealed that while bacterial composition is highly diverse on healthy skin, there is a dramatic loss of skin microbial diversity during AD flares, and the proportion of *Staphylococcus* shifts from ~20% in normal skin to a dominant ~90% in AD flare [82]. The main consequence of increased colonization of *S. aureus* in AD skin is the exacerbation of the allergic Th2 inflammatory response by staphylococcal enterotoxins (also known as "superantigens") and phenol-soluble modulins (PSMs) [83–85] and the disruption of epidermal barrier integrity mediated by other virulence factors of *S. aureus* (e.g., *S. aureus* proteases, such as aureolysin and V8 protease) [86]. A recent study from the Gallo lab has also shown that *S. aureus*-derived PSMα also induces the expression of endogenous protease activity in keratinocytes, further contributing to the disruption of barrier homeostasis [87]. Lack of early childhood exposure to beneficial microbes likely promotes dysbiosis of the skin microbiome. Indeed, studies from the Gallo group have shown that the commensal bacteria *S. epidermidis* can secrete antimicrobial peptide or DNA analog to suppress the growth of pathogenic bacteria *S. aureus* or *group A Streptococcus* (GAS), and furthermore, the commensal bacteria *S. hominis* can suppress toxin production from *S. aureus* through an autoinducing peptide [87–89]. Together, these evidences suggest that imbalanced skin microbiome composition and overgrowth of *S. aureus* are key triggering factors for the pathogenesis of atopic dermatitis.

Impaired TLR2 function has been associated with the pathogenesis of atopic dermatitis (AD) (Figure 2). Genetic polymorphisms of TLR2 have been identified to be associated with AD [90, 91], and TLR2 was also found to be downregulated in macrophages or peripheral blood mononuclear cells (PBMC) isolated from peripheral blood from AD patients [92–94]. Additionally, macrophages or PBMC from AD patients treated with TLR2 ligands produce significantly less TH1/TH17 cytokines such as interleukin 6 (IL6), IL1β, IFNγ, IL12, and IL17F and IL22, but more TH2 cytokine IL5 [93, 95]. *S. aureus*-mediated TLR2 activation is also strongly impaired in Langerhans cells from AD skin [48]. Confocal microscopy of skin sections from normal or AD patients revealed that TLR2 is normally expressed throughout the epidermis but limited to the basal keratinocytes in AD skin [92]. In normal keratinocytes, activation of TLR2 rapidly increases the expression of tight junction (TJ) protein claudin1 and antimicrobial peptide (AMP) genes such as β-defensins and cathelicidin in differentiated epidermal layers [96]. However, the lesional skin of AD patients expresses significantly decreased levels of TJ proteins as well as AMPs [96, 97], indicating that TLR2 signaling is impaired in the suprabasal layers of the epidermis where these genes are expressed. Therefore, impaired TLR2 signaling in various skin cells from AD patients may ultimately skew the immune response to *S. aureus* toward a TH2-dominant immune phenotype, a hallmark of allergic diseases such as AD. Cytokines produced by TH2 lymphocytes including IL4, IL5, and IL13 are central to the pathogenesis of atopic diseases [98].

While TLR2 signaling is impaired during the acute phase of AD, it has also been suggested that aberrant activation of TLR2 may play a role in promoting the development of the Th1 immune pathway that leads to the exacerbation and persistence of inflammation during the chronic phase of AD [99, 100]. Thymic stromal lymphopoietin (TSLP), a cytokine highly expressed by epidermal keratinocytes in AD skin, has been recognized as the master regulator linking innate response at the barrier surface to TH2-skewed adaptive immune response in atopic diseases [101, 102]. The expression of TSLP can be triggered by exposure to environmental factors, such as allergens and microorganisms, and elevated TSLP expression is observed before the development of clinical AD phenotypes in both human and mice [101, 103], suggesting that TSLP is the early initiating factor driving AD pathogenesis. In vitro, TLR ligands (including TLR3 ligand poly (I:C), TLR2-6 ligand FSL1, and TLR5 ligand flagellin) or isolated *S. aureus* membrane components induce TSLP expression and release from primary human keratinocytes, and TSLP expression can also be regulated by vitamin D3

and TH2 cytokines (IL4 and IL13) in human KCs [104]. Considering that TSLP can be induced upon activation of several TLRs (including TLR2 and TLR3) or by TLR-independent mechanisms [101], it is still unclear whether aberrant activation of TLR2 contributes to high TSLP expression in AD. Future studies are needed to define the role of TLR2 in TSLP expression and in converting AD from a Th2-dominant acute phase to a Th2-Th1 mixed chronic inflammation phase.

4. TLR-Targeted Therapies

TLRs play important roles in linking innate and adaptive immune responses to initiate immediate as well as long-term host defense against danger signals, and dysregulations of TLRs are responsible for the pathogenesis of several inflammatory skin diseases, and therefore, targeting TLRs is of great therapeutic potential to treat skin diseases. Several TLR agonists or antagonists or TLR modulators have been approved or are currently in development to treat skin diseases [105]. We will next review TLR's therapeutic implication, recent advances, and future prospects in treating skin diseases.

4.1. Therapeutic Use of TLR Ligands to Boost Host Immunity against Pathogens or Cancer. TLR agonists have been used to treat infectious skin diseases by boosting host innate immune defense against pathogens. *Candida albicans*, a fungal member of the normal human skin microbiome, is normally harmless, but in immunodeficient patients, it can cause life-threatening infections. Amphotericin B (AmB), a commonly used antifungal agent, stimulates several TLRs (TLR1, TLR2, and TLR4) followed by the production of pro-inflammatory cytokines such IL6, IL8, and TNF, boosting the host's immunity against *C. albicans* [106]. Caspofungin (echinocandins), a new class of antifungal drugs, inhibits the synthesis of β-glucan in the fungal cell wall by influencing the interactions between Dectin1 and TLR2, TLR4, or TLR9 [107]. TLR ligands have also been used for the treatment or vaccine development for herpes simplex virus (HSV) [105]. In mice, HSV vaccines adjuvanted with the TLR9 agonist unmethylated CpG are superior to the unadjuvanted vaccine at eliciting a robust HSV-specific cell-mediated immune response [108].

TLR agonists have also been used to boost locoregional and systemic immunity against cancer. Imiquimod (IMQ), a TLR7/8 ligand, is the first US FDA-approved drug to treat external genital and perianal warts and then approved for actinic keratosis and basal cell carcinoma (BCC), the most common skin cancer worldwide [109]. The effect of IMQ is mediated by recruitment and activation of pDC, cDC, or macrophages through TLR7/TLR8, leading to the production of cytokines including type 1 IFNs, IL1, IL6, and TNF followed by the development of cell-mediated adaptive immunity against cancer cells [109]. Due to its autoimmune-stimulatory capacity, a known side effect of IMQ is the development of psoriasis-like skin inflammation in both human and mice, and therefore, topical application of IMQ has been commonly used as a method to trigger psoriasis-like skin inflammation in mice. Synthetic unmethylated CpG type B oligodeoxynucleotide CpG 7909, the TLR9 agonist that stimulates DC, macrophages, or NK cells, has been shown to be effective against BCC and metastatic melanoma [110, 111]. Other TLR ligands, such as TLR3 ligand poly (I:C), a synthetic analog of viral dsRNA, can be used in combination with antitumor nanoparticles to promote melanoma regression in mice by promoting melanocyte apoptosis and shifting macrophages to a proinflammatory and tumoricidal phenotype [112].

4.2. Therapeutic Effects of TLR Inhibition in Psoriasis. While the TLR7-8 agonist imiquimod triggers psoriasis, synthetic oligonucleotides, antagonists for TLR7-9, can suppress Th1 and Th17 immune development in a mouse model of IL23-induced psoriasis [113]. In addition, several oligonucleotide-based antagonists of TLR7-9 such as IMO-3100 and IMO-8400 have been shown to be safe and effective in phase 2 clinical trials in patients with moderate-to-severe plaque psoriasis by blocking the activation of the IL17 pathway [114].

Conventional psoriasis therapies, including topical applications of vitamin D analogs or vitamin A analogs, have also been shown to exert their anti-inflammatory effects by modulating TLR function. Vitamin D3 downregulates the expression of TLR2, TLR4, and TLR9 and suppresses TLR9-mediated cytokine production in human monocytes [115], and the vitamin D analog calcipotriol attenuates CpG-mediated elevation of TLR9 and MyD88 expression in pDCs [116]. Retinoids, namely, vitamin A and its metabolites, have been used to treat psoriasis since the 1980s. Retinoid-mediated activation of retinoic acid receptors (RAR) and retinoid X receptors (RXR) improves the symptoms of psoriasis by regulating cell proliferation/-differentiation as well as by suppressing inflammation [117]. Retinoid analog can reduce the expression of TLR2 and its coreceptor CD14 in human monocytes and therefore prevent TLR2-mediated innate immune response to microbes [118, 119].

Together, inhibition of TLRs by specific TLR antagonists or by natural compounds such as vitamin A or D analogs attenuates the activation of the innate immune system that initiates the autoimmune cascade in psoriasis. Although new biological drugs targeting T cell activation molecules such as TNFα (such as etanercept, adalimumab, and infliximab), IL12 and IL23 (such as ustekinumab), IL23 (such as guselkumab, tildrakizumab, and risankizumab), IL17A (such as secukinumab and ixekizumab), or IL17 receptor A (such as brodalumab) have shown to be safe and efficacious in recent psoriasis clinical trials, however, lack of long-term efficacy and rapid regain of psoriasis upon removal of these drugs suggest that preventing adaptive immune activation alone is not sufficient to treat psoriasis. Targeting TLRs or PRRs in combination with T cell therapy may result in more sustainable effect to treat psoriasis.

4.3. TLRs and Atopic Dermatitis. As we have described earlier, impaired TLR2 function plays a role in driving loss of barrier integrity and the immune system imbalance (Th2 dominance) during the acute phase of AD, but

aberrant activation of TLR2 may lead to Th1 immune development during the chronic phase of AD and may also lead to the production of keratinocyte-specific cytokine TSLP that drives the allergic immune responses. Therefore, strategies that finely modulate TLR2 expression or function hold promise in restoring barrier function and immune balance in AD.

Topical calcineurin inhibitors (TCIs), including tacrolimus and pimecrolimus, are FDA-approved drugs for the treatment of AD. TCIs block the activity of the enzyme calcineurin, to prevent the activation of the nuclear factor of activated T cells (NFAT), which in turn blocks cytokine IL2 production as well as the subsequent T cell activation and proliferation [120–122]. It has also been reported that the abnormal expression of TLR1 and TLR2 can be normalized after a 3-week treatment with tacrolimus ointment [123], suggesting that TCIs may exert their therapeutic effects by restoring normal function of TLR2 signaling in AD.

5. Conclusion

Skin, located at the first line of defense, is constantly exposed to pathogenic or danger factors from the environment. TLRs, the key pattern recognition receptors, are involved in the recognition of PAMPs or DAMPs, initiation of innate immune responses, regulation of adaptive immune responses, and ultimately development of immediate and long-term immunity against pathogens. There is a growing body of evidence demonstrating that TLRs play indispensable roles in the pathogenesis of several inflammatory skin diseases, and therefore, therapeutic strategies have been developed and studied to target TLRs to either boost immunity against pathogens or cease aberrant activation of TLRs that drives autoimmune activation. But with recent success in the new biological drugs targeting T cells, the effector cell type at the downstream of disease progression, therapeutic approaches targeting innate immune activation during early stages of disease progression become less favorable. However, inhibiting the activation of the adaptive immune activation alone, without blocking the early innate immune events, can only alleviate disease symptoms but cannot cure the disease and may lead to rapid regain of inflammation upon drug removal. Future studies will be needed to develop targeted therapies for TLRs or PRRs which may be used in combination with T cell-targeted therapy to achieve more sustainable interventions to treat inflammatory skin diseases, such as psoriasis or atopic dermatitis.

Conflicts of Interest

The authors have nothing to disclose.

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References

[1] T. Kawai and S. Akira, "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors," *Nature Immunology*, vol. 11, no. 5, pp. 373–384, 2010.

[2] H. Kumar, T. Kawai, and S. Akira, "Pathogen recognition by the innate immune system," *International Reviews of Immunology*, vol. 30, no. 1, pp. 16–34, 2011.

[3] T. H. Mogensen, "Pathogen recognition and inflammatory signaling in innate immune defenses," *Clinical Microbiology Reviews*, vol. 22, no. 2, pp. 240–273, 2009.

[4] T. Kawai and S. Akira, "The roles of TLRs, RLRs and NLRs in pathogen recognition," *International Immunology*, vol. 21, no. 4, pp. 317–337, 2009.

[5] F. L. Rock, G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan, "A family of human receptors structurally related to Drosophila Toll," *Proceedings of the National Academy of Sciences*, vol. 95, no. 2, pp. 588–593, 1998.

[6] B. Lemaitre, E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann, "The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in Drosophila adults," *Cell*, vol. 86, no. 6, pp. 973–983, 1996.

[7] R. L. Gallo and L. V. Hooper, "Epithelial antimicrobial defence of the skin and intestine," *Nature Reviews. Immunology*, vol. 12, no. 7, pp. 503–516, 2012.

[8] L. J. Zhang and R. L. Gallo, "Antimicrobial peptides," *Current Biology*, vol. 26, no. 1, pp. R14–R19, 2016.

[9] S. X. Chen, L. J. Zhang, and R. L. Gallo, "Dermal white adipose tissue: a newly recognized layer of skin innate defense," *The Journal of Investigative Dermatology*, vol. 139, no. 5, pp. 1002–1009, 2019.

[10] R. R. Driskell, B. M. Lichtenberger, E. Hoste et al., "Distinct fibroblast lineages determine dermal architecture in skin development and repair," *Nature*, vol. 504, no. 7479, pp. 277–281, 2013.

[11] A. T. Ermertcan, F. Öztürk, and K. Gündüz, "Toll-like receptors and skin," *Journal of the European Academy of Dermatology and Venereology*, vol. 25, no. 9, pp. 997–1006, 2011.

[12] L. S. Miller, "Toll-like receptors in skin," *Advances in Dermatology*, vol. 24, pp. 71–87, 2008.

[13] L. S. Miller and R. L. Modlin, "Toll-like receptors in the skin," *Seminars in Immunopathology*, vol. 29, no. 1, pp. 15–26, 2007.

[14] J. I. Godfroy III, M. Roostan, Y. S. Moroz, I. V. Korendovych, and H. Yin, "Isolated Toll-like receptor transmembrane domains are capable of oligomerization," *PLoS One*, vol. 7, no. 11, p. e48875, 2012.

[15] T. Kawasaki and T. Kawai, "Toll-like receptor signaling pathways," *Frontiers in Immunology*, vol. 5, p. 461, 2014.

[16] C. C. Lee, A. M. Avalos, and H. L. Ploegh, "Accessory molecules for Toll-like receptors and their function," *Nature Reviews. Immunology*, vol. 12, no. 3, pp. 168–179, 2012.

[17] R. L. Gallo and J. J. Bernard, "Innate immune sensors stimulate inflammatory and immunosuppressive responses to UVB radiation," *The Journal of Investigative Dermatology*, vol. 134, no. 6, pp. 1508–1511, 2014.

[18] L. Yu, L. Wang, and S. Chen, "Endogenous toll-like receptor ligands and their biological significance," *Journal of Cellular and Molecular Medicine*, vol. 14, no. 11, pp. 2592–2603, 2010.

[19] M. Yamamoto and K. Takeda, "Current views of toll-like receptor signaling pathways," *Gastroenterology Research and Practice*, vol. 2010, Article ID 240365, 8 pages, 2010.

[20] Z. Zhang, U. Ohto, T. Shibata et al., "Structural analysis reveals that Toll-like receptor 7 is a dual receptor for guanosine and single-stranded RNA," *Immunity*, vol. 45, no. 4, pp. 737–748, 2016.

[21] T. Shibata, U. Ohto, S. Nomura et al., "Guanosine and its modified derivatives are endogenous ligands for TLR7," *International Immunology*, vol. 28, no. 5, pp. 211–222, 2016.

[22] L. J. Zhang, G. L. Sen, N. L. Ward et al., "Antimicrobial peptide LL37 and MAVS signaling drive interferon-β production by epidermal keratinocytes during skin injury," *Immunity*, vol. 45, no. 1, pp. 119–130, 2016.

[23] D. Zhang, G. Zhang, M. S. Hayden et al., "A toll-like receptor that prevents infection by uropathogenic bacteria," *Science*, vol. 303, no. 5663, pp. 1522–1526, 2004.

[24] I. Botos, D. M. Segal, and D. R. Davies, "The structural biology of Toll-like receptors," *Structure*, vol. 19, no. 4, pp. 447–459, 2011.

[25] M. Triantafilou, F. G. J. Gamper, R. M. Haston et al., "Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting," *The Journal of Biological Chemistry*, vol. 281, no. 41, pp. 31002–31011, 2006.

[26] W. Piao, L. W. Ru, and V. Y. Toshchakov, "Differential adapter recruitment by TLR2 co-receptors," *Pathogens and Disease*, vol. 74, no. 5, p. ftw043, 2016.

[27] I. Zanoni, R. Ostuni, L. R. Marek et al., "CD14 controls the LPS-induced endocytosis of Toll-like receptor 4," *Cell*, vol. 147, no. 4, pp. 868–880, 2011.

[28] K. A. Fitzgerald, S. M. McWhirter, K. L. Faia et al., "IKKε and TBK1 are essential components of the IRF3 signaling pathway," *Nature Immunology*, vol. 4, no. 5, pp. 491–496, 2003.

[29] S. M. Joung, Z. Y. Park, S. Rani, O. Takeuchi, S. Akira, and J. Y. Lee, "Akt contributes to activation of the TRIF-dependent signaling pathways of TLRs by interacting with TANK-binding kinase 1," *Journal of Immunology*, vol. 186, no. 1, pp. 499–507, 2011.

[30] J. M. Cooper, Y. H. Ou, E. A. McMillan et al., "TBK1 provides context-selective support of the activated AKT/mTOR pathway in lung cancer," *Cancer Research*, vol. 77, no. 18, pp. 5077–5094, 2017.

[31] M. K. Crow, M. Olferiev, and K. A. Kirou, "Type I interferons in autoimmune disease," *Annual Review of Pathology*, vol. 14, no. 1, pp. 369–393, 2018.

[32] Y. Lai and R. Gallo, "Toll-like receptors in skin infections and inflammatory diseases," *Infectious Disorders Drug Targets*, vol. 8, no. 3, pp. 144–155, 2008.

[33] Y. Lai, A. di Nardo, T. Nakatsuji et al., "Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury," *Nature Medicine*, vol. 15, no. 12, pp. 1377–1382, 2009.

[34] J. J. Bernard, C. Cowing-Zitron, T. Nakatsuji et al., "Ultraviolet radiation damages self noncoding RNA and is detected by TLR3," *Nature Medicine*, vol. 18, no. 8, pp. 1286–1290, 2012.

[35] A. W. Borkowski, K. Park, Y. Uchida, and R. L. Gallo, "Activation of TLR3 in keratinocytes increases expression of genes involved in formation of the epidermis, lipid accumulation, and epidermal organelles," *The Journal of Investigative Dermatology*, vol. 133, no. 8, pp. 2031–2040, 2013.

[36] A. W. Borkowski, I. H. Kuo, J. J. Bernard et al., "Toll-like receptor 3 activation is required for normal skin barrier repair following UV damage," *Journal of Investigative Dermatology*, vol. 135, no. 2, pp. 569–578, 2015.

[37] F. Meisgen, N. Xu Landén, A. Wang et al., "MiR-146a negatively regulates TLR2-induced inflammatory responses in keratinocytes," *The Journal of Investigative Dermatology*, vol. 134, no. 7, pp. 1931–1940, 2014.

[38] K. Yamasaki, K. Kanada, D. T. Macleod et al., "TLR2 expression is increased in rosacea and stimulates enhanced serine protease production by keratinocytes," *Journal of Investigative Dermatology*, vol. 131, no. 3, pp. 688–697, 2011.

[39] T. Yuki, H. Yoshida, Y. Akazawa, A. Komiya, Y. Sugiyama, and S. Inoue, "Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes," *Journal of Immunology*, vol. 187, no. 6, pp. 3230–3237, 2011.

[40] Q. Lin, L. Wang, Y. Lin et al., "Toll-like receptor 3 ligand polyinosinic:polycytidylic acid promotes wound healing in human and murine skin," *The Journal of Investigative Dermatology*, vol. 132, no. 8, pp. 2085–2092, 2012.

[41] L. J. Zhang, S. X. Chen, C. F. Guerrero-Juarez et al., "Age-related loss of innate immune antimicrobial function of dermal fat is mediated by transforming growth factor beta," *Immunity*, vol. 50, no. 1, pp. 121–136.e5, 2019, e5.

[42] L. J. Zhang, C. F. Guerrero-Juarez, T. Hata et al., "Dermal adipocytes protect against invasive Staphylococcus aureus skin infection," *Science*, vol. 347, no. 6217, pp. 67–71, 2015.

[43] S. Morizane, A. Kajita, K. Mizuno, T. Takiguchi, and K. Iwatsuki, "Toll-like receptor signalling induces the expression of serum amyloid A in epidermal keratinocytes and dermal fibroblasts," *Clinical and Experimental Dermatology*, vol. 44, no. 1, pp. 40–46, 2019.

[44] J. F. Wang, K. Hori, J. Ding et al., "Toll-like receptors expressed by dermal fibroblasts contribute to hypertrophic scarring," *Journal of Cellular Physiology*, vol. 226, no. 5, pp. 1265–1273, 2011.

[45] S. Bhattacharyya, W. Wang, W. Qin et al., "TLR4-dependent fibroblast activation drives persistent organ fibrosis in skin and lung," *JCI Insight*, vol. 3, no. 13, 2018.

[46] M. Haniffa, M. Gunawan, and L. Jardine, "Human skin dendritic cells in health and disease," *Journal of Dermatological Science*, vol. 77, no. 2, pp. 85–92, 2015.

[47] C. N. Renn, D. J. Sanchez, M. T. Ochoa et al., "TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response," *Journal of Immunology*, vol. 177, no. 1, pp. 298–305, 2006.

[48] K. Iwamoto, T. J. Nümm, S. Koch, N. Herrmann, N. Leib, and T. Bieber, "Langerhans and inflammatory dendritic epidermal cells in atopic dermatitis are tolerized toward TLR2 activation," *Allergy*, vol. 73, no. 11, pp. 2205–2213, 2018.

[49] V. Flacher, M. Bouschbacher, E. Verronèse et al., "Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria," *Journal of Immunology*, vol. 177, no. 11, pp. 7959–7967, 2006.

[50] B. E. Clausen and P. Stoitzner, "Functional specialization of skin dendritic cell subsets in regulating T cell responses," *Frontiers in Immunology*, vol. 6, p. 534, 2015.

[51] F. O. Nestle, C. Conrad, A. Tun-Kyi et al., "Plasmacytoid predendritic cells initiate psoriasis through interferon-α

production," *The Journal of Experimental Medicine*, vol. 202, no. 1, pp. 135–143, 2005.

[52] N. Kadowaki, S. Ho, S. Antonenko et al., "Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens," *The Journal of Experimental Medicine*, vol. 194, no. 6, pp. 863–870, 2001.

[53] M. J. Portou, D. Baker, D. Abraham, and J. Tsui, "The innate immune system, toll-like receptors and dermal wound healing: a review," *Vascular Pharmacology*, vol. 71, pp. 31–36, 2015.

[54] C. K. Thind and A. D. Ormerod, "Recent advances in inflammatory skin diseases," *Scottish Medical Journal*, vol. 53, no. 2, pp. 30–34, 2008.

[55] C. E. Griffiths and J. N. Barker, "Pathogenesis and clinical features of psoriasis," *Lancet*, vol. 370, no. 9583, pp. 263–271, 2007.

[56] X. Yin, H. Q. Low, L. Wang et al., "Genome-wide meta-analysis identifies multiple novel associations and ethnic heterogeneity of psoriasis susceptibility," *Nature Communications*, vol. 6, no. 1, 2015.

[57] C. Conrad and M. Gilliet, "Psoriasis: from pathogenesis to targeted therapies," *Clinical Reviews in Allergy and Immunology*, vol. 54, no. 1, article 8668, pp. 102–113, 2018.

[58] M. Xu, H. Lu, Y. H. Lee et al., "An interleukin-25-mediated autoregulatory circuit in keratinocytes plays a pivotal role in psoriatic skin inflammation," *Immunity*, vol. 48, no. 4, pp. 787–798.e4, 2018, e4.

[59] H. Li, Q. Yao, A. G. Mariscal et al., "Epigenetic control of *IL-23* expression in keratinocytes is important for chronic skin inflammation," *Nature Communications*, vol. 9, no. 1, 2018.

[60] C. Albanesi, S. Madonna, P. Gisondi, and G. Girolomoni, "The interplay between keratinocytes and immune cells in the pathogenesis of psoriasis," *Frontiers in Immunology*, vol. 9, 2018.

[61] R. Lande, J. Gregorio, V. Facchinetti et al., "Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide," *Nature*, vol. 449, no. 7162, pp. 564–569, 2007.

[62] D. Ganguly, G. Chamilos, R. Lande et al., "Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8," *Journal of Experimental Medicine*, vol. 206, no. 9, pp. 1983–1994, 2009.

[63] S. Garcia-Rodriguez, S. Arias-Santiago, R. Perandrés-López et al., "Increased gene expression of Toll-like receptor 4 on peripheral blood mononuclear cells in patients with psoriasis," *Journal of the European Academy of Dermatology and Venereology*, vol. 27, no. 2, pp. 242–250, 2013.

[64] S. Carrasco, F. S. Neves, M. H. Fonseca et al., "Toll-like receptor (TLR) 2 is upregulated on peripheral blood monocytes of patients with psoriatic arthritis: a role for a gram-positive inflammatory trigger?," *Clinical and Experimental Rheumatology*, vol. 29, no. 6, pp. 958–962, 2011.

[65] R. L. Smith, H. L. Hébert, J. Massey et al., "Association of Toll-like receptor 4 (TLR4) with chronic plaque type psoriasis and psoriatic arthritis," *Archives of Dermatological Research*, vol. 308, no. 3, pp. 201–205, 2016.

[66] S. Shao, H. Fang, E. Dang et al., "Neutrophil extracellular traps promote inflammatory responses in psoriasis via activating epidermal TLR4/IL-36R crosstalk," *Frontiers in Immunology*, vol. 10, 2019.

[67] P. Besgen, P. Trommler, S. Vollmer, and J. C. Prinz, "Ezrin, maspin, peroxiredoxin 2, and heat shock protein 27: potential targets of a streptococcal-induced autoimmune response in psoriasis," *Journal of Immunology*, vol. 184, no. 9, pp. 5392–5402, 2010.

[68] S. P. Jariwala, "The role of dendritic cells in the immunopathogenesis of psoriasis," *Archives of Dermatological Research*, vol. 299, no. 8, pp. 359–366, 2007.

[69] M. Kakeda, M. Arock, C. Schlapbach, and N. Yawalkar, "Increased expression of heat shock protein 90 in keratinocytes and mast cells in patients with psoriasis," *Journal of the American Academy of Dermatology*, vol. 70, no. 4, pp. 683–690.e1, 2014.

[70] M. Masson Regnault, M. P. Konstantinou, A. Khemis et al., "Early relapse of psoriasis after stopping brodalumab: a retrospective cohort study in 77 patients," *Journal of the European Academy of Dermatology and Venereology*, vol. 31, no. 9, pp. 1491–1496, 2017.

[71] A. Khemis, M. Cavalié, H. Montaudié, J. P. Lacour, and T. Passeron, "Rebound pustular psoriasis after brodalumab discontinuation," *The British Journal of Dermatology*, vol. 175, no. 5, pp. 1065-1066, 2016.

[72] A. Blauvelt, R. Langley, J. Szepietowski et al., "Secukinumab withdrawal leads to loss of treatment responses in a majority of subjects with plaque psoriasis with retreatment resulting in rapid regain of responses: a pooled analysis of two phase 3 trials," *Journal of the American Academy of Dermatology*, vol. 74, no. 5, pp. Ab273–Ab273, 2016.

[73] L. Sharma, "Diagnostic clinical features of atopic dermatitis," *Indian Journal of Dermatology, Venereology and Leprology*, vol. 67, no. 1, pp. 25–27, 2001.

[74] T. Bieber, "Atopic dermatitis," *The New England Journal of Medicine*, vol. 358, no. 14, pp. 1483–1494, 2008.

[75] R. Beasley et al., "Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC," *Lancet*, vol. 351, no. 9111, article S0140673697073029, pp. 1225–1232, 1998.

[76] H. Williams, C. Robertson, A. Stewart et al., "Worldwide variations in the prevalence of symptoms of atopic eczema in the international study of asthma and allergies in childhood," *Journal of Allergy and Clinical Immunology*, vol. 103, no. 1, pp. 125–138, 1999.

[77] S. K. Bantz, Z. Zhu, and T. Zheng, "The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma," *Journal of Clinical & Cellular Immunology*, vol. 05, no. 02, 2014.

[78] S. F. Bloomfield, R. Stanwell-Smith, R. W. R. Crevel, and J. Pickup, "Too clean, or not too clean: the hygiene hypothesis and home hygiene," *Clinical and Experimental Allergy*, vol. 36, no. 4, pp. 402–425, 2006.

[79] J. Q. Gong, L. Lin, T. Lin et al., "Skin colonization by Staphylococcus aureus in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial," *British Journal of Dermatology*, vol. 155, no. 4, pp. 680–687, 2006.

[80] P. Meylan, C. Lang, S. Mermoud et al., "Skin colonization by *Staphylococcus aureus* precedes the clinical diagnosis of atopic dermatitis in infancy," *Journal of Investigative Dermatology*, vol. 137, no. 12, pp. 2497–2504, 2017.

[81] G. Baviera, M. C. Leoni, L. Capra et al., "Microbiota in healthy skin and in atopic eczema," *Biomed Research International*, vol. 2014, Article ID 436921, 6 pages, 2014.

[82] H. H. Kong, J. Oh, C. Deming et al., "Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis," *Genome Research*, vol. 22, no. 5, pp. 850–859, 2012.

[83] B. S. Baker, "The role of microorganisms in atopic dermatitis," *Clinical and Experimental Immunology*, vol. 144, no. 1, pp. 1–9, 2006.

[84] T. Nakatsuji, T. H. Chen, A. M. Two et al., "*Staphylococcus aureus* exploits epidermal barrier defects in atopic dermatitis to trigger cytokine expression," *Journal of Investigative Dermatology*, vol. 136, no. 11, pp. 2192–2200, 2016.

[85] Y. Nakamura, J. Oscherwitz, K. B. Cease et al., "Staphylococcus δ-toxin induces allergic skin disease by activating mast cells," *Nature*, vol. 503, no. 7476, pp. 397–401, 2013.

[86] L. Blicharz, L. Rudnicka, and Z. Samochocki, "Staphylococcus aureus: an underestimated factor in the pathogenesis of atopic dermatitis?," *Advances in Dermatology and Allergology*, vol. 36, no. 1, pp. 11–17, 2019.

[87] M. R. Williams, S. K. Costa, L. S. Zaramela et al., "Quorum sensing between bacterial species on the skin protects against epidermal injury in atopic dermatitis," *Science Translational Medicine*, vol. 11, no. 490, p. eaat8329, 2019.

[88] T. Nakatsuji, T. H. Chen, A. M. Butcher et al., "A commensal strain of Staphylococcus epidermidis protects against skin neoplasia," *Science Advances*, vol. 4, no. 2, p. eaao4502, 2018.

[89] A. L. Cogen, K. Yamasaki, K. M. Sanchez et al., "Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin," *Journal of Investigative Dermatology*, vol. 130, no. 1, pp. 192–200, 2010.

[90] Y. A. Tyurin, A. F. Shamsutdinov, N. N. Kalinin, A. A. Sharifullina, and I. D. Reshetnikova, "Association of Toll-like cell receptors TLR2 (p.Arg753GLN) and TLR4 (p.Asp299GLY) polymorphisms with indicators of general and local immunity in patients with atopic dermatitis.," *Journal of Immunology Research*, vol. 2017, 6 pages, 2017.

[91] C. Salpietro, L. Rigoli, M. M. del Giudice et al., "Tlr2 and Tlr4 gene polymorphisms and atopic dermatitis in Italian children: a multicenter study," *International Journal of Immunopathology and Pharmacology*, vol. 24, 4_suppl, pp. 33–40, 2011.

[92] R. Panzer, C. Blobel, R. Fölster-Holst, and E. Proksch, "TLR2 and TLR4 expression in atopic dermatitis, contact dermatitis and psoriasis," *Experimental Dermatology*, vol. 23, no. 5, pp. 364–366, 2014.

[93] M. Niebuhr, C. Lutat, S. Sigel, and T. Werfel, "Impaired TLR-2 expression and TLR-2-mediated cytokine secretion in macrophages from patients with atopic dermatitis," *Allergy*, vol. 64, no. 11, pp. 1580–1587, 2009.

[94] H. Hasannejad, R. Takahashi, M. Kimishima, K. Hayakawa, and T. Shiohara, "Selective impairment of Toll-like receptor 2-mediated proinflammatory cytokine production by monocytes from patients with atopic dermatitis," *Journal of Allergy and Clinical Immunology*, vol. 120, no. 1, pp. 69–75, 2007.

[95] Y. Yu, Y. Zhang, J. Zhang et al., "Impaired Toll-like receptor 2-mediated Th1 and Th17/22 cytokines secretion in human peripheral blood mononuclear cells from patients with atopic dermatitis," *Journal of Translational Medicine*, vol. 13, no. 1, article 744, 2015.

[96] I. H. Kuo, A. Carpenter-Mendini, T. Yoshida et al., "Activation of epidermal Toll-like receptor 2 enhances tight junction function: implications for atopic dermatitis and skin barrier repair," *Journal of Investigative Dermatology*, vol. 133, no. 4, pp. 988–998, 2013.

[97] P. Y. Ong, T. Ohtake, C. Brandt et al., "Endogenous antimicrobial peptides and skin infections in atopic dermatitis," *New England Journal of Medicine*, vol. 347, no. 15, pp. 1151–1160, 2002.

[98] N. A. Gandhi, G. Pirozzi, and N. M. H. Graham, "Commonality of the IL-4/IL-13 pathway in atopic diseases," *Expert Review of Clinical Immunology*, vol. 13, no. 5, pp. 425–437, 2017.

[99] S. Kaesler, T. Volz, Y. Skabytska et al., "Toll-like receptor 2 ligands promote chronic atopic dermatitis through IL-4-mediated suppression of IL-10," *Journal of Allergy and Clinical Immunology*, vol. 134, no. 1, pp. 92–99.e6, 2014.

[100] H. Jin, L. Kumar, C. Mathias et al., "Toll-like receptor 2 is important for the T_H1 response to cutaneous sensitization," *Journal of Allergy and Clinical Immunology*, vol. 123, no. 4, pp. 875–882.e1, 2009.

[101] Z. Wang, L. J. Zhang, G. Guha et al., *PLoS One*, vol. 7, no. 12, p. e51262, 2012.

[102] Y. J. Liu, "Thymic stromal lymphopoietin: master switch for allergic inflammation," *Journal of Experimental Medicine*, vol. 203, no. 2, pp. 269–273, 2006.

[103] J. Kim, B. E. Kim, J. Lee et al., "Epidermal thymic stromal lymphopoietin predicts the development of atopic dermatitis during infancy," *Journal of Allergy and Clinical Immunology*, vol. 137, no. 4, pp. 1282–1285.e4, 2016.

[104] Y. Xie, T. Takai, X. Chen, K. Okumura, and H. Ogawa, "Long TSLP transcript expression and release of TSLP induced by TLR ligands and cytokines in human keratinocytes," *Journal of Dermatological Science*, vol. 66, no. 3, pp. 233–237, 2012.

[105] N. Matin, O. Tabatabaie, P. Mohammadinejad, and N. Rezaei, "Therapeutic targeting of Toll-like receptors in cutaneous disorders," *Expert Opinion on Therapeutic Targets*, vol. 19, no. 12, pp. 1651–1663, 2015.

[106] M. R. Mihu, R. Pattabhi, and J. D. Nosanchuk, "The impact of antifungals on toll-like receptors," *Frontiers in Microbiology*, vol. 5, 2014.

[107] S. Moretti, S. Bozza, C. D'Angelo et al., "Role of innate immune receptors in paradoxical caspofungin activity in vivo in preclinical aspergillosis," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 8, pp. 4268–4276, 2012.

[108] M. T. Hensel, J. D. Marshall, M. R. Dorwart et al., "Prophylactic herpes simplex virus 2 (HSV-2) vaccines adjuvanted with stable emulsion and Toll-like receptor 9 agonist induce a robust HSV-2-specific cell-mediated immune response, protect against symptomatic disease, and reduce the latent viral reservoir," *Journal of Virology*, vol. 91, no. 9, 2017.

[109] E. Hanna, R. Abadi, and O. Abbas, "Imiquimod in dermatology: an overview," *International Journal of Dermatology*, vol. 55, no. 8, pp. 831–844, 2016.

[110] B. G. Molenkamp, B. J. R. Sluijter, P. A. M. van Leeuwen et al., "Local administration of PF-3512676 CpG-B instigates tumor-specific $CD8^+$ T-cell reactivity in melanoma patients," *Clinical Cancer Research*, vol. 14, no. 14, pp. 4532–4542, 2008.

[111] B. D. Koster, M. F. C. M. van den Hout, B. J. R. Sluijter et al., "Local adjuvant treatment with low-dose CpG-B offers durable protection against disease recurrence in clinical stage I-II

melanoma: data from two randomized phase II trials," *Clinical Cancer Research*, vol. 23, no. 19, pp. 5679–5686, 2017.

[112] J. zhao, Z. Zhang, Y. Xue et al., "Anti-tumor macrophages activated by ferumoxytol combined or surface-functionalized with the TLR3 agonist poly (I : C) promote melanoma regression," *Theranostics*, vol. 8, no. 22, pp. 6307–6321, 2018.

[113] W. Jiang, F. G. Zhu, L. Bhagat et al., "A Toll-like receptor 7, 8, and 9 antagonist inhibits Th1 and Th17 responses and inflammasome activation in a model of IL-23-induced psoriasis," *The Journal of Investigative Dermatology*, vol. 133, no. 7, pp. 1777–1784, 2013.

[114] M. Suarez-Farinas, M. Suarez-Farinas, J. Belasco, T. Sullivan, R. Arbeit, and J. Krueger, "Treatment of psoriasis patients with IMO-3100 shows improvement in gene expression patterns of meta-analysis derived-3 transcriptome and IL-17 pathway," *Arthritis and Rheumatism*, vol. 65, pp. S495–S495, 2013.

[115] L. J. Dickie, L. D. Church, L. R. Coulthard, R. J. Mathews, P. Emery, and M. F. McDermott, "Vitamin D3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes," *Rheumatology (Oxford)*, vol. 49, no. 8, pp. 1466–1471, 2010.

[116] T. Suzuki, J. Sakabe, K. Kamiya, A. Funakoshi, and Y. Tokura, "The vitamin D3 analogue calcipotriol suppresses CpG-activated TLR9-MyD88 signalling in murine plasmacytoid dendritic cells," *Clinical and Experimental Dermatology*, vol. 43, no. 4, pp. 445–448, 2018.

[117] L. Beckenbach, J. M. Baron, H. F. Merk, H. Löffler, and P. M. Amann, "Retinoid treatment of skin diseases," *European Journal of Dermatology*, vol. 25, no. 5, pp. 384–391, 2015.

[118] M. C. Dispenza, E. B. Wolpert, K. L. Gilliland et al., "Systemic isotretinoin therapy normalizes exaggerated TLR-2-mediated innate immune responses in acne patients," *Journal of Investigative Dermatology*, vol. 132, no. 9, pp. 2198–2205, 2012.

[119] P. T. Liu, S. R. Krutzik, J. Kim, and R. L. Modlin, "Cutting edge: all-trans retinoic acid down-regulates TLR2 expression and function," *Journal of Immunology*, vol. 174, no. 5, pp. 2467–2470, 2005.

[120] M. Caproni, D. Torchia, E. Antiga, W. Volpi, E. D. Bianco, and P. Fabbri, "The effects of tacrolimus ointment on regulatory T lymphocytes in atopic dermatitis," *Journal of Clinical Immunology*, vol. 26, no. 4, pp. 370–375, 2006.

[121] J. Cury Martins, C. Martins, V. Aoki, A. F. T. Gois, H. A. Ishii, and E. M. K. da Silva, "Topical tacrolimus for atopic dermatitis," *Cochrane Database of Systematic Reviews*, vol. 7, 2015.

[122] M. Czarnecka-Operacz and D. Jenerowicz, "Topical calcineurin inhibitors in the treatment of atopic dermatitis – an update on safety issues," *Journal der Deutschen Dermatologischen Gesellschaft*, vol. 10, no. 3, pp. 167–172, 2012.

[123] E. Antiga, W. Volpi, D. Torchia, P. Fabbri, and M. Caproni, "Effects of tacrolimus ointment on Toll-like receptors in atopic dermatitis," *Clinical and Experimental Dermatology*, vol. 36, no. 3, pp. 235–241, 2011.

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Research Article

Innate Immune Responses Associated with Resistance against *Haemonchus contortus* in Morada Nova Sheep

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The immune response against *Haemonchus contortus* infections is primarily associated with the Th2 profile. However, the exact mechanisms associated with increased sheep resistance against this parasite remains poorly elucidated. The present study is aimed at evaluating mediators from the innate immune response in lambs of the Morada Nova Brazilian breed with contrasting *H. contortus* resistance phenotypes. Briefly, 287 lambs were characterized through fecal egg counts (FEC) and packed cell volume (PCV) after two independent experimental parasitic challenges with 4,000 H. *contortus* L_3. 20 extreme resistance phenotypes (10 most resistant and 10 most susceptible) were selected, subjected to a third artificial infection with 4,000 L_3, and euthanized 7 days later. Tissue samples were collected from abomasal fundic and pyloric mucosa and abomasal lymph nodes. Blood samples were collected at days 0 and 7 of the third parasitic challenge. RNA was extracted from tissue and blood samples for relative quantification of innate immune-related genes by RT-qPCR. For the abomasal fundic mucosa, increased *TNFα* and *IL1β* expression levels ($P < 0.05$) were found in the susceptible animals, while resistant animals had *IL33* superiorly expressed ($P < 0.05$). Higher levels ($P < 0.05$) of *TLR2* and *CFI* were found in the abomasal pyloric mucosa of resistant animals. *TNFα* was at higher levels ($P < 0.05$) in the blood of susceptible lambs, at day 0 of the third artificial infection. The exacerbated proinflammatory response observed in susceptible animals, at both local and systemic levels, may be a consequence of high *H. contortus* parasitism. This hypothesis is corroborated by the higher blood levels of *TNFα* before the onset of infection, which probably remained elevated from the previous parasitic challenges. On the other hand, resistant lambs had an enhanced response mediated by TLR recognition and complement activation. Nevertheless, this is the first study to directly associate sheep parasitic resistance with IL33, an innate trigger of the Th2-polarized response.

1. Introduction

Haemonchus contortus infections are the main cause of economic losses to sheep farming in tropical countries. This gastrointestinal nematode (GIN) is considered the most pathogenic sheep parasite, and it is the prevalent species in most of the Brazilian territory [1–4]. The losses are due to decreased productivity, sheep mortality, and expenses with anthelmintic treatments [1, 5]. The inadequate use of anthelmintics led to a widespread multiple resistance against most of the commercially available molecules [6–9], which highlights the importance of alternative control methods, such as selection of genetically resistant animals, and the development of immunotherapeutic or imunoprophylactic tools.

Therefore, it is essential to understand the genetic or immune-related mechanisms involved in the development of host resistance against GIN infections.

The immune response of sheep against GIN infections is primarily associated with the adaptive Th2-polarized profile, with local release of the interleukins IL4, IL5, and IL13, in addition to IgE production, eosinophilia, and mastocytosis [10–13]. However, the exact mechanisms associated with increased sheep resistance against *H. contortus* infections remains poorly elucidated, especially regarding the involvement of the innate immunity.

The activation of Toll-like receptor (TLR) genes (especially *TLR2*, *TLR4*, and *TLR10*) has been associated with host defense against *H. contortus* [14, 15]. In addition, the activation of the nuclear factor κB (NFκB) pathway induces inflammatory response in the early stages of parasitic infection, with increased production of proinflammatory cytokines, such as TNFα and IL-1β [16–18]. In resistant animals, this response is rapidly replaced by the induction of anti-inflammatory activity, with increased levels of IL10 and TGFβ [14, 19]. On the other hand, susceptible animals present a persistent inflammatory response, with a high expression of NFκB signaling pathway molecules (*IKBKB* and *NFKBIA*) and proinflammatory cytokines (*IL1β*, *IL6*, and *TNFα*), followed by a late expression of regulatory markers (*IL10* and *TGFβ*) [14].

The reactive oxygen species, as nitric oxide, are well known for its antimicrobial activity and are associated with cytotoxic effect against GIN [20]. This molecule is stimulated by inducible nitric oxide synthase (iNOS) released by activated effector cells. In murine models, both iNOS and nitric oxide were proved to be involved in resistance against parasitic nematodes [21]. The activation of genes responsible for producing reactive oxygen species (*NOS2A*) was directly associated with increased resistance of sheep against *H. contortus* and *Trichostrongylus colubriformis* [14].

GIN infection leads to the activation of the alternative pathway of the complement system [22, 23], and the action of the resulting opsonins has been proved to be lethal to GIN larvae [24]. This pathway involves the spontaneous cleavage of C3 into active forms, C3a and C3b, with strong opsonizing properties. Besides, like the other pathways, alternative activation of the complement results in the formation of the terminal complex (C5-C9) [25]. Although, due to the high abundance of C3 at mucosal surfaces, regulatory mechanisms are required to avoid hyperactivation of this pathway, in which complement factor I (CFI) plays an essential role [26]. Superior activation of genes directly associated with complement activation (*C7* and *CFI*) has been observed in sheep resistant to *H. contortus* [27].

Recent studies have shown the importance of interleukins IL25 and IL33 in the early phase of defense against GIN [28–30]. These "alarmins" are constitutively expressed in epithelial cells of the mucosal barriers, the first cells to have contact with the invading pathogens. In response to tissue injury, there is a release of IL25 and IL33 [31], potent inducers and enhancers of Th2 profile immune response, by stimulating type 2 innate lymphoid cells (ILC2) and CD4+Th2-polarized cells [31–33]. As for sheep, *Trichostrongylus colubriformis* infection was previously associated with upregulation of *IL33* in the intestinal mucosa [34]. However, regarding the role of IL25, there are no previous studies evaluating this cytokine in GIN-infected sheep.

The Morada Nova, a Brazilian hair sheep breed, is well known for its improved natural resistance against GIN infections compared with other breeds such as Dorper, Texel, Ile de France, and Santa Inês [35–37]. Most of studies have compared immune profiles between sheep breeds with different levels of parasitic resistance, while studies targeting immune responses inside breeds are scarce and absent for the Morada Nova breed. Therefore, the present study is aimed at evaluating innate immune mediators in the abomasum and abomasal lymph nodes of Morada Nova lambs with opposing resistance phenotypes against *H. contortus* infections. Furthermore, the systemic inflammatory profile was also assessed in the blood of these animals. Nevertheless, this is the first study to investigate the role of the "alarmins" in the *H. contortus* resistance.

2. Materials and Methods

2.1. Experimental Lambs and Animal Management. 287 Morada Nova lambs, 146 males and 141 females, were weaned at approximately a hundred days of age. The lambs were kept with their mothers in 3 hectares of pasture covered with Aruana grass (*Panicum maximum* cv. Aruana) and fed in a creep feeding system until weaning. Newly weaned lambs were allocated to four paddocks having the same pasture composition described above and were separated by sex. In the summer (rainy season), they were fed exclusively at the pasture. During dry season, they were supplemented with corn or grass silage added with pelleted citrus pulp. Water and mineral salt were supplied *ad libitum* throughout the experiment.

2.2. Phenotyping for Haemonchus contortus Resistance. At weaning, lambs were naturally infected with GIN, with a mean fecal egg count (FEC) of 6,643 ± 8,994 eggs per gram of feces (EPG). *Haemonchus* (96.4%) was the predominant genus in fecal cultures, followed by *Cooperia* (2.1%) and *Trichostrongylus* (1.5%). To eliminate the natural infection, all animals were dewormed with monepantel (Zolvix®, Novartis Animal Health, Brazil) at a 2.5 mg/kg dose. Nematode-free status was confirmed after two negative FECs (days 7 and 14 post-treatment). 15 days after deworming, the lambs were experimentally infected with a single oral dose of 4,000 H. *contortus* L_3 (day zero: D0). In this occasion, blood samples were collected for packed cell volume (PCV) determination. Individual FECs were performed every seven days from D21, and PCV was determined every fourteen days from D14. On D42 of the first parasitic challenge, the lambs were dewormed another time and, 15 days later, submitted to a second parasitic challenge, following the same chronogram previously described.

The lambs were classified according to their parasitic resistance level based on the averages of FEC and PCV after artificial infection (excluding post-deworming values: D0). Among these, the 10 most resistant (lowest FEC and highest

PCV) and the 10 most susceptible (highest FEC and lowest PCV) were identified. These animals were dewormed on D42 of the second parasitic challenge and placed in previously decontaminated cemented stalls, in order to avoid natural GIN infections. After 15 days, they were once again infected with 4,000 H. *contortus* L_3 and euthanized seven days later. Necropsy was performed and tissue samples were collected from the abomasal mucosa (fundic and pyloric regions) and abomasal lymph nodes, which were immediately snap frozen in liquid nitrogen (-196°C) and stored at -80°C until processing. Blood samples were collected in PAXgene Blood RNA tubes (Preanalytix, Valencia, USA) at D0 and D7 of the third parasitic challenge. These samples were kept at room temperature (25°C) for 12 h and then frozen at -20°C until process.

2.3. Target Gene Selection and Primer Design. A total of 15 target genes related to the innate immune responses were selected for relative quantification by reverse transcription followed by real-time quantitative PCR (RT-qPCR): pattern recognition receptors (*TLR2*, *TLR4*, *TLR7*, and *TLR10*); molecules of the NFκB signaling pathway (*NFKBIA*, *IKBKB*); inducible nitric oxide synthase (*NOS2A*), alarmin cytokines (*IL25* and *IL33*); proinflammatory cytokines (*TNFα* and *IL1β*); anti-inflammatory cytokines (*TGFβ* and *IL10*); and complement system components (*C7* and *CFI*). All target genes were quantified in the tissue samples, whereas, only the proinflammatory cytokines *TNFα* and *IL1β* were evaluated in blood samples, due to the lower yield of RNA extraction.

All primer pairs were designed using Primer3 (http://primer3.ut.ee/), based on messenger RNA (mRNA) sequences deposited in Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and gene sequences from Ensembl (http://www.ensembl.org/index.html). Whenever possible, primer spanning at least one junction between two adjacent exons were selected in order to avoid genomic DNA amplification. Primer sequences were analyzed by Netprimer (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimerServlet) and Oligoanalizer (https://www.idtdna.com/calc/analyzer), to avoid secondary structures formation. The specificity of primer pairs was verified by aligning the sequences with those deposited on international databases, using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. RNA Extraction and Complementary DNA (cDNA) Synthesis. Total RNA was extracted from tissue samples using QIAzol® Lysis Reagent (Qiagen) and Tissue Ruptor (Qiagen), followed by RNA purification in silica columns using RNeasy Mini Kit (Qiagen). Blood samples collected in PAXgene tubes were submitted to RNA extraction using the PAXgene Blood RNA Kit (Preanalytix), following the manufacturer's recommendations. The concentration and purity of RNA samples were estimated in the spectrophotometer NanoDrop™ 2000 (Thermo Scientific, Cleveland, USA), by absorbance readings at 260 nm (A_{260}) and A_{260}/A_{280} ratios, respectively. The RNA integrity was confirmed by 1.0% agarose gel electrophoresis.

Complementary DNA (cDNA) synthesis was performed using 1,500 ng of total RNA, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA), and oligo(dT) primers (IDT) in T100™ Thermal Cycler (Bio-Rad, Redmond, USA). For the amplification of intronless genes (*TLR2* and *TLR10*) and *TLR7* (primers designed out of exon-exon junction), RNA samples were treated with DNAse I (Thermo Fisher) before reverse transcription (RT).

2.5. Real-Time Quantitative PCR (qPCR). Real-time quantitative PCR (qPCR) was performed based on SYBR Green I DNA intercalating dye system, using Quantifast SYBR Green PCR kit (Qiagen), in MicroAmp® Optical 96-Well Reaction Plates (Applied Biosystems) and sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems). The reaction mix consisted of 7.5 μL of 2X Quantifast SYBR Green PCR Master Mix, 0.3 μL each primer at 10 μM concentration, 1.9 μL of ultrapure water, and 5 μL of cDNA at 4 ng/μL concentration (20 ng per reaction), in a final volume of 15 μL. The qPCR assays were carried out in a 7500 Real-Time PCR System Thermal Cycler (Applied Biosystems), and cycling conditions consisted of preincubation at 95°C for five minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 35 seconds, followed by melting curve analysis with temperature ranging between 65°C and 95°C, at 0.5°C increments every five seconds. All samples were tested in duplicate. No-template controls (NTC) were included for each qPCR run.

In addition, the amplified products were subjected to 2.5% agarose gel electrophoresis to confirm the expected fragment sizes. The efficiencies of the optimized qPCR reactions were determined from linear regression by plotting Cq values and respective cDNA concentration from seven five-fold serial dilutions of cDNA (75 ng/μL).

The relative gene expression was normalized by the reference gene *YWHAZ* (abomasal pyloric mucosa) and by the geometric mean of the Cq values of *GAPDH* and *YWHAZ* (abomasal fundic mucosa and abomasal lymph nodes), according to the stability test previously performed [38]. For the blood samples, five different genes (*GAPDH*, *G6PDH*, *YWHAZ*, *ACTB*, and *B2M*) were tested with the programs BestKeeper, NormFinder, and RefFinder, which identified *YWHAZ* as the most stable reference gene. The expression levels were calculated according to Pfaffl [39]. For each gene/tissue combination, the sample with the lowest expression level (highest ΔCq) was used as a calibrator.

2.6. Statistical Analyses. The relative gene expression levels for the different target genes between the extremes of infection by *H. contortus* in the abomasal mucosa and respective lymph nodes were compared by the Mann-Whitney *U* test. Gene expression levels of systemic inflammatory responses were compared with a generalized linear model (GLM) with repeated measures of the same animal, considering the effects of phenotypic group, collection date, and interaction. Statistical analyses were performed with the software RStudio (version 1.1.463), at a 5% significance level. All the graphs were plotted using GraphPad Prism (version 7.0a).

Table 1: Sequences of the primers used for relative gene quantification (RT-qPCR) in Morada Nova lambs resistant or susceptible to *Haemonchus contortus* infection, including: accession number, amplicon size, exon boundary covered, efficiency (E), determination coefficient (R^2), and slope.

Gene	Access number	Sequence (5′-3′)	Amplicon size (bp)	Exon boundary	E (%)	R^2	Slope
GAPDH	NM_001190390.1	F: CAAGCTCATTTCCTGGTACGAC R: TCTCTCTTCCTCTCGTGCTCCT	131	10/11	99.136	0.999	-3.343
YWHAZ	NM_001267887.1	F: CTGAGAAAGCCTGCTCTCTTGC R: GGTATCCGATGTCCACAATGTC	143	5/6	102.340	0.999	-3.267
TLR2	NM_001048231.1	F: CTCCCACTTCCGTCTCTTTGAT R: CTCCAGGTAGGTCCTGGTGTTC	133	Intronless	96.378	0.999	-3.412
TLR4	NM_001135930	F: ACCCTTGCGTACAGGTTGTTC R: ATGGCTGCCTAAATGTCTCAGG	137	1/2	100.710	0.964	-3.300
TLR7	NM_001135059.1	F: TTGAGAAGCCCCTTCAGAAGTC R: TCAGACACTGCCAGAAGTACGG	117	None	101.823	0.994	-3.279
TLR10	NM_001135925.1	F: GTGGTTATCATGCTCGTTCTGG R: TCTTCCTAACCCTGAGCCATGT	118	Intronless	95.298	0.992	-3.440
NFKBIA	NM_001166184.1	F: CGAGACTTTCGAGGAAATACCC R:GACACGTGTGGCCATTGTAGTT	141	3/4	95.848	0.999	-3.420
IKBKB	XM_015104530.1	F: AGGCTGCCGAGAAGAGTGAC R: CAAACTCTGGTCCTGCTCCTTC	104	23/24	92.421	0.986	-3.518
iNOS	AF223942.1	F: CACCTCTACTGGGAGGAGATGC R: GAACATAGACCTTGGGCTGGTC	102	24/25	99.905	0.999	-3.324
IL1β	NM_001009465.2	F: AGTGGTGTTCTGCATGAGCTTC R: CAGGGTCGGTGTATCACCTTTT	124	4/5	100.440	0.997	-3.311
TNFα	NM_001024860	F: CTCAGGTCATCTTCTCAAGCCT R: GAGGGCATTGGCATACGAG	108	2/3	94.086	0.983	-3.472
IL10	NM_001009327.1	F: CTTTAAGGGTTACCTGGGTTGC R: TCACGTGCTCCTTGATGTCAG	110	2/3	102.305	0.995	-3.268
TGFβ	NM_001009400	F: CAGCTCCACAGAAAAGAACTGC R: GTGTCCAGGCTCCAGATGTAGG	144	5/6	98.993	0.994	-3.346
C7	XM_012096998.2	F: CTATGAATGTGGGTCCTCCTTG R: CTCCCTACCAGCCACAGTGTAA	130	16/17	102.74	0.990	-3.258
CFI	XM_004009622.3	F: ATGGAGTGTGCAGGTACAGATG R: CTCACAATACCCCAAACGTAAG	113	14/15	99.818	0.997	-3.326

3. Results

3.1. Selection of the Extreme Resistance Phenotypes. 20 animals classified as extreme resistance phenotypes (10 most resistant and 10 most susceptible) were selected. The overall mean of FEC of the two parasitic challenges for resistant lambs was quite low (192.2 ± 48.85 EPG) and significantly lower ($P < 0.001$) than that of susceptible lambs (14,981 ± 1,938 EPG). There was also a significant difference ($P < 0.001$) for PCV between resistant (36.77 ± 0.68%) and susceptible (29.25 ± 0.75%) lambs.

3.2. Specificity and Efficiency of the Designed Primers. All primer pairs were specific. Electrophoresis confirmed the amplification of single products of the expected sizes, and a single peak was observed at melting curve analysis. The efficiencies of the qPCR assays ranged from 92.421% to 102.740%, and the correlation coefficients (R^2) ranged between 0.964 and 0.999. Primer's sequences and other information are shown in Table 1.

3.3. Relative Quantification of Genes Associated with Innate Immune Responses

3.3.1. Local Immune Responses (Tissue Samples). The comparison of gene expression levels of the immune-related mediators in the different tissues between the groups are represented in Figure 1. *TLR2* and *CFI* were superiorly expressed ($P < 0.05$) in the abomasal pyloric mucosa of the resistant animals, while *IL33* was at higher levels in the same group ($P < 0.05$), but in the abomasal fundic mucosa. The susceptible group, in turn, presented an exacerbated inflammation of the abomasal mucosa, represented by superior expression of both *TNFα* and *IL1β* ($P < 0.05$) in the abomasal fundic

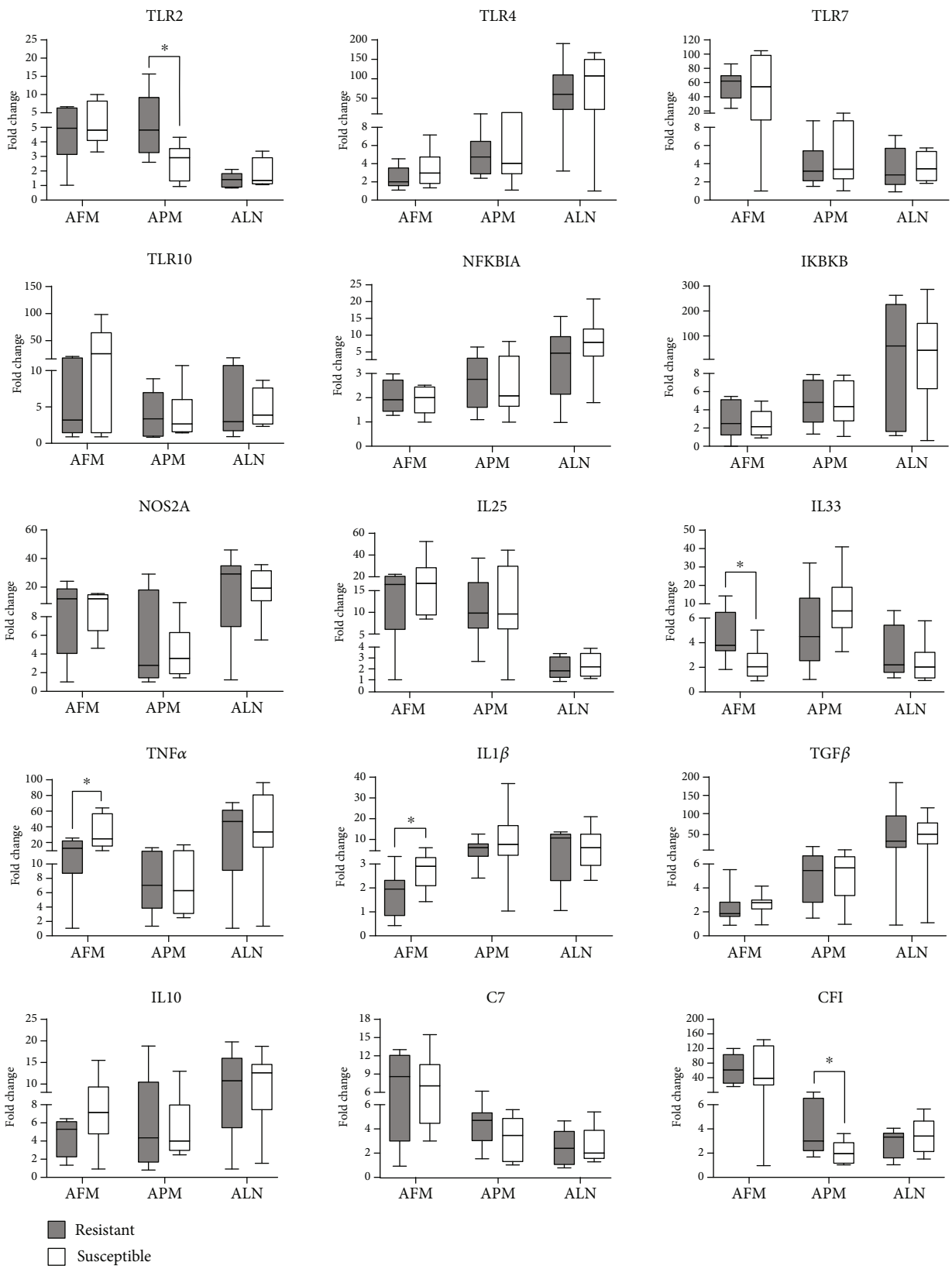
TLR2
TLR4
TLR7
TLR10
NFKBIA
IKBKB
NOS2A
IL25
IL33
TNFα
IL1β
TGFβ
IL10
C7
CFI
Fold change
AFM
APM
ALN
*
Resistant
Susceptible

Figure 1

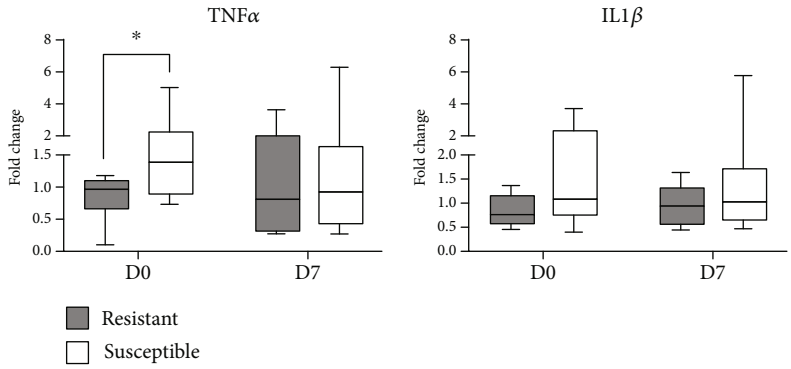

Figure 2

mucosa. No significant differences ($P > 0.05$) were observed for the other tested genes in any of the evaluated tissues.

3.3.2. Systemic Immune Responses (Blood Samples). Expression levels of the proinflammatory cytokines (*TNFα* and *IL1β*) between the groups in the blood samples are shown in Figure 2. A significant difference was observed only for *TNFα*, which was found superiorly expressed ($P < 0.05$) in the susceptible animals, at D0-3. *IL1β*, in turn, did not differ ($P > 0.05$) between groups. No significant differences were observed between experimental dates ($P > 0.05$), nor significant interaction with the phenotypic group ($P > 0.05$).

4. Discussion

The present study evaluated gene expression levels of the innate immune response mediators in Morada Nova lambs with contrasting *H. contortus* resistance phenotypes. Susceptible lambs had increased levels of the proinflammatory cytokines at both local (abomasal mucosa and lymph nodes) and systemic levels (blood). Resistant animals, on the other hand, presented an enhanced local immune response mediated by TLR recognition, IL33 synthesis, and complement activation.

TLR recognition is the main mechanism of identification of invading agents by the sentinel cells [40], and it was associated with effective responses against helminths [41–43]. Ingham et al. [14] found higher levels of *TLR2*, *TLR4*, *TLR7*, and *TLR10* transcripts in the abomasal mucosa of sheep with increased resistance to *H. contortus* infections. In the present study, we found similar results, and *TLR2* was at higher levels in the abomasal pyloric region of the resistant lambs, which suggests an enhanced sensitivity on the recognition of the invading parasites, resulting in earlier effective immune response.

NFκB is the main signaling pathway involved in the inflammatory response induced by TLR recognition [44]. Ingham et al. [14] observed that susceptible sheep had increased levels of *NFKBIA* and *IKBKB* transcripts in the jejunal mucosa three days after artificial infection with *T. colubriformis*. The authors hypothesized that increased levels of these transcripts could be associated with the lower *TLR* expression, resulting in lower sensitivity in parasite recognition and consequent delayed induction of the initial inflammatory response. However, in our study, no significant differences in the *NFKBIA* and *IKBKB* transcript levels were observed between groups. This difference may be due to the later time of sampling performed in our experiment, at the 7th dpi.

The reactive oxygen species, well-known antimicrobials, were previously associated with lethal effects against nematodes, including *H. contortus* [20, 45, 46]. The iNOS induction of NO synthesis was associated with defense against parasitic infections [21]. Higher levels of *NOS2A* transcripts were found in the abomasal mucosa of sheep with increased resistance to *H. contortus* [14, 47]. On the other hand, *NOS2A* knocked out mice were not affected on their resistance against GIN infection [48]. In the present study, it was not possible to associate a differential profile for *NOS2A* mRNA levels with resistance to *H. contortus* infection.

IL25 and IL33 are constitutively expressed cytokines, released by epithelial cells from mucosal barriers in response to cellular damage. These alarmins are known to be natural triggers and enhancers of the Th2 type immune response [28, 32, 49, 50], resulting in local and systemic induction of IL4, IL5, and IL13 synthesis, eosinophilia, and high levels of IgE [51–53]. Depletion of IL25 or IL33 was proved to abrogate natural resistance to several helminth species [29, 54–59]. Regarding sheep resistance against GIN infections, this is the first study, to our knowledge, to investigate the involvement of IL25, although no differential expression profile was observed between resistant and susceptible lambs. However, the importance of this alarmin in sheep resistance against parasitic infections cannot be ruled out, since this cytokine may be activated at an earlier stage of infection.

The involvement of IL33 in sheep response to GIN was recently evidenced. Andronicos et al. [34] and Corvan et al. [60] cultured human epithelial cells or ovine intestinal epithelium with infecting *T. colubriformis* larvae. Cellular necrosis induced by parasitism or stimulation with excretory and secretory products from larvae was associated with a marked increase in *IL33* transcript levels. Also, a 15-fold increase in the *IL33* expression was observed in the small intestine mucosa of sheep artificially infected with *T. colubriformis*, at 14th dpi [34]. The present study evidenced, at the 7th

dpi, superior expression of *IL33* in the abomasal fundic mucosa of the resistant lambs. This is the first study to associate higher levels of this cytokine with sheep resistance against GIN infections. In addition, this difference between the extremes of resistance was earlier detected when compared to the findings observed by Andronicos et al. [34]. We hypothesized that resistant sheep are able to respond in an early and enhanced manner for alarmin releasing after infection.

During GIN infections, the lesion resulting from the parasitism causes inflammation of the gut mucosa, with consequence release of proinflammatory cytokines, such as TNFα and IL1β, at both local [16, 18, 61] and systemic levels [62]. However, in sheep that develop an effective response against these parasites, this initial inflammatory response is followed by induction of regulatory activity, characterized by increased expression levels of *IL10* and *TGFβ* [16, 19, 63]. Susceptible sheep, on the other hand, usually present higher mRNA levels of *TNFα* and *IL1β* in the gut mucosa and respective lymph nodes, at the early [17, 19] and late infection [12]. When comparing the kinetics of this cytokines, it was shown that susceptible sheep have a delayed and prolonged proinflammatory activity, compared to resistant animals [19]. In the present study, the results were consistent with previous reports, with higher expression levels of the proinflammatory cytokines *TNFα* and *IL1β* in the abomasal mucosa of susceptible lambs. Even, higher levels of TNFα were also found in the blood of lambs from this group. The pronounced inflammatory activity is probably due to an incomplete defense against parasites at the onset of infection. Thus, a larger number of surviving helminths continue to cause lesions in the gastrointestinal mucosa, leading to enhanced local inflammatory process. Nevertheless, the exacerbated systemic inflammation before the onset of infection may be a consequence of residual response induced by previous two parasitic challenges performed in these animals.

Activation of the complement system by the alternative pathway is one of the first immune events following GIN infection, resulting in opsonin activation and the formation of the terminal complex [22, 23, 64, 65]. The opsonization of the parasite surface with complement proteins plays an important role in the destruction of larval forms at the beginning of infection, due to eosinophils attraction and stimulation [24, 65, 66]. The alternative complement pathway involves a "tick over" process leading to spontaneous cleavage of C3 into its active forms, C3a and C3b [67]. The complement factor I (CFI) is the main regulatory component involved on the alternative pathway, inactivating C3 convertase and avoiding excessive production of C3b, given the abundance of C3 in plasma and mucous membranes [26, 67]. In the present study, two complement factor genes were evaluated: *CFI*, related to the control of alternative route activation and *C7*, a membrane attack complex (MAC) protein. Higher levels of *CFI* transcripts were found in the abomasal pyloric mucosa of the resistant lambs, which indicates the participation of this mediator in the initial response against *H. contortus* larval forms. Recent studies have also demonstrated superior expression of *C7* and *CFI* in sheep with increased resistance to *H. contortus* infection, at the early [68] or late infection [27].

5. Conclusion

Susceptible lambs had increased transcripts levels of proinflammatory cytokines *TNFα* and *IL1β*. This exacerbated inflammatory response, both locally (abomasal fundic mucosa) and systemically (blood), may be a consequence of the higher *H. contortus* parasitism in this group. Resistant animals, on the other hand, presented an enhanced local (abomasum) immune response mediated by TLR recognition, IL33 synthesis, and complement activation. Nevertheless, this is the first study to directly associate sheep resistance against *H. contortus* with higher levels of *IL33*, an innate inducer of the Th2-polarized response.

Data Availability

Previously reported reference gene stability test data were used to support this study and are available at 10.1007/s11033-018-4281-x. These prior study is cited at relevant places within the text as reference [38]. All remaining data are included within the article.

Ethical Approval

This experiment was approved by the Embrapa Southeast livestock Ethics Committee on Animal Experimentation (process number 04/2017) and is in accordance with national and international ethical principles and guidelines for animal experimentation.

Conflicts of Interest

The authors have no conflict of interest.

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References

[1] A. F. T. Amarante, P. A. Bricarello, R. A. Rocha, and S. M. Gennari, "Resistance of Santa Ines, Suffolk and Ile de France sheep to naturally acquired gastrointestinal nematode infections," *Veterinary Parasitology*, vol. 120, no. 1-2, pp. 91–106, 2004.

[2] H. Louvandini, C. F. M. Veloso, G. R. Paludo, A. Dell'Porto, S. M. Gennari, and C. M. McManus, "Influence of protein supplementation on the resistance and resilience on young hair sheep naturally infected with gastrointestinal nematodes during rainy and dry seasons," *Veterinary Parasitology*, vol. 137, no. 1-2, pp. 103–111, 2006.

[3] A. C. S. Chagas, M. C. S. Oliveira, S. N. Esteves et al., "Parasitismo por nematóides gastrintestinais em matrizes e cordeiros criados em São Carlos, São Paulo," *Revista Brasileira de Parasitologia Veterinária*, vol. 17, pp. 126–132, 2008.

[4] A. C. S. Chagas, L. M. Katiki, I. C. Silva et al., "*Haemonchus contortus*: a multiple-resistant Brazilian isolate and the costs for its characterization and maintenance for research use," *Parasitology International*, vol. 62, no. 1, pp. 1–6, 2013.

[5] L. J. O'Connor, L. P. Kahn, and S. W. Walkden-Brown, "The effects of amount, timing and distribution of simulated rainfall on the development of *Haemonchus contortus* to the infective larval stage," *Veterinary Parasitology*, vol. 146, no. 1-2, pp. 90–101, 2007.

[6] G. C. Coles, F. Jackson, W. E. Pomroy et al., "The detection of anthelmintic resistance in nematodes of veterinary importance," *Veterinary Parasitology*, vol. 136, no. 3-4, pp. 167–185, 2006.

[7] F. A. Almeida, K. C. O. D. Garcia, P. R. Torgerson, and A. F. T. Amarante, "Multiple resistance to anthelmintics by *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep in Brazil," *Parasitology International*, vol. 59, no. 4, pp. 622–625, 2010.

[8] M. C. R. Cintra, V. N. Teixeira, L. V. Nascimento, and C. S. Sotomaior, "Lack of efficacy of monepantel against *Trichostrongylus colubriformis* in sheep in Brazil," *Veterinary Parasitology*, vol. 216, pp. 4–6, 2016.

[9] A. C. Martins, P. L. F. Bergamasco, G. Felippelli et al., "*Haemonchus contortus* resistance to monepantel in sheep: fecal egg count reduction tests and randomized controlled trials," *Semina: Ciências Agrárias*, vol. 38, no. 1, pp. 231–238, 2017.

[10] C. Lacroux, T. H. C. Nguyen, O. Andreoletti et al., "*Haemonchus contortus* (Nematoda: Trichostrongylidae) infection in lambs elicits an unequivocal Th_2 immune response," *Veterinary Research*, vol. 37, no. 4, pp. 607–622, 2006.

[11] K. P. Shakya, J. E. Miller, and D. W. Horohov, "A Th2 type of immune response is associated with increased resistance to *Haemonchus contortus* in naturally infected Gulf Coast Native lambs," *Veterinary Parasitology*, vol. 163, no. 1-2, pp. 57–66, 2009.

[12] L. G. Zaros, M. R. M. Neves, C. L. Benvenuti et al., "Response of resistant and susceptible Brazilian Somalis crossbreed sheep naturally infected by *Haemonchus contortus*," *Parasitology Research*, vol. 113, no. 3, pp. 1155–1161, 2014.

[13] K. M. MacKinnon, S. A. Bowdridge, I. Kanevsky-Mullarky, A. M. Zajac, and D. R. Notter, "Gene expression profiles of hair and wool sheep reveal importance of Th2 immune mechanisms for increased resistance to *Haemonchus contortus*," *Journal of Animal Science*, vol. 93, no. 5, pp. 2074–2082, 2015.

[14] A. Ingham, A. Reverter, R. Windon, P. Hunt, and M. Menzies, "Gastrointestinal nematode challenge induces some conserved gene expression changes in the gut mucosa of genetically resistant sheep," *International Journal for Parasitology*, vol. 38, no. 3-4, pp. 431–442, 2008.

[15] K. M. MacKinnon, J. L. Burton, A. M. Zajac, and D. R. Notter, "Microarray analysis reveals difference in gene expression profiles of hair and wool sheep infected with *Haemonchus contortus*," *Veterinary Immunology and Immunopathology*, vol. 130, no. 3-4, pp. 210–220, 2009.

[16] N. M. Craig, H. R. P. Miller, W. D. Smith, and P. A. Knight, "Cytokine expression in naïve and previously infected lambs after challenge with *Teladorsagia circumcincta*," *Veterinary Immunology and Immunopathology*, vol. 120, no. 1-2, pp. 47–54, 2007.

[17] J. R. Jacobs, K. N. Sommers, A. M. Zajac, D. R. Notter, and S. A. Bowdridge, "Early IL-4 gene expression in abomasum is associated with resistance to *Haemonchus contortus* in hair and wool sheep breeds," *Parasite Immunology*, vol. 38, no. 6, pp. 333–339, 2016.

[18] S. El-Ashram, C. Li, F. Abouhajer et al., "An *ex vivo* abomasal ovine model to study the immediate immune response in the context of *Haemonchus contortus* larval-stage," *Veterinary Parasitology*, vol. 254, pp. 105–113, 2018.

[19] M. Hassan, J. P. Hanrahan, B. Good, G. Mulcahy, and T. Sweeney, "A differential interplay between the expression of Th1/Th2/Treg related cytokine genes in *Teladorsagia circumcincta* infected *DRB1*1101* carrier lambs," *Veterinary Research*, vol. 42, no. 1, p. 45, 2011.

[20] M. Colasanti, L. Gradoni, M. Mattu et al., "Molecular bases for the anti-parasitic effect of NO (review)," *International Journal of Molecular Medicine*, vol. 9, no. 2, pp. 131–134, 2002.

[21] T. V. Rajan, P. Porte, J. A. Yates, L. Keefer, and L. D. Shultz, "Role of nitric oxide in host defense against an extracellular, metazoan parasite, *Brugia malayi*," *Infection and Immunity*, vol. 64, no. 8, pp. 3351–3353, 1996.

[22] Y. Hong, C. W. Kim, and B. Ghebrehiwet, "*Trichinella spiralis*: activation of complement by infective larvae, adults, and newborn larvae," *Experimental Parasitology*, vol. 74, no. 3, pp. 290–299, 1992.

[23] L. A. Kerepesi, J. A. Hess, T. J. Nolan, G. A. Schad, and D. Abraham, "Complement component C3 is required for protective innate and adaptive immunity to *Larval Strongyloides stercoralis* in mice," *Journal of Immunology*, vol. 176, no. 7, pp. 4315–4322, 2006.

[24] A. R. E. Anwar, S. R. Smithers, and A. B. Kay, "Killing of schistosomula of *Schistosoma mansoni* coated with antibody and/or complement by human leukocytes *in vitro*: requirement for complement in preferential killing by eosinophils," *Journal of Immunology*, vol. 122, no. 2, pp. 628–637, 1979.

[25] C. D. Mackenzie, M. Jungery, P. M. Taylor, and B. M. Ogilvie, "Activation of complement, the induction of antibodies to the surface of nematodes and the effect of these factors and cells on worm survival *in vitro*," *European Journal of Immunology*, vol. 10, no. 8, pp. 594–601, 1980.

[26] A. Naik, S. Sharma, and R. J. Quigg, "Complement regulation in renal disease models," *Seminars in Nephrology*, vol. 33, no. 6, pp. 575–585, 2013.

[27] Z. Guo, J. F. González, J. N. Hernandez et al., "Possible mechanisms of host resistance to *Haemonchus contortus* infection in sheep breeds native to the Canary Islands," *Scientific Reports*, vol. 6, no. 1, article 26200, 2016.

[28] J. L. Barlow, S. Peel, J. Fox et al., "IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction," *Journal of Allergy and Clinical Immunology*, vol. 132, no. 4, pp. 933–941, 2013.

[29] C. Pei, C. Zhao, A. J. Wang et al., "Critical role for interleukin-25 in host protective Th2 memory response against *Heligmosomoides polygyrus bakeri*," *Infection and Immunity*, vol. 84, no. 12, pp. 3328–3337, 2016.

[30] S. Koyasu and K. Moro, "Th2-type innate immune responses mediated by natural helper cells," *Annals of the New York Academy of Sciences*, vol. 1283, no. 1, pp. 43–49, 2013.

[31] K. Yasuda, T. Muto, T. Kawagoe et al., "Contribution of IL-33-activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 9, pp. 3451–3456, 2012.

[32] Y. Kondo, T. Yoshimoto, K. Yasuda et al., "Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system," *International Immunology*, vol. 20, no. 6, pp. 791–800, 2008.

[33] D. R. Neill, S. H. Wong, A. Bellosi et al., "Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity," *Nature*, vol. 464, no. 7293, pp. 1367–1370, 2010.

[34] N. M. Andronicos, J. McNally, A. C. Kotze, P. W. Hunt, and A. Ingham, "*Trichostrongylus colubriformis* larvae induce necrosis and release of IL33 from intestinal epithelial cells in vitro: implications for gastrointestinal nematode vaccine design," *International Journal for Parasitology*, vol. 42, no. 3, pp. 295–304, 2012.

[35] C. McManus, P. Hermuche, S. Paiva, J. Ferrugem Moraes, C. de Melo, and C. Mendes, "Geographical distribution of sheep breeds in Brazil and their relationship with climatic and environmental factors as risk classification for conservation," *Brazilian Journal of Science and Technology*, vol. 1, no. 1, p. 3, 2014.

[36] J. Issakowicz, A. C. K. S. Issakowicz, M. S. Bueno et al., "Parasitic infection, reproductive and productive performance from Santa Inês and Morada Nova ewes," *Small Ruminant Research*, vol. 136, pp. 96–103, 2016.

[37] J. B. Ferreira, A. C. D. S. Bezerra, M. M. Guilhermino et al., "Performance, endoparasitary control and blood values of ewes locally adapted in semiarid region," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 52, pp. 23–29, 2017.

[38] J. H. B. Toscano, L. G. Lopes, L. A. Giraldelo, M. H. da Silva, C. H. Okino, and A. C. de Souza Chagas, "Identification of appropriate reference genes for local immune-related studies in Morada Nova sheep infected with *Haemonchus contortus*," *Molecular Biology Reports*, vol. 45, no. 5, pp. 1253–1262, 2018.

[39] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, no. 9, article 45e, 2001.

[40] L. A. J. O'Neill, "How Toll-like receptors signal: what we know and what we don't know," *Current Opinion in Immunology*, vol. 18, no. 1, pp. 3–9, 2006.

[41] D. van der Kleij, E. Latz, J. F. H. M. Brouwers et al., "A novel host–parasite lipid cross-talk," *The Journal of Biological Chemistry*, vol. 277, no. 50, pp. 48122–48129, 2002.

[42] S. J. Jenkins, J. P. Hewitson, S. Ferret-Bernard, and A. P. Mountford, "Schistosome larvae stimulate macrophage cytokine production through TLR4-dependent and independent pathways," *International Immunology*, vol. 17, no. 11, pp. 1409–1418, 2005.

[43] L. A. Kerepesi, J. A. Hess, O. Leon, T. J. Nolan, G. A. Schad, and D. A. Abraham, "Toll-like receptor 4 (TLR4) is required for protective immunity to larval *Strongyloides stercoralis* in mice," *Microbes and Infection*, vol. 9, no. 1, pp. 28–34, 2007.

[44] A. Hoffmann and D. Baltimore, "Circuitry of nuclear factor κB signaling," *Immunological Reviews*, vol. 210, no. 1, pp. 171–186, 2006.

[45] J. E. Albina and J. S. Reichner, "Nitric oxide in inflammation and immunity," *New Horizons*, vol. 3, no. 1, pp. 46–64, 1995.

[46] A. C. Kotze and S. J. McClure, "*Haemonchus contortus* utilises catalase in defence against exogenous hydrogen peroxide in vitro," *International Journal for Parasitology*, vol. 31, no. 14, pp. 1563–1571, 2001.

[47] M. Menzies, A. Reverter, N. Andronicos, P. Hunt, R. Windon, and A. Ingham, "Nematode challenge induces differential expression of oxidant, antioxidant and mucous genes down the longitudinal axis of the sheep gut," *Parasite Immunology*, vol. 32, no. 1, pp. 36–46, 2010.

[48] L. Ganley, S. Babu, and T. V. Rajan, "Course of *Brugia malayi* Infection in C57BL/6J NOS2 +/+ and −/− Mice," *Experimental Parasitology*, vol. 98, no. 1, pp. 35–43, 2001.

[49] S. A. Saenz, M. C. Siracusa, J. G. Perrigoue et al., "IL25 elicits a multipotent progenitor cell population that promotes T_H2 cytokine responses," *Nature*, vol. 464, no. 7293, pp. 1362–1366, 2010.

[50] K. M. Kroeger, B. M. Sullivan, and R. M. Locksley, "IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88-and p38α-dependent pathway," *Journal of Leukocyte Biology*, vol. 86, no. 4, pp. 769–778, 2009.

[51] G. Pan, D. French, W. Mao et al., "Forced expression of murine IL-17E induces growth retardation, jaundice, a Th2-biased response, and multiorgan inflammation in mice," *Journal of Immunology*, vol. 167, no. 11, pp. 6559–6567, 2001.

[52] M. M. Fort, J. Cheung, D. Yen et al., "IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo," *Immunity*, vol. 15, no. 6, pp. 985–995, 2001.

[53] M. R. Kim, R. Manoukian, R. Yeh et al., "Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production," *Blood*, vol. 100, no. 7, pp. 2330–2340, 2002.

[54] P. G. Fallon, S. J. Ballantyne, N. E. Mangan et al., "Identification of an interleukin (IL)-25–dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion," *The Journal of Experimental Medicine*, vol. 203, no. 4, pp. 1105–1116, 2006.

[55] A. Zhao, J. F. Urban Jr., R. Sun et al., "Critical role of IL-25 in nematode infection-induced alterations in intestinal function," *The Journal of Immunology*, vol. 185, no. 11, pp. 6921–6929, 2010.

[56] P. Angkasekwinai, P. Srimanote, Y. H. Wang et al., "Interleukin-25 (IL-25) promotes efficient protective immunity against *Trichinella spiralis* infection by enhancing the antigen-specific IL-9 response," *Infection and Immunity*, vol. 81, no. 10, pp. 3731–3741, 2013.

[57] A. E. Price, H. E. Liang, B. M. Sullivan et al., "Systemically dispersed innate IL-13-expressing cells in type 2 immunity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 25, pp. 11489–11494, 2010.

[58] L. Y. Hung, I. P. Lewkowich, L. A. Dawson et al., "IL-33 drives biphasic IL-13 production for noncanonical type 2 immunity against hookworms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 1, pp. 282–287, 2013.

[59] N. E. Humphreys, D. Xu, M. R. Hepworth, F. Y. Liew, and R. K. Grencis, "IL-33, a potent inducer of adaptive immunity to intestinal nematodes," *Journal of Immunology*, vol. 180, no. 4, pp. 2443–2449, 2008.

[60] S. M. Corvan, L. Agnew, and N. M. Andronicos, "*Trichostrongylus colubriformis* induces IgE-independent CD13, CD164 and CD203c mediated activation of basophils in an in vitro intestinal epithelial cell co-culture model," *Veterinary Parasitology*, vol. 207, no. 3-4, pp. 285–296, 2015.

[61] S. El-Ashram, I. Al Nasr, M. El-Kemary, R. Mehmood, M. Hu, and X. Suo, "Early and late gene expression profiles of the ovine mucosa in response to *Haemonchus contortus* infection employing Illumina RNA-seq technology," *Parasitology International*, vol. 66, no. 5, pp. 681–692, 2017.

[62] A. S. Schafer, M. L. R. Leal, M. B. Molento et al., "Immune response of lambs experimentally infected with *Haemonchus contortus* and parenterally treated with a combination of zinc and copper," *Small Ruminant Research*, vol. 123, no. 1, pp. 183–188, 2015.

[63] R. M. Maizels, "Infections and allergy—helminths, hygiene and host immune regulation," *Current Opinion in Immunology*, vol. 17, no. 6, pp. 656–661, 2005.

[64] M. Stankiewicz, W. Jonas, and D. Elliott, "Alternative pathway activation of complement in fetal lamb serum by *Trichostrongylus vitrinus* larvae," *Parasite Immunology*, vol. 3, no. 4, pp. 309–318, 1981.

[65] V. Desakorn, P. Suntharasamai, S. Pukrittayakamee, S. Migasena, and D. Bunnag, "Adherence of human eosinophils to infective filariform larvae of *Necator americanus in vitro*," *The Southeast Asian Journal of Tropical Medicine and Public Health*, vol. 18, no. 1, pp. 66–72, 1987.

[66] M. A. Rainbird, D. Macmillan, and E. N. T. Meeusen, "Eosinophil-mediated killing of Haemonchus contortus larvar: effect of eosinophil activation and role of antobody, complement and interleukin-5," *Parasite Immunology*, vol. 20, no. 2, pp. 93–103, 1998.

[67] P. J. Lachmann, E. Lay, and D. J. Seilly, "Experimental confirmation of the C3 tickover hypothesis by studies with an Ab (S77) that inhibits tickover in whole serum," *The FASEB Journal*, vol. 32, no. 1, pp. 123–129, 2018.

[68] R. Zhang, F. Liu, P. Hunt et al., "Transcriptome analysis unraveled potential mechanisms of resistance to *Haemonchus contortus* infection in Merino sheep populations bred for parasite resistance," *Veterinary Research*, vol. 50, no. 1, pp. 7–19, 2019.

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Research Article

Differential Expression of IFN-γ, IL-10, TLR1, and TLR2 and Their Potential Effects on Downgrading Leprosy Reaction and Erythema Nodosum Leprosum

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Leprosy reactions are acute immunological events that occur during the evolution of chronic infectious disease causing neural damage and disabilities. A study using blood samples of 17 leprosy reaction patients and 17 reaction-free was carried out by means of associations between antigens, receptors, and expression of cytokines, using path analysis providing new insights into the immunological mechanisms involved in triggering leprosy reactions. Toll-like receptors (TLR) such as TLR1 and TLR2, presented balanced expression in the reaction-free multibacillary (MB) group (TLR1: 1.01 ± 0.23, TLR2: 1.22 ± 0.18; $p = 0.267$). On the other hand, downgrading type 1 reaction (T1R) (TLR1: 1.24 ± 0.17, TLR2: 2.88 ± 0.37; $p = 0.002$) and erythema nodosum leprosum (ENL) (TLR1: 1.93 ± 0.17, TLR2: 2.81 ± 0.15; $p = 0.004$) revealed an unbalance in relation to the expression of these receptors. When the path analysis was approached, it was noted that interleukin 10 (IL-10) expression showed a dependence relation with phenolic glycolipid I (PGL-I) in downgrading T1R (direct effect = 0.503 > residual effect = 0.364), whereas in ENL, such relationship occurred with lipoarabinomannan (LAM) (direct effect = 0.778 > residual effect = 0.280). On the contrary, in the reaction-free leprosy group, interferon-gamma (IFN-γ) levels were dependent on the association between TLR2 and TLR1 (0.8735). The high TLR2 expression associated with IL-10 levels, in the leprosy reaction groups, may be hypothetically related to the formation of TLR2/2 homodimers and/or TLR2/6 heterodimers linked to evasion mechanisms in downgrading reactions and pathophysiology of ENL.

1. Introduction

Leprosy reactions are acute immunological events that overlap the chronic infection caused by *Mycobacterium leprae* (*M. leprae*). The antigenic components of this bacillus are the potential triggers of these reactions that affect in different degrees the peripheral nerves causing physical disabilities [1]. These immunological events are classified into type 1 (T1R) and erythema nodosum leprosum (ENL), affecting different clinical forms of the disease before, during, and after treatment [2].

The type 1 reaction (T1R), subdivided in upgrading and downgrading, is a delayed hypersensitivity reaction against components of *M. leprae*, whose the affected clinical forms are borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL) [3].

The upgrading and downgrading reactions are clinically indistinguishable, characterized by the presence of oedema

and erythema in preexisting skin lesions, appearance of new skin lesions with classic inflammatory signs, and neuritis associated with sensory and motor alterations [4]. On the other hand, such reactions may be differentiated by histopathology, the profile of the immunological response, and temporality of the occurrence of these events [4].

The upgrading reaction, also called reverse reaction, occurs after administration of multidrug therapy (MDT), in which the type 1 helper (Th1) cytokine pattern (interleukin-1β [IL-1β], tumor necrosis factor-alpha [TNF-α] IL-2, and interferon-gamma [IFN-γ]) is found in patient lesions, in addition to elevation of TNF-α, IFN-γ, and IL-17F in the serum of these patients and other markers such as interferon gamma-induced protein 10 (IP-10), vascular endothelial growth factor (VEGF), and chemokine 10 (CXCL10) [5–8]. The T1R guarantees resistance against *M. leprae*, leading to migration in the clinical spectrum of the disease of those borderline individuals to the tuberculoid pole, reducing, finally, the bacilloscopic and morphological indices [9].

On the contrary, the downgrading reaction occurs before MDT and after treatment in relapse cases, representing an immunological activity directed against nonessential antigenic determinants of *M. leprae* survival. Thus, it may be observed in downgrading reaction the increase in the number of bacilli, B lymphocyte levels, and immunoglobulin gamma (IgG) antibodies, besides the low levels of natural killers and T cells [4, 10, 11]. Furthermore, the immunological profile of this reaction allows evasion mechanisms of the bacillus favoring the migration of borderline individuals towards the lepromatous leprosy (LL) pole in the clinical spectrum of the disease [9, 12].

Regarding the type 2 reaction, also called erythema nodosum leprosum (ENL), it represents a type III hypersensitivity reaction caused by the deposition of immune complexes in the joints, skin, endothelium, and other body structures, affecting 10% of BL and 50% of LL [13]. The main clinical presentation of this reaction are erythematous nodules in the skin, in addition to systemic symptoms such as fever, malaise, arthralgia, myositis, iridocyclitis, orchitis, glomerulonephritis, and laboratory abnormalities, such as neutrophilia and high C-reactive protein [13–15]. The proinflammatory component was associated with the immunopathology of ENL in several studies, since patients presented an increasing of $CD4^+$ T lymphocytes and a reduction in the levels of $CD8^+$ T cells in the blood when compared with reaction-free LL controls [16]. Elevated levels of circulating TNF-α as well as expression of IL-6 and IFN-γ were present in the serum and cutaneous lesions of ENL patients [17–19].

The *M. leprae* presents in its cell wall a glycolipid called lipoarabinomannan (LAM) and in its capsule the phenolic glycolipid I (PGL-I), two important surface molecules that are recognized mainly by Toll-like receptor 1 (TLR1) and 2 (TLR2) that associate to form the heterodimer TLR1/2. This heterodimer activates pathways that control dissemination of intracellular microorganisms influencing, therefore, components of innate and adaptive immunities [20].

A probable TLR2/2 homodimer was hypothesized and associated with IL-10 synthesis in mycobacteria. Although IL-10 plays an important role in the control of the inflammatory process, its elevation inhibits the synthesis of proinflammatory cytokines, which facilitates the survival and persistence of pathogens such as *M. leprae*, functioning, thereby, as an evasion mechanism [21].

Therefore, by means of gene expressions, serological data, and a causal model, this study has aimed hypothesizing the presence of an unbalance between the TLR1 and TLR2 expressions associated to high bacillary loading and IL-10 expression in leprosy reactions, which, consequently, are favorable to survival of bacillus and the occurrence of these events.

2. Material and Methods

2.1. Type of the Study and the Sample. This is a cross-sectional study, in which the sample was composed of 34 leprosy patients, being 17 with leprosy reactions (7 T1R and 10 ENL) and 17 reaction-free leprosy patients (8 paucibacillaries and 9 multibacillaries). All patients selected to this research were diagnosed by experts on leprosy according to the clinical, histological, and immunological criteria of Ridley and Jopling [2].

2.2. Place of the Study. The sample was selected according to inclusion and exclusion criteria for a long and sufficient period. The data collection was performed at the National Reference Center for Sanitary Dermatology and Leprosy (CREDESH) of the Federal University of Uberlândia (UFU), MG, Brazil, from 2014 to 2016.

2.3. Inclusion and Exclusion Criteria. The inclusion criteria were participants older than 18 years, leprosy patients affected by leprosy reactions before, during, or after treatment, and leprosy patients not affected by leprosy reactions (composing the reaction-free leprosy group).

Exclusion criteria were individuals with comorbidities as other chronic or acute diseases, the use of thalidomide and/or steroid therapy, and the use of immunotherapies and analogues.

2.4. Data Collection. In the reaction group, biological samples from each patient were collected once on the first day of clinical exacerbation of the leprosy reaction. Therefore, samples were obtained before, during, and/or after MDT.

Regarding the reaction-free group, all patients had biological samples collected before starting MDT.

2.5. Clinical and Epidemiological Variables. Variables such as clinical form; operational classification; sex, age group; bacilloscopic index; disability grade (DG); number, distribution and characteristics of cutaneous lesions and affected nerves in the diagnosis were obtained. The clinical evaluation of leprologists allowed quantifying the number of cutaneous lesions and affected nerves (evidenced by physical examination and electroneuromyography) in the diagnosis, besides classifying the disability grade of individuals from 0 to 2 [22].

2.6. ELISA anti-PGL-I Serology. The presence of anti-PGL-I and LAM antibodies reflects bacillary load and helps classifying clinical forms. The detection of IgM antibodies in anti-

PGL-I serological tests, instead of IgG, increases sensitivity and influences performance in the serological test among patients with PB and MB leprosy, besides IgM to be produced in acute phase of infection [23].

The native PGL-I isolated by organic extraction of *M. leprae*-infected armadillo tissues from which the bacteria had been purified and utilized in PGL-I ELISA was obtained from Colorado State University through the NIH/NIAID Leprosy Contract N 01 AI 25469.

For the PGL-I antibody detection ELISA assays, microtiter plates (MaxiSorp-NUNC®) were covered with native PGL-I diluted in phosphate-buffered saline (PBS), at concentration of 0.2 μg/ml. Serum samples were added in duplicate using a dilution of 1 : 100 (native PGL-I) in PBS/BSA 1%, incubated for 1 hour at 37.8°C, and subsequently washed. The anti-human IgM-peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) was added to the plates in the dilution of 1 : 10.000 (PGL-I ELISA) and 1 : 2.000 (ND-O-HSA). The substrate o-phenylenediamine dihydrochloride (OPD, Sigma) enzyme substrate was added to the plates and incubated at room temperature for 10 minutes in the dark chamber. The reaction was stopped by the addition of H_2SO_4 4 N. The optical density (OD) was obtained in a microplate reader (Thermo Plate, TP-Reader, Rayto Life and Analytical Sciences Co. Ltd, Germany) at 492 nm. Two positive and three negative controls were included in each plate.

2.7. ELISA anti-LAM Serology. Regarding anti-LAM, studies have revealed that IgG is the predominant circulating antibodies against *M. leprae* antigens [24, 25].

About the method, the LAM antibody, a monoclonal antibody derived from the cell wall of *M. leprae* extracted from a pool of an infected armadillo liver and spleen tissue, was sensitized 96-well high affinity plates (MaxiSorp, Nunc®) with 50 μl Native LAM (BEI Resources, NR-19348) diluted in carbonate/bicarbonate buffer (50 μl Native LAM 100 μg/ml diluted in 4950 μl of carbonate/bicarbonate buffer, pH 9.6); the plates were incubated overnight in a cold room at 4°C; four washes were performed with 0.05% PBST (200 μl/well), and serum samples diluted in 5% PBS/BSA (1 : 5) were added in triplicate. The plates were incubated for 1 h at 37°C and, after five washes with 0.05% PBST, were added 50 μl of peroxidase-labeled anti-IgG diluted 1: 1000 in PBS/BSA and incubated for 1 h at 37°C; after six washes with 0.05% PBST, the plates were exposed 50 μl of OPD solution for 5 min (2 mg OPD+5000 μl buffer citrate+2 μl H_2O_2), and the reaction was then quenched with 20 μl/well of sulfuric acid (H_2SO_4 2N). The plates were read on a microplate reader (TP-Reader, Thermo Plate®) at a wavelength of 492 nm.

Antibody titers were expressed as direct values of optical density and subsequently subjected to statistical normalization for a percentage scale that maintained the ratio between differences in antigen expression levels.

2.8. RNA Isolation, cDNA Synthesis, and Real Time qPCR. RNA from blood was extracted using the TRIzol® LS reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and quality of the RNA were determined by ultraviolet absorbance and electrophoresis. Complementary DNA (cDNA) was generated by reverse transcription (MMLV, Life Technologies, Carlsbad, CA, USA) using 1 μg of RNA, according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (real-time qPCR) with the thermocycler ABI PRISM 7300 (Applied Biosystems, USA) was used for gene expression analyses by using the TaqMan Universal PCR Master Mix to quantify the TLR1, TLR2, IFN-γ, and IL-10 genes in the peripheral blood of patients. The qPCR reaction was developed with a final volume of 12 μl, with the following reaction mix: 6 μl of Master Mix, 0.2 μl of specific set of TaqMan primers and probe, 5 μl of the cDNA, and 0.8 of distilled water-free RNase. Amplification conditions were those recommended by the manufacturer. All reactions were performed in triplicate, and probes used were TLR1 (Hs00413978_m1), TLR2 (Hs01014511_m1), IL-10 (Hs00961619_m1), IFN-γ (Hs00989291_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs03929097_q1) which was used as the endogenous control. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For mRNA quantification, we have used the method $2^{-\Delta\Delta Ct}$ as described elsewhere [26] and the expression data are represented in fold change.

2.9. Sample Size Calculation. We calculate the sample size of this study using the software G* Power (version 3.1.9.2, for windows) to reduce the costs and prove the statistical hypothesis. In order to obtain the effect size, it used the correlation coefficient (0.47) obtained from the relation between the bacilloscopic index and the anti-PGL-I IgM ELISA index of a previous pilot study. The statistical significance level alpha was 5% (0.05), and the power of the test was 0.85 (85%). Our sample size, considering the above parameters, was 34 individuals.

2.10. Statistical Analyses. The Shapiro-Wilk test was used to test the normality of data distribution. In comparing the two groups, reactional and reaction-free, the Student's *t*-test was performed to detect differences between means of serological markers and immunological variables. Regarding these previously quoted variables, the Analysis of Variance (ANOVA test) was chosen to analyze the differences between more than three groups. The binomial test was employed to prove the control of confounding factors related to epidemiological variables, by means of a comparison among the proportion of reactional and reaction-free cases. To verify the magnitude of the association among variables in the sample, the Pearson's correlation matrix was calculated.

2.11. Path Analysis. The path analysis, based on multiple linear regression, is the most robust test used in multivariate statistics [27]. Thus, direct and indirect effects were quantified between the dependent and independent variables. Interpretation of the path model was done as follows: direct effect is represented by unidirectional arrows (←), with their

respective values (estimates), starting in the independent variable towards the dependent variable; bidirectional arrows (↔) and their respective values represent the correlation between two independent variables; the indirect effect of two independent variables on the dependent is represented by the combination of both bidirectional and unidirectional arrows (↔ . ←), whose exact values can be calculated by multiplication of these two numerical estimates [28].

According to Singh and Chaudhary criteria [29], the independent variable (x) influences the dependent variable (y) indirectly only, if the direct effect of variable (x) on (y) was less than the residual effect ($p\varepsilon$) which is less than the total effect (r_{yx}), summed up by $|p_{yx}| < p\varepsilon < r_{yx}$, or the independent variable (x) can influence the dependent (y) inferring direct causal relation, if the direct effect of the variable x on y is bigger than the residual effect, finally represented as $|p_{yx}| > p\varepsilon$.

When one or more variables are considered independent and dependent variables, concomitantly, it means that there is more than one causal model, this way we have a path analysis in the chain [30].

It was used for statistical calculations the GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) and the Software for Experimental Statistics in Genetics® (GENES Software, Lavras, MG, Brazil) specifically designed for path analysis [31]. The significance level α was 5% for all analyses.

2.12. Ethical Considerations. This study was carried out in accordance with the recommendations of "Guidelines of the National Board on Research Ethics (CONEP)" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by UFU Research Ethics Committee under the number 633.052/2014.

3. Results

3.1. Clinical and Epidemiological Characterization. The study was formed by 34 patients with leprosy, divided into two groups, reactional and reaction-free patients, each one with 17 individuals. Among all samples, 76.4% (26/34) were classified as multibacillary (MB) and 23.6% (8/34) as paucibacillary (PB), in which 100% of leprosy reaction cases were MB (17/17) and 47% (8/17), in the reaction-free group, were PB (Table 1).

In the leprosy reaction group, the most frequent clinical forms were the LL 53% (9/17) followed by BB with 23.5% (4/17) according to Table 1. Still, analyzing this group, 41.2% (7/17) showed T1R, while 58.8% (10/17) had ENL (Table 1). It was noted that 29.4% (5/17) of the reactions occurred before treatment, all of T1R, and 70.6% (12/17) after treatment (Table 1). Regarding the disability grade, the zero degree predominated in 61.7% (21/34) of the individuals in the sample, whose highest frequency was present in the reaction-free group representing 38.2% (13/34) of the sample (Table 1). There was a predominance of males in both groups (58.8%, 10/17) (Table 1).

The mean age of the leprosy reaction group was 45 years, while the reaction-free group was 47 years. In the sample, ages from 35 to 44 years and from 45 to 54 years were predominant, with frequencies of 29.4% and 23.6%, respectively. However, in comparison between leprosy reaction and reaction-free groups, concerning the age group, the binomial test showed that there was no difference between age group proportions, which represents, therefore, the control of confounding factors associated with this variable (25-34, $p = 0.527$; 35-44, $p = 1.00$; 45-54, $p = 1.00$; 55-64, $p = 0.302$; and ≥65, $p = 0.392$).

3.2. Laboratorial Analyses. It was verified that the mean (m) and the standard error of the mean (SEM) of anti-LAM in the leprosy reaction group (2.30 ± 0.26) was significantly higher than reaction-free group (1.46 ± 0.25) ($p = 0.032$) (Figure 1(a)). The anti-PGL-I presented significantly higher levels in the leprosy reaction group (3.88 ± 0.52) than reaction-free (1.93 ± 0.44) ($p = 0.008$) (Figure 1(b)).

The Figure 1(c) shows the results after stratifications of the groups. The groups with ENL (3.10 ± 0.18) and reaction-free leprosy MB (1.82 ± 0.28) presented higher levels of anti-LAM IgG; in addition, the levels of this antibody in ENL group differed from all others as observed in Figure 1(c).

As for the levels of anti-PGL-I IgM, the comparative analysis between reaction-free leprosy PB (0.61 ± 0.18) and the groups T1R (3.25 ± 0.63), ENL (4.43 ± 0.79) and reaction-free leprosy MB (2.96 ± 0.58) showed a significant difference among levels expression of that antibody as shown in Figure 1(d).

The TLR1 and TLR2 expression levels were compared in all groups showing that these receptors in reaction-free leprosy MB group there were balanced expression (TLR1: 1.01 ± 0.23, TLR2: 1.22 ± 0.18; $p = 0.267$) (Figure 1(e)).

However, the groups with T1R (TLR1: 1.24 ± 0.17, TLR2: 2.88 ± 0.37; $p = 0.002$) and ENL presented unbalance among the expressions of these receptors (TLR1: 1.93 ± 0.17, TLR2: 2.81 ± 0.15; $p = 0.004$) (Figure 1(e)).

For the cytokine analyses, the IL-10 expression was relatively higher in the leprosy reaction group (4.31 ± 0.83) when confronted with reaction-free leprosy patients (1.25 ± 0.53) ($p = 0.003$) (Figure 2(a)). In relation to the expression of IFN-γ in the peripheral blood, higher levels were observed in the reaction-free leprosy patients (2.05 ± 0.32) when compared to the leprosy reaction group (0.43 ± 0.12) ($p < 0.001$) (Figure 2(b)). To better understand the expression levels of IL-10, all groups were subdivided, as previously mentioned. It was observed, according to Figure 2(c), that both the group with T1R (2.31 ± 0.09) and ENL (3.93 ± 0.46) expressed relatively higher levels of IL-10 with significant differences when compared to reaction-free leprosy PB (0.25 ± 0.21) and MB (1.30 ± 0.72).

IFN-γ was also analyzed in the 4 groups (Figure 2(d)), with high expression in the reaction-free leprosy PB (2.70 ± 0.37) and MB (1.49 ± 0.30) in contrast to the low expression in the groups with T1R (0.39 ± 0.14) and ENL (0.46 ± 0.20), with significant differences among the reaction-free leprosy PB group and the groups with T1R ($p < 0.001$) and ENL ($p < 0.001$).

Table 1: Clinical and epidemiological variables of reactional and reaction-free leprosy patients.

		I/TT				BT (PB)				BT(MB)				BB				BL				LL				Total of groups				Total	
		Leprosy reaction		Reaction-free		Leprosy reaction		Reaction-free		Leprosy reaction		Reaction-free		Leprosy reaction		Reaction-free		Leprosy reaction		Reaction-free		Leprosy reaction		Reaction-fee		Leprosy reaction		Reaction-free			
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Operational classification	PB	0	*0*	6	*100*	0	*0*	2	*100*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	**0**	*0*	**8**	*47*	*8*	*23.6*
	MB	0	*0*	0	*0*	0	*0*	0	*0*	1	*20*	4	*80*	4	*80*	1	*20*	3	*60*	2	*40*	9	*81.8*	2	*18*	**17**	*100*	**9**	*53*	*23*	*67.6*
Leprosy reaction	T1R									1	*5.9*			4	*23.5*			2	*11.8*			0	*0*			**7**	*41.2*			7	20.5
	ENL									0	*0*			0	*0*			1	*5.9*			9	*53*			**10**	*58.8*			10	29.4
Period of occurrence of leprosy reaction	Before treatment									1	*5.9*			3	*17.6*			1	*5.9*			0	*0*			**5**	*29.4*			5	14.7
	During treatment									0	*0*			0	*0*			0	*0*			0	*0*			**0**	*0*			0	0
	After treatment									0	*0*			1	*5.9*			2	*11.8*			9	*53*			**12**	*70.6*			12	35.2
Disability grade	0	0	*0*	6	*100*	0	*0*	2	*100*	1	*20*	3	*60*	1	*20*	0	*0*	1	*20*	2	*40*	5	*46*	0	*0*	**8**	23.5	**13**	38.2	21	61.7
	1	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	1	*20*	1	*20*	1	*20*	1	*20*	0	*0*	3	*27*	2	*18*	**5**	14.7	**4**	11.8	9	26.4
	2	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	2	*40*	0	*0*	1	*20*	0	*0*	1	*9*	0	*0*	**4**	11.8	**0**	0	4	11.7
Sex	Male	0	*0*	0	*0*	0	*0*	2	*100*	1	*20*	4	*80*	2	*40*	1	*20*	2	*40*	2	*40*	5	*45.5*	1	*9.1*	**10**	58.8	**10**	58.8	20	58.8
	Female	0	*0*	6	*100*	0	*0*	0	*0*	0	*0*	0	*0*	2	*40*	0	*0*	1	*20*	0	*0*	4	*36.3*	1	*9.1*	**7**	41.2	**7**	41.2	14	41.2
Age group	18-24	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	1	*20*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	**1**	2.9	**0**	0.0	1	2.9
	25-34	0	*0*	2	*33*	0	*0*	0	*0*	0	*0*	1	*20*	1	*20*	0	*0*	0	*0*	0	*0*	1	*9.09*	0	*0*	**2**	5.9	**3**	8.8	5	14.7
	35-44	0	*0*	3	*50*	0	*0*	0	*0*	0	*0*	0	*0*	1	*20*	1	*20*	2	*40*	0	*0*	2	*19*	1	*9*	**5**	14.7	**5**	14.7	10	29.4
	45-54	0	*0*	0	*0*	0	*0*	1	*50*	0	*0*	1	*20*	1	*20*	0	*0*	0	*0*	1	*20*	3	*27.3*	1	*9*	**4**	11.8	**4**	11.8	8	23.6
	55-64	0	*0*	1	*17*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	3	*27.3*	0	*0*	**3**	8.8	**1**	2.9	4	11.7
	≥65	0	*0*	0	*0*	0	*0*	1	*50*	1	*20*	2	*40*	0	*0*	0	*0*	1	*20*	1	*20*	0	*0*	0	*0*	**2**	5.9	**4**	11.8	6	17.7
	Total	**0**	**0**	**6**	**35.2**	**0**	**0**	**2**	**11.8**	**1**	**5.9**	**4**	**23.5**	**4**	**23.5**	**1**	**5.9**	**3**	**17.6**	**2**	**11.8**	**9**	**53**	**2**	**11.8**	***17***	*50*	***17***	*50*	*34*	*100*

I: indeterminate leprosy; TT: tuberculoid leprosy; BT: borderline tuberculoid; BB: borderline borderline; BL: borderline lepromatous; LL: lepromatous leprosy; PB: paucibacillary; MB: multibacillary;

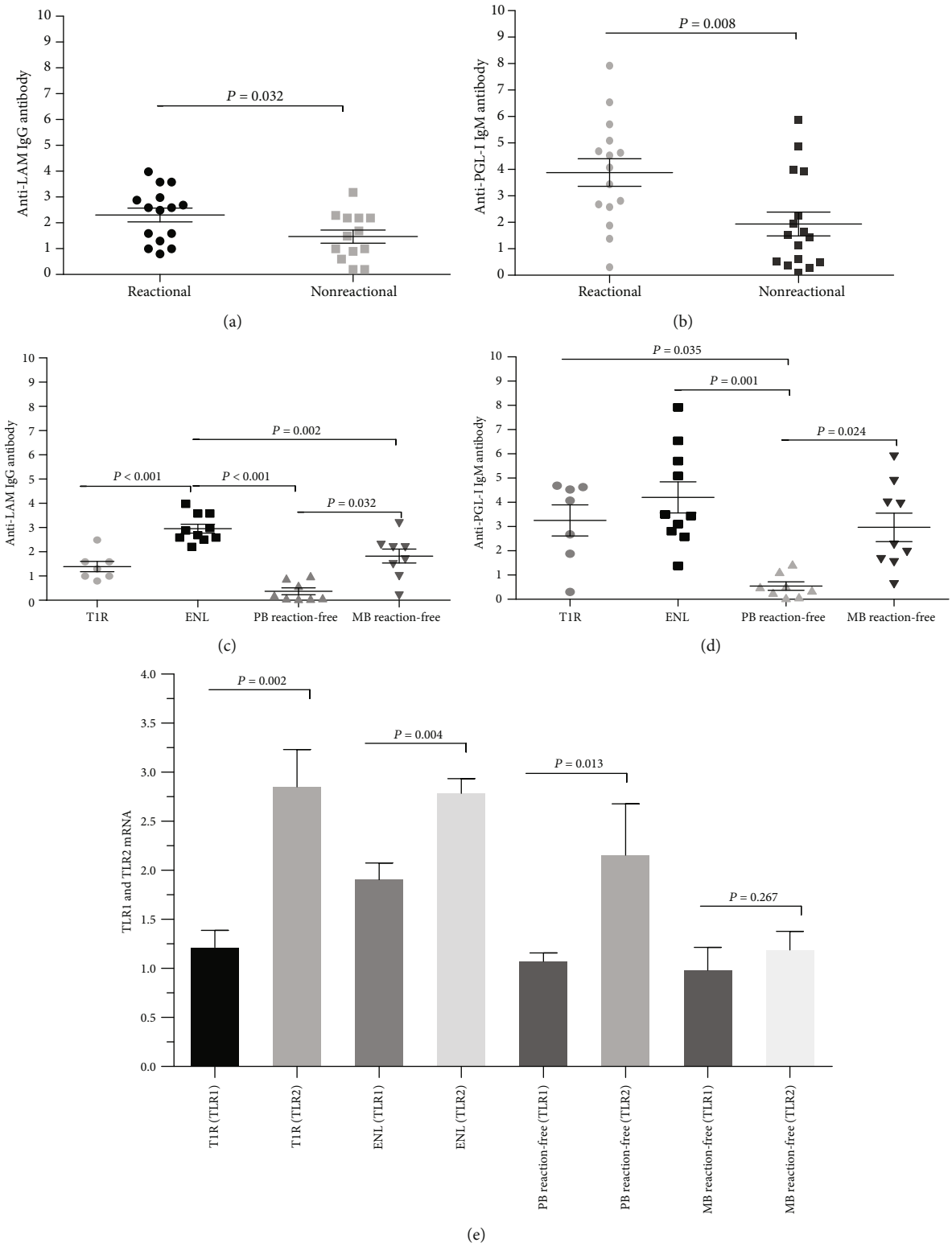

Figure 1: Serological markers (LAM and PGL-I) and TLR 1 and 2 in the peripheral blood of leprosy reaction and reaction-free leprosy patients. (a) Comparison between mean levels of anti-LAM IgG by ELISA (Enzyme-Linked Immunosorbent Assay) in the leprosy reaction and reaction-free leprosy patients. (b) Comparison between mean levels of anti-PGL-I IgM by ELISA in the leprosy reaction and reaction-free leprosy patients. (c) Comparison between mean levels of anti-LAM IgG by ELISA in the reactional and reaction-free leprosy patients PB and MB. (d) Comparison between mean levels of anti-PGL-I IgM by ELISA in the reactional and reaction-free leprosy patients PB and MB. (e) Comparison between TLR1 and TLR2 mRNA gene expression in the T1R, ENL, reaction-free leprosy patients PB and MB. The RNA expression was represented in fold change in relation to the endogenous control.

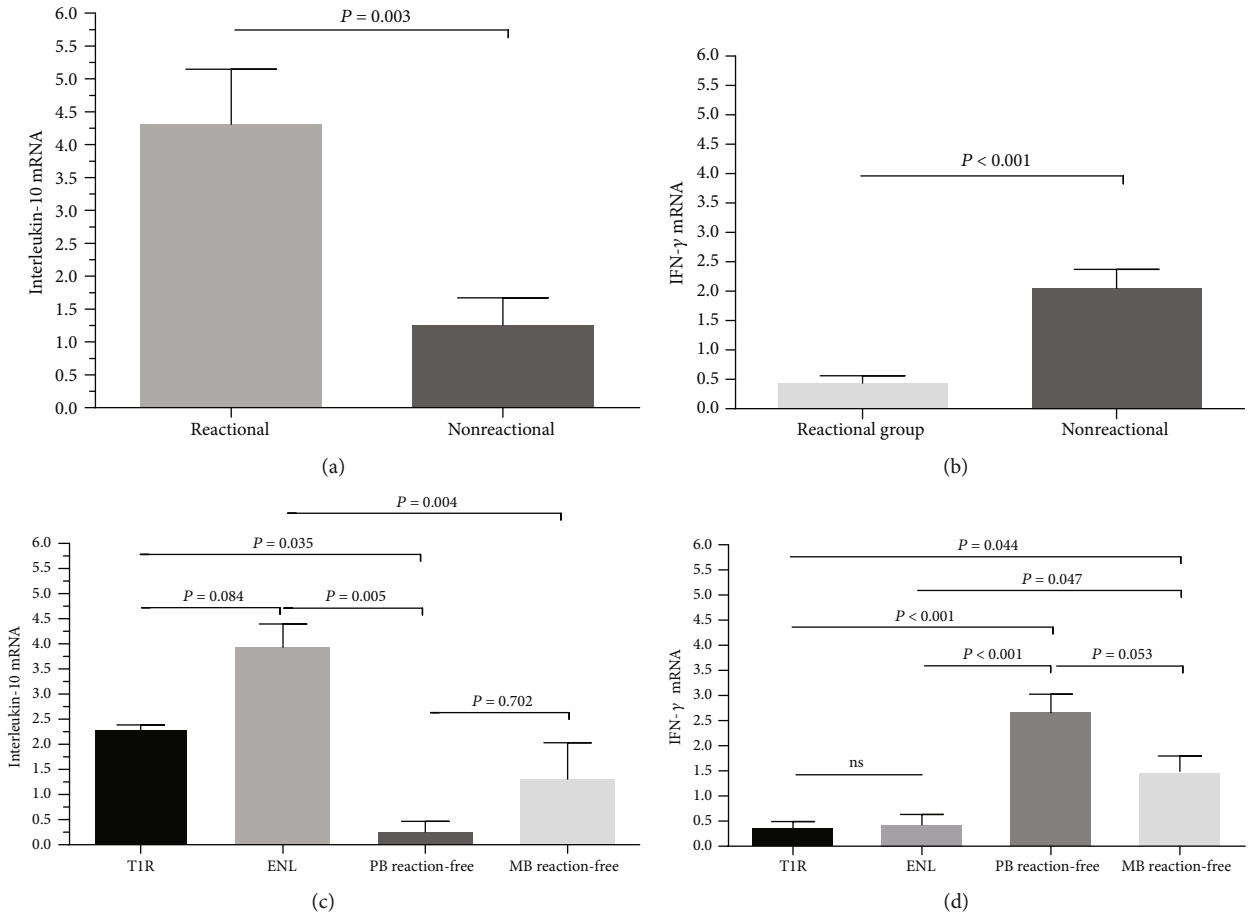

Figure 2: IL-10 and IFN-γ expression in leprosy reaction and reaction-free leprosy patients. (a) Comparison between IL-10 expression in the reactional and reaction-free leprosy. (b) Comparison between IFN-γ expression in the reactional and reaction-free leprosy patients. (c) Comparison between IL-10 expression in the reactional and reaction-free leprosy patients PB and MB. (d) Comparison between IFN-γ expression in the reactional and reaction-free leprosy patients PB and MB.

3.3. Path Analysis. The *Pearson's* correlation matrix in the leprosy reaction group demonstrated positive correlations among the dependent variable IL-10 and the independent variables TLR2 ($r = 0.89$; $p < 0.001$), anti-LAM ($r = 0.55$; $p = 0.043$), anti-PGL-I ($r = 0.70$; $p = 0.004$), number of injured nerves ($r = 0.62$; $p = 0.018$), and number of skin lesions ($r = 0.69$; $p = 0.005$) (Table 2).

Positive and significant correlations between IFN-γ expression and both TLR1 ($r = 0.74$; $p = 0.023$) and TLR2 ($r = 0.78$; $p = 0.013$) in the reaction-free leprosy group were shown in Table 3.

Figure 3(a) demonstrated the direct effects of anti-LAM (0.407) and anti-PGL-I (0.474) on IL-10 (dependent variable), which were greater than the residual effect (0.372), demonstrating the causal relationship among these variables and IL-10 expression in the leprosy reaction groups.

Figure 3(b) shows the direct effects of anti-LAM (0.623) and anti-PGL-I (0.605) on TLR2 (second dependent variable in the reactional group), which were greater than the residual effect of this model (0.255).

On the other hand, Figure 3(c) demonstrates that the IFN-γ expression can be influenced by association between TLR1 and TLR2 (0.873) that was greater than the residual effect (0.612) of this model.

Figure 4(a) shows the causal diagram in T1R, whose direct effect of anti-PGL-I (0.503) and indirect effect of TLR2 via anti-PGL-I (0.488) on IL-10 were greater than the residual effect (0.364). Figure 4(b) shows the causal model in ENL, whose direct effect of anti-LAM (0.778) and indirect effect of TLR2 via anti-LAM (0.721) on IL-10 expression were greater than the residual effect of this model (0.280).

4. Discussion

The present study demonstrates, through path analysis, the dependence relationship between the major antigens of *M. leprae* and receptors of innate immunity (especially TLR2) indicating a possible key role in triggering the reactional states, in fact contributing with an immunosuppressive immune response favorable to survival and bacillary multiplication.

The association between the leprosy reaction group and the operational classification in the present study showed that MB patients presented the highest potential to develop

Table 2: Correlation matrix among dependent and independent variables of the leprosy reaction cases, based on Pearson's correlation.

Variables	Leprosy reaction					
	IL-10			Toll-like receptor 2		
	r_{xy}	CI (95%)	p value	r_{xy}	CI (95%)	p value
*Number of injured nerves	0.62	0.13–0.87	0.018	0.47	-0.08–0.80	0.087
*Number of skin lesions	0.69	0.26–0.90	0.005	0.69	0.26–0.90	0.005
*DG	0.28	0.29–0.71	0.331	0.13	-0.42–0.62	0.639
Age	-0.36	-0.75–0.20	0.193	-0.30	-0.72–0.27	0.282
Toll-like receptor 1	0.43	-0.13–0.78	0.121	0.44	-0.11–0.79	0.109
Toll-like receptor 2	0.89	0.68–0.96	<0.001			
IL-10				0.89	0.68–0.96	<0.001
IFN-γ	0.05	-0.54–0.61	0.877	0.37	0.52–0.94	0.233
IL-4	-0.17	-0.64–0.40	0.561	0.09	-0.46–0.60	0.747
TNF-α	-0.031	-0.55–0.51	0.916	-0.15	-0.63–0.41	0.605
Anti-LAM	0.55	0.03–0.84	0.043	0.70	0.29–0.90	0.004
Anti-PGL-I	0.70	0.08–0.85	0.004	0.68	0.14–0.87	0.007
Bacterial index	0.21	-0.36–0.67	0.468	0.20	-0.36–0.66	0.478

r_{xy}: Pearson's correlation coefficient; CI (95%): confidence interval of 95%, DG: disability grade; IL-10: interleukin 10; IFN-γ: interferon gamma; IL-4: interleukin 4; TNF-α: tumor necrosis factor (alpha); anti-LAM: anti-lipoarabinomannan antibody; anti-PGL-I: anti-phenolic glycolipid-I antibody. *Diagnostic data.

Table 3: Correlation matrix among dependent and independent variables of the reaction-free leprosy patients group, based on Pearson's correlation.

Variables	Reaction-free group								
	IFN-γ			Toll-like receptor 1			Toll-like receptor 2		
	r_{xy}	CI (95%)	p value	r_{xy}	CI (95%)	p value	r_{xy}	CI (95%)	p value
*Number of injured nerves	0.04	-0.68–0.72	0.905	-0.20	-0.77–0.62	0.576	-0.18	-0.63–0.76	0.606
*Number of skin lesions	-0.35	-0.85–0.47	0.265	-0.26	-0.74–0.67	0.473	-0.47	-0.86–0.43	0.142
DG	-0.06	-0.78–0.61	0.869	-0.09	-0.82–0.54	0.795	-0.11	-0.71–0.70	0.741
Age	0.24	-0.55–0.81	0.513	0.07	-0.64–0.76	0.845	-0.06	-0.62–0.77	0.865
Toll-like receptor 1	0.73	0.03–0.94	0.023				0.87	0.43–0.98	0.002
Toll-like receptor 2	0.78	0.45–0.98	0.013	0.87	0.43–0.98	0.002			
IL-10	0.66	-0.13–0.95	0.053	0.32	-0.62–0.84	0.401	0.43	-0.55–0.87	0.249
IFN-γ				0.74	0.03–0.94	0.023	0.78	0.45–0.98	0.013
IL-4	-0.31	-0.85–0.47	0.384	-0.25	-0.84–0.49	0.485	-0.34	-0.86–0.44	0.309
TNF-α	-0.28	-0.85–0.45	0.426	-0.31	-0.87–0.38	0.386	-0.36	-0.87–0.41	0.279
Anti-LAM	-0.29	-0.84–0.49	0.419	-0.28	-0.85–0.47	0.429	-0.28	-0.82–0.54	0.399
Anti-PGL-I	-0.51	-0.89–0.31	0.132	-0.33	-0.78–0.62	0.357	-0.35	-0.86–0.42	0.294
Bacterial index	-0.45	-0.87–0.38	0.197	-0.38	-0.81–0.55	0.283	-0.74	-0.89–0.33	0.144

r_{xy}: Pearson's correlation coefficient; CI (95%): confidence interval of 95%, DG: disability grade; IL-10: interleukin 10; IFN-γ: interferon gamma; IL-4: interleukin 4; TNF-α: tumor necrosis factor (alpha); anti-LAM: anti-lipoarabinomannan antibody; anti-PGL-I: anti-phenolic glycolipid-1 antibody. *Diagnostic data.

leprosy reactions, independently of the type of reaction, which is corroborated by other research [32]. In this study, the ENL was the most frequent in the leprosy reaction group, probably because most MB patients are from the borderline leprosy (BL) and LL clinical forms. This study differs from the prevalence demonstrated in other studies, in which the borderline tuberculoid (BT) and T1R were the most prevalent [33]. It is noteworthy that most of the T1R occurred before treatment, which confirms a downgrading T1R and the presence of an immunosuppressive profile associated to an increase in IL-10. With respect to ENL, this reactions occurred after treatment related to the presence of IL-10 playing an important role in the immunopathogenesis of this event [4, 11, 34]. Regarding the disability grade in diagnosis, those individuals not affected by leprosy reactions will mostly have a degree of disability of zero due to the relationship

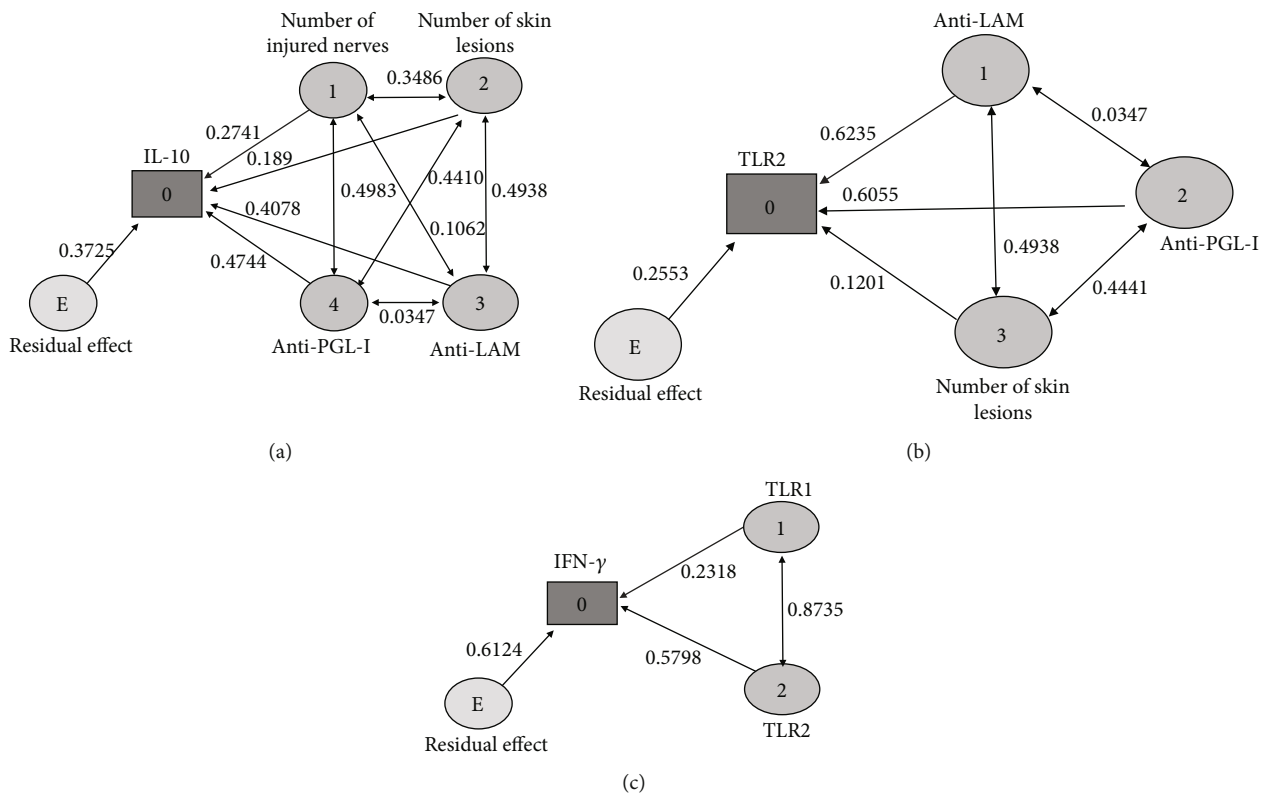

Figure 3: Path diagrams of the leprosy reaction and reaction-free leprosy groups. (a) A path diagram indicating the relationships among independent variables involved in diagnosis, number of skin lesions at diagnosis, anti-LAM, and anti-PGL-I influencing the variation of the dependent variable IL-10 in the reaction group. (b) A path diagram showing the relationships among independent variables, number of skin lesions at diagnosis, anti-LAM, and anti-PGL-I promoting the variation in TLR2 expression in the reaction group. (c) A path diagram involving independent variables TLR1 and TLR2, which induced variation in the dependent variable IFN-γ in the reaction-free leprosy group. The path analysis demonstrated dependence relation among dependent variable (0) and independent variables with direct effect greater than the residual effect.

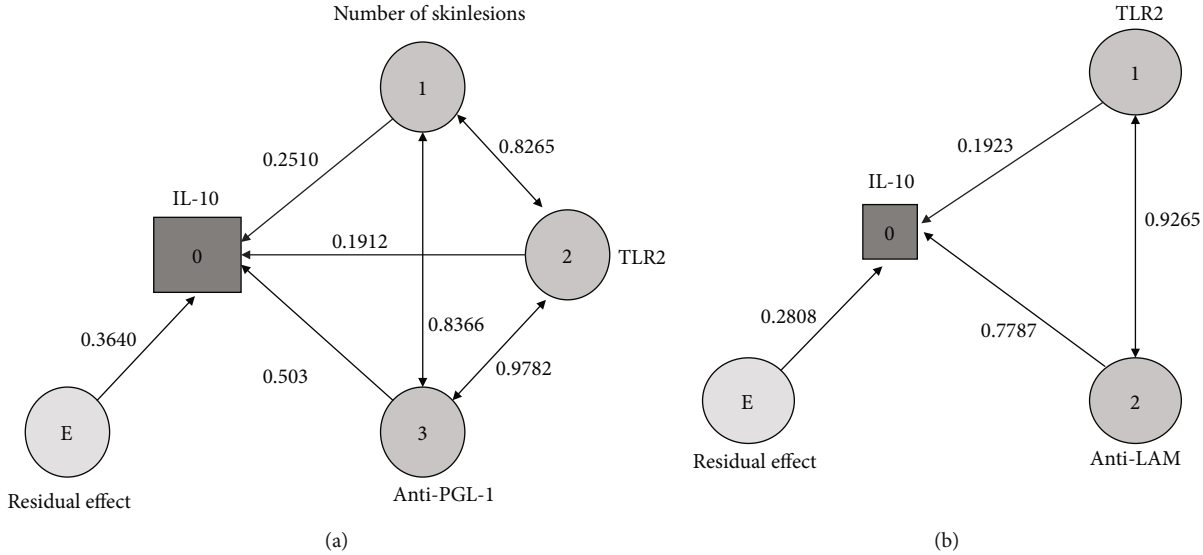

Figure 4: Path diagrams of type 1 leprosy reactions and ENL. (a) A path diagram indicating the dependence relationship between IL-10 (dependent variable) and anti-PGL-I in the group with T1R after extratification of the groups. (b) Path analysis pointing to the dependence relationship between IL-10 (dependent variable) and anti-LAM levels in ENL after extratification of groups.

between leprosy reactions and neural damage [35]. The prevalence of males in the groups is in agreement with another research that associates the concern of women with health status to the early diagnosis of the disease [36].

The mean age in the leprosy reaction group in this study is also concordant with previous reports [37, 38].

Higher levels of anti-LAM in the leprosy reaction group is due to the presence of great number of individuals with ENL, since LAM is involved in the formation of immune complexes and in the pathogenesis of erythema nodosum leprosum [39]. High levels of anti-PGL-I in leprosy reactions, independently on the type of reaction, ratify that this antigen is a risk marker for the occurrence of reactions, during treatment and after discharge from MDT, corroborating with previous studies that showed positive serology anti-PGL-I as a risk factor to the reactional condition [3, 7, 40].

When analyzing and comparing the expression of TLR1 and TLR2 in the same group, our findings demonstrated differences in these expressions, mainly in the reactional groups, in contrast to the quantitative balance of these receptors in the reaction-free leprosy MB group. These differences can indicate that there may be a signaling pathway-dependent heterodimer TLR1/2 in these patients determining the immune response to the pathogen [20].

As an additional evidence, we have also shown that TLR1 and TLR2 expression levels presented no differences in the reaction-free leprosy MB group, a high-risk group to develop leprosy reaction; however, they were not affected by this reactions during the research.

Studies have quoted the importance of the physical interaction between TLR1 and TLR2 in the recognition of mycobacterial antigens and consequent activation of nuclear factor kappa B (NF-κB) inducing the synthesis of proinflammatory cytokines [20, 41]. On the contrary, using knockout macrophages ($TLR1^{-/-}$ or $TLR2^{-/-}$), authors demonstrated that in the absence of one of these receptors there was damage in the activation of the NF-κB and, thus, low levels of TNF-α [42]. This association between TL1/2 corroborates with our results, mainly in the reaction-free groups, which suggests heterodimer formation and activation that leads to IFN-γ expression, a specific response to mycobacterial antigens [43, 44].

The differential expression among TLRs in the leprosy reaction groups, T1R and ENL, hypothetically suggests that the TLR2/2 homodimer formation may mediate the production of IL-10 [21]. Authors have hypothesised that prolonged TLR2/2 homodimer signaling, induced by mycobacterial components, limits the activation of mitogen-activating protein kinase (MAPK) pathways by inhibiting phagolysosome fusion and antigen presentation by MHC class II. In addition, this mechanism promotes the synthesis of anti-inflammatory cytokines, such as IL-10 and transforming growth factor β (TGF-β), which in turn, block the activation of NF-κB [21].

Although the hypothetical TLR2/2 homodimer mechanism has been proposed for *M. tuberculosis*, it still requires a functional validation. However, current molecular techniques do not allow to prove the existence of homodimers involving Toll-like receptors [21].

These events are represented in a hypothetical immunological pathway in Figure 5.

Interestingly, using cause and effect diagrams in the leprosy reaction group, we have shown that there may be a hypothetical immunological pathway involving TLR2, LAM, and PGL-I antigens, which was associated with the presence of IL-10, leading to a cellular immune response associated with the lepromatous leprosy pole of the disease [45]. Even though proinflammatory cytokines may contribute to demyelination, in our study, the number of injured nerves was associated with IL-10 expression according to previous studies using a rat Schwann cells (SCs)/axon coculture system and T and B cell-deficient ($Rag1^{-/-}$) mice, which reported rapid demyelination following adherence of *M. leprae* to SCs in the absence of immune cells. Nerve injury may be related to a mechanism dependent on PGL1 as observed in this present study, that is, *M. leprae* is sufficient to induce demyelination [46, 47].

According to previous results and hypothesis, in this present study, higher TLR2 expression in the reactional group, mostly in T1R, may be associated to TLR2/2 homodimer formation and association of TLR2/6 inducing a Th2 profile, while the TRL2/1 heterodimers may be occurring in reaction-free leprosy patients [28, 41, 48, 49]. The unbalanced immune response may explain why some patients with the same clinical form and bacilloscopic indexes will present different clinical outcomes.

To reinforce our hypotheses, studies have also shown that viable *M. leprae* can influence the formation of lipid droplets that lead to prostaglandin E_2 (PGE_2) production, which is involved in the synthesis of IL-10 in a TLR2-dependent pathway [48, 50, 51].

We cannot rule out that live bacilli can still induce IL-10 expression by interacting with other receptors, like the leukocyte-Ig-like receptors (LILR) and dendritic cell-specific ICAM-grabbing nonintegrin (DC-SIGN) [52, 53].

Although the predominance of IL-10 rather than IFN-γ in these reactions seems incomprehensible, most of the patients with T1R had downgrading reaction, whose bacillary viability may favor cell-mediated immunity, but not as effective as in those individuals affected by upgrading reaction [4, 6, 11, 54]. Regarding ENL, studies have reported the elevation of IL-10 in this reaction that may be explained by its ability to stimulate B cell proliferation and differentiation, which in turn secrete immunoglobulins in its membrane and subsequently, will activate components in the formation of immune complexes. In accordance with the above, high levels of anti-PGL-I IgM, anti-LAM IgG, B lymphocytes in skin lesions, and peripheral blood were pointed out as markers for ENL [55, 56]. In spite of this hypothesis is not proven for leprosy, we cannot fail to highlight another possible role of IL-10 related to its proinflammatory activity, which such cytokine under the action of IFN-α will activate the signal transducer and activator of transcription 1 (STAT1) inducing synthesis of chemokine 9 (CXCL9) and chemokine 10 (CXCL10) [57–59].

An important study reported, after cell stimulation with IL-10, the synthesis and elevation of neopterin, whose concentrations increase in the presence of IFN-γ, a proinflammatory cytokine [59]. This idea can be reinforced with studies that showed the presence of neopterin

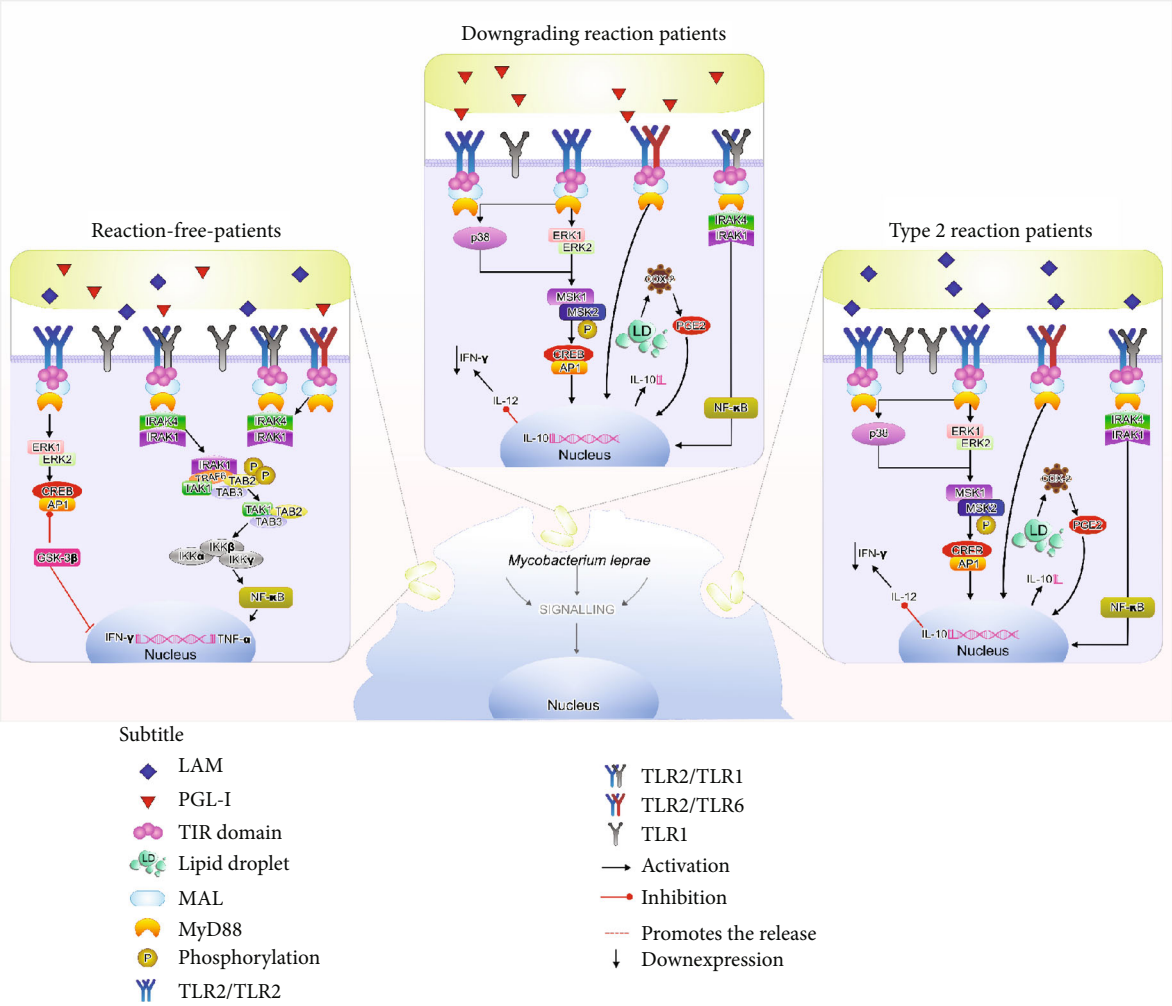

Figure 5: *M. leprae* and TLR interactions. Hypothetical mechanism of leprosy reactions according to evidences of associations between TLR2 and TLR1 receptors, the recognition of LAM and PGL-I markers, and the induction of IL-10 and IFN-γ expression. Reaction-free leprosy patients, mainly MB, present balanced production of TLRs (TLR1/2) that induces IFN-γ production through NF-κB activation, consequently with a better control of bacillary dissemination. On the other hand, patients with downgrading and ENL present an increased levels of TLR2, which may favor TLR2/2 homodimer formation and association of TLR2/6, which leads to IL-10 production, inhibiting the proinflammatory response and consequently causing bacillary spreading and higher risk of leprosy reactions.

as a marker for the occurrence of T1R upgrading and ENL reactions [60].

In T1R, PGL-I was the main agonist that was associated with TLR2 and IL-10 expression. A previous study has narrated that patients with T1R have higher levels of cluster of differentiation 14 (CD14), a macrophage surface marker that concentrates and distributes triacyl-lipopeptides to TLR2 and TLR1, which leads to the hypothesis that this molecular marker, CD14, facilitates the association between PGL-I and TLR2 [61, 62]. The associations among LAM, TLR2, and IL-10 in ENL are in agreement with other studies that have indicated LAM as the main molecule associated to the pathogen that activates the complement and acting in an active way in the formation of these immune complexes [63].

These reactions can be triggered by multiplication of persistent bacilli before and after MDT. On the other hand, during treatment, these events can be related to fragmented mycobacterial products unleashing reactional states [64].

5. Conclusions

Finally, we showed an unbalance in the expressions of TLR1 and TLR2, in the leprosy reaction groups, in contrast to reaction-free leprosy MB, the group which presented a balance in these expressions. Thus, we conclude and hypothesized, in reactional groups, a possible signaling pathway favoring the formation of TLR2/2 homodimers, association of TLR2/6, and consequently, greater expression of IL-10, which may favor bacillary survival and the occurrence of these events. The understanding of this unbalanced response may lead us to novel therapeutic strategies to prevent leprosy reactions.

Data Availability

The path analysis and laboratory data of all patients used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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References

[1] W. H. Van Brakel, I. B. Khawas, and S. B. Lucas, "Reactions in leprosy: an epidemiological study of 3 86 patients in west Nepal," *Leprosy Review*, vol. 65, no. 3, pp. 190–203, 1994.

[2] D. S. Ridley and W. H. Jopling, "Classification of leprosy according to immunity. A five-group system," *International Journal of Leprosy and Other Mycobacterial Diseases*, vol. 34, no. 3, pp. 255–273, 1966.

[3] J. A. Nery, F. Bernardes Filho, J. Quintanilha, A. M. Machado, S. Oliveira Sde, and A. M. Sales, "Understanding the type 1 reactional state for early diagnosis and treatment: a way to avoid disability in leprosy," *Anais Brasileiros de Dermatologia*, vol. 88, no. 5, pp. 787–792, 2013.

[4] V. N. Sehgal, S. N. Bhattacharya, and S. Jain, "Relapse or late reversal reaction?," *International Journal of Leprosy and Other Mycobacterial Diseases*, vol. 58, no. 1, pp. 118–121, 1990.

[5] S. Chaitanya, M. Lavania, R. P. Turankar, S. R. Karri, and U. Sengupta, "Increased serum circulatory levels of interleukin 17F in type 1 reactions of leprosy," *Journal of Clinical Immunology*, vol. 32, no. 6, pp. 1415–1420, 2012.

[6] A. D. Moubasher, N. A. Kamel, H. Zedan, and D. D. Raheem, "Cytokines in leprosy, I. Serum cytokine profile in leprosy," *International Journal of Dermatology*, vol. 37, no. 10, pp. 733–740, 1998.

[7] M. M. Stefani, J. G. Guerra, A. L. Sousa et al., "Potential plasma markers of type 1 and type 2 leprosy reactions: a preliminary report," *BMC Infectious Diseases*, vol. 9, p. 75, 2009.

[8] S. Khadge, S. Banu, K. Bobosha et al., "Longitudinal immune profiles in type 1 leprosy reactions in Bangladesh, Brazil, Ethiopia and Nepal," *BMC Infectious Diseases*, vol. 15, p. 477, 2015.

[9] J. Cuevas, J. L. Rodriguez-Peralto, R. Carrillo, and F. Contreras, "Erythema nodosum leprosum: reactional leprosy," *Seminars in Cutaneous Medicine and Surgery*, vol. 26, no. 2, pp. 126–130, 2007.

[10] K. Linder, M. Zia, W. V. Kern, R. K. Pfau, and D. Wagner, "Relapses vs. reactions in multibacillary leprosy: proposal of new relapse criteria," *Tropical Medicine & International Health*, vol. 13, no. 3, pp. 295–309, 2008.

[11] B. Naafs and C. L. van Hees, "Leprosy type 1 reaction (formerly reversal reaction)," *Clinics in Dermatology*, vol. 34, no. 1, pp. 37–50, 2016.

[12] B. Naafs, "Leprosy reactions. New knowledge," *Tropical and Geographical Medicine*, vol. 46, no. 2, pp. 80–84, 1994.

[13] A. Polycarpou, S. L. Walker, and D. N. Lockwood, "A systematic review of immunological studies of erythema nodosum leprosum," *Frontiers in Immunology*, vol. 8, p. 233, 2017.

[14] S. L. Walker, M. Balagon, J. Darlong et al., "ENLIST 1: an international multi-centre cross-sectional study of the clinical features of erythema nodosum leprosum," *PLoS Neglected Tropical Diseases*, vol. 9, no. 9, article e0004065, 2015.

[15] N. T. Foss, E. B. de Oliveira, and C. L. Silva, "Correlation between TNF production, increase of plasma C-reactive protein level and suppression of T lymphocyte response to concanavalin a during erythema nodosum leprosum," *International Journal of Leprosy and Other Mycobacterial Diseases*, vol. 61, no. 2, pp. 218–226, 1993.

[16] M. A. Bach, L. Chatenoud, D. Wallach, F. P. D. Tuy, and F. Cottenot, "Studies on T cell subsets and functions in leprosy," *Clinical and Experimental Immunology*, vol. 44, no. 3, pp. 491–500, 1981.

[17] E. N. Sarno, G. E. Grau, L. M. Vieira, and J. A. Nery, "Serum levels of tumour necrosis factor-alpha and interleukin-1 β during leprosy reactional srates," *Clinical & Experimental Immunology*, vol. 84, no. 1, pp. 103–108, 1991.

[18] A. L. Sousa, V. M. Fava, L. H. Sampaio et al., "Genetic and immunological evidence implicates interleukin 6 as a susceptibility gene for leprosy type 2 reaction," *The Journal of Infectious Diseases*, vol. 205, no. 9, pp. 1417–1424, 2012.

[19] M. O. Moraes, E. N. Sarno, A. S. Almeida et al., "Cytokine mRNA expression in leprosy: a possible role for interferon-gamma and interleukin-12 in reactions (RR and ENL)," *Scandinavian Journal of Immunology*, vol. 50, no. 5, pp. 541–549, 1999.

[20] S. R. Krutzik, M. T. Ochoa, P. A. Sieling et al., "Activation and regulation of Toll-like receptors 2 and 1 in human leprosy," *Nature Medicine*, vol. 9, no. 5, pp. 525–532, 2003.

[21] I. Saraav, S. Singh, and S. Sharma, "Outcome of Mycobacterium tuberculosis and Toll-like receptor interaction: immune response or immune evasion?," *Immunology and Cell Biology*, vol. 92, no. 9, pp. 741–746, 2014.

[22] J. W. Brandsma and W. H. Van Brakel, "WHO disability grading: operational definitions," *Leprosy Review*, vol. 74, no. 4, pp. 366–373, 2003.

[23] M. M. Stefani, A. B. Grassi, L. H. Sampaio et al., "Comparison of two rapid tests for anti-phenolic glycolipid-I serology in Brazil and Nepal," *Memórias do Instituto Oswaldo Cruz*, vol. 107, Suppl 1, pp. 124–131, 2012.

[24] R. Melsom, M. Harboe, B. Myrvang, T. Godal, and A. Belehu, "Immunoglobulin class specific antibodies to M. leprae in leprosy patients, including the indeterminate group and healthy contacts as a step in the development of methods for serodiagnosis of leprosy," *Clinical and Experimental Immunology*, vol. 47, no. 2, pp. 225–233, 1982.

[25] J. Touw, E. M. Langendijk, G. L. Stoner, and A. Belehu, "Humoral immunity in leprosy: immunoglobulin G and M antibody responses to Mycobacterium leprae in relation to various disease patterns," *Infection and Immunity*, vol. 36, no. 3, pp. 885–892, 1982.

[26] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[27] S. Greenland, J. Pearl, and J. M. Robins, "Causal diagrams for epidemiologic research," *Epidemiology*, vol. 10, no. 1, pp. 37–48, 1999.

[28] S. Wright, "Correlation and causation," *Journal of Agricultural Research*, vol. 20, pp. 557–585, 1921.

[29] R. K. Singh and B. D. Chaudhary, "Biometrical methods in quantitative genetic analysis," *Biometrics*, vol. 34, pp. 723-724, 1979.

[30] R. B. Kline, "Latent variable path analysis in clinical research: a beginner's tour guide," *Journal of Clinical Psychology*, vol. 47, no. 4, pp. 471–484, 1991.

[31] C. D. Cruz, "GENES: a software package for analysis in experimental statistics and quantitative genetics," *Acta Scientiarum, Agronomy*, vol. 35, no. 3, pp. 271–276, 2013.

[32] B. Kumar, S. Dogra, and I. Kaur, "Epidemiological characteristics of leprosy reactions: 15 years experience from North India," *International Journal of Leprosy and Other Mycobacterial Diseases*, vol. 72, no. 2, pp. 125–133, 2004.

[33] D. E. Antunes, S. Araujo, G. P. Ferreira et al., "Identification of clinical, epidemiological and laboratory risk factors for leprosy reactions during and after multidrug therapy," *Memórias do Instituto Oswaldo Cruz*, vol. 108, no. 7, pp. 901–908, 2013.

[34] N. K. Madan, K. Agarwal, and R. Chander, "Serum cytokine profile in leprosy and its correlation with clinico-histopathological profile," *Leprosy Review*, vol. 82, no. 4, pp. 371–382, 2011.

[35] K. E. Leon, J. T. Jacob, C. Franco-Paredes, P. E. Kozarsky, H. M. Wu, and J. K. Fairley, "Delayed diagnosis, leprosy reactions, and nerve injury among individuals with Hansen's disease seen at a United States clinic," *Open Forum Infectious Diseases*, vol. 3, no. 2, article ofw063, 2016.

[36] A. Le Grand, "Women and leprosy: a review," *Leprosy Review*, vol. 68, no. 3, pp. 203–211, 1997.

[37] G. Mastrangelo, J. da Silva Neto, G. V. da Silva et al., "Leprosy reactions: the effect of gender and household contacts," *Memórias do Instituto Oswaldo Cruz*, vol. 106, no. 1, pp. 92–96, 2011.

[38] P. Suchonwanit, S. Triamchaisri, S. Wittayakornrerk, and P. Rattanakaemakorn, "Leprosy reaction in Thai population: a 20-year retrospective study," *Dermatology Research and Practice*, vol. 2015, Article ID 253154, 5 pages, 2015.

[39] C. Verhagen, W. Faber, P. Klatser, A. Buffing, B. Naafs, and P. Das, "Immunohistological analysis of in situ expression of mycobacterial antigens in skin lesions of leprosy patients across the histopathological spectrum. Association of Mycobacterial lipoarabinomannan (LAM) and Mycobacterium leprae phenolic glycolipid-I (PGL-I) with leprosy reactions," *The American Journal of Pathology*, vol. 154, no. 6, pp. 1793–1804, 1999.

[40] D. E. Antunes, G. P. Ferreira, M. V. Nicchio et al., "Number of leprosy reactions during treatment: clinical correlations and laboratory diagnosis," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 49, no. 6, pp. 741–745, 2016.

[41] R. I. Tapping and P. S. Tobias, "Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling," *Journal of Endotoxin Research*, vol. 9, no. 4, pp. 264–268, 2003.

[42] P. Y. Bochud, T. R. Hawn, M. R. Siddiqui et al., "Toll-like receptor 2 (TLR2) polymorphisms are associated with reversal reaction in leprosy," *The Journal of Infectious Diseases*, vol. 197, no. 2, pp. 253–261, 2008.

[43] O. Takeuchi, S. Sato, T. Horiuchi et al., "Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins," *The Journal of Immunology*, vol. 169, no. 1, pp. 10–14, 2002.

[44] M. J. Jimenez-Dalmaroni, M. E. Gerswhin, and I. E. Adamopoulos, "The critical role of toll-like receptors—from microbial recognition to autoimmunity: a comprehensive review," *Autoimmunity Reviews*, vol. 15, no. 1, pp. 1–8, 2016.

[45] T. B. Geijtenbeek, S. J. Van Vliet, E. A. Koppel et al., "Mycobacteria target DC-SIGN to suppress dendritic cell function," *The Journal of Experimental Medicine*, vol. 197, no. 1, pp. 7–17, 2003.

[46] A. Rambukkana, G. Zanazzi, N. Tapinos, and J. L. Salzer, "Contact-dependent demyelination by Mycobacterium leprae in the absence of immune cells," *Science*, vol. 296, no. 5569, pp. 927–931, 2002.

[47] A. Rambukkana, "Mycobacterium leprae-induced demyelination: a model for early nerve degeneration," *Current Opinion in Immunology*, vol. 16, no. 4, pp. 511–518, 2004.

[48] K. A. Mattos, H. D'Avila, L. S. Rodrigues et al., "Lipid droplet formation in leprosy: Toll-like receptor-regulated organelles involved in eicosanoid formation and Mycobacterium leprae pathogenesis," *Journal of Leukocyte Biology*, vol. 87, no. 3, pp. 371–384, 2010.

[49] A. Ozinsky, D. M. Underhill, J. D. Fontenot et al., "The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13766–13771, 2000.

[50] K. A. de Mattos, E. N. Sarno, M. C. Pessolani, and P. T. Bozza, "Deciphering the contribution of lipid droplets in leprosy: multifunctional organelles with roles in Mycobacterium leprae pathogenesis," *Memórias do Instituto Oswaldo Cruz*, vol. 107, Suppl 1, pp. 156–166, 2012.

[51] N. Misra, M. Selvakumar, S. Singh et al., "Monocyte derived IL 10 and PGE2 are associated with the absence of Th 1 cells and in vitro T cell suppression in lepromatous leprosy," *Immunology Letters*, vol. 48, no. 2, pp. 123–128, 1995.

[52] L. B. Barreiro, H. Quach, J. Krahenbuhl et al., "DC-SIGN interacts with Mycobacterium leprae but sequence variation in this lectin is not associated with leprosy in the Pakistani population," *Human Immunology*, vol. 67, no. 1-2, pp. 102–107, 2006.

[53] K. J. Anderson and R. L. Allen, "Regulation of T-cell immunity by leucocyte immunoglobulin-like receptors: innate immune receptors for self on antigen-presenting cells," *Immunology*, vol. 127, no. 1, pp. 8–17, 2009.

[54] T. J. Kang, C. E. Yeum, B. C. Kim, E. Y. You, and G. T. Chae, "Differential production of interleukin-10 and interleukin-12 in mononuclear cells from leprosy patients with a Toll-like receptor 2 mutation," *Immunology*, vol. 112, no. 4, pp. 674–680, 2004.

[55] A. K. Chakrabarty, A. Kashyap, V. N. Sehgal, and K. Saha, "Solubilization of preformed immune complexes in sera of patients with type 1 and type 2 lepra reactions," *International Journal of Leprosy and Other Mycobacterial Diseases*, vol. 56, no. 4, pp. 559–565, 1988.

[56] V. N. Sehgal, R. K. Gautam, and V. K. Sharma, "Immunoprofile of reactions in leprosy," *International Journal of Dermatology*, vol. 25, no. 4, pp. 240–244, 1986.

[57] M. Bachmann, S. Ulziibat, L. Hardle, J. Pfeilschifter, and H. Muhl, "IFNα converts IL-22 into a cytokine efficiently activating STAT1 and its downstream targets," *Biochemical Pharmacology*, vol. 85, no. 3, pp. 396–403, 2013.

[58] H. Muhl, "Pro-inflammatory signaling by IL-10 and IL-22: bad habit stirred up by interferons?," *Frontiers in Immunology*, vol. 4, p. 18, 2013.

[59] C. Huber, J. R. Batchelor, D. Fuchs et al., "Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma," *The Journal of Experimental Medicine*, vol. 160, no. 1, pp. 310–316, 1984.

[60] F. F. Hamerlinck, P. R. Klatser, D. S. Walsh, J. D. Bos, G. P. Walsh, and W. R. Faber, "Serum neopterin as a marker for reactional states in leprosy," *FEMS Immunology and Medical Microbiology*, vol. 24, no. 4, pp. 405–409, 1999.

[61] D. R. Ranoa, S. L. Kelley, and R. I. Tapping, "Human lipopolysaccharide-binding protein (LBP) and CD14 independently deliver triacylated lipoproteins to Toll-like receptor 1 (TLR1) and TLR2 and enhance formation of the ternary signaling complex," *The Journal of Biological Chemistry*, vol. 288, no. 14, pp. 9729–9741, 2013.

[62] D. O. Santos, H. C. Castro, S. C. Bourguignon et al., "Expression of B7-1 costimulatory molecules in patients with multibacillary leprosy and reactional states," *Clinical and Experimental Dermatology*, vol. 32, no. 1, pp. 75–80, 2007.

[63] N. Bahia El Idrissi, P. K. Das, K. Fluiter et al., "M. leprae components induce nerve damage by complement activation: identification of lipoarabinomannan as the dominant complement activator," *Acta Neuropathologica*, vol. 129, no. 5, pp. 653–667, 2015.

[64] D. V. Opromolla, "Some considerations on the origin of type 1 reactions in leprosy," *International Journal of Leprosy and Other Mycobacterial Diseases*, vol. 73, no. 1, pp. 33-34, 2005.

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Research Article

Genetic Polymorphisms of *Toll-like receptors 2* and *9* as Susceptibility Factors for the Development of Ankylosing Spondylitis and Psoriatic Arthritis

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Introduction. Ankylosing spondylitis (AS) and psoriatic arthritis (PsA) are classified as spondyloarthritis (SpA), a group of inflammatory rheumatic diseases with complex genetic etiology. Toll-like receptors (TLRs) have an important role in the mechanism of innate immunity and may influence inflammatory responses. Polymorphisms in *TLR* genes that lead to changes in these receptors or that interfere with the transcription rates of mRNA TLR may be involved in the chronic inflammatory immune response observed in SpA. Currently, there is a lack of studies associating genetic polymorphisms in *TLRs* and SpA. *Objective*. Therefore, this case-control study is aimed at analyzing the influence of the respective SNPs on *TLR2 rs5743708*, *TLR6 rs5743810*, and *TLR9 rs5743836* and *rs187084* in the immunopathogenesis of SpA. *Methods*. The polymorphisms genotyped by PCR-RFLP were *TLR2 rs5743708*, *TLR6 rs5743810*, and *TLR9 rs5743836* and *rs187084*. The *HLA-B*27* was performed by PCR-SSP. *Results*. Logistic regression analysis showed a strong association between SNPs in *TLR2* and *TLR9* and susceptibility to SpA (OR = 12.56; CI = 6.5-25.9 and OR = 1.62; CI = 1.20-2.21, respectively). No association was observed among *HLA-B*27* and *TLR* polymorphisms ($p = 0.72$), nor among BASDAI and *TLR* polymorphisms ($p = 0.85$). *Discussion*. Our findings suggest that polymorphisms in *TLR2* and *TLR9* genes may contribute to the immunopathogenesis of the SpA. The *rs187084*, *rs5743836*, and *rs5743708* polymorphisms were associated with the risk of SpA development, in this study, and lead to significant changes in the innate and adaptive immune response profile, as well as the maintenance of the regulation of immunological mechanisms. *Conclusion*. The polymorphism *rs5743708* for the *TLR2* and the *rs187084_rs5743836 TLR9* haplotypes appear to be involved in the development of clinical forms of SpA and can be a possible therapeutic target for the spondyloarthritis.

1. Introduction

Spondyloarthritis (SpA) is a group of rheumatic diseases (RD) with immunological origin that presents chronic inflammatory and autoimmune conditions, and SpA shares clinical, serological, and genetic features, besides presenting a complex pathogenesis [1–3]. This group of diseases includes ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), arthritis associated with inflammatory bowel disease (IBD), and undifferentiated arthritis (USpA) [1]. AS is the most prevalent clinical form of SpA. In Brazil, 65.1% of the cases of SpA are classified as AS while PsA represents 18.3% [4, 5]. A high number of SpA patients are HLA-B27 positive [1], a molecular marker already associated with AS.

In the Brazilian population, the presence of this antigen is around 69.5% [5, 6].

The innate immune response in RD can be stimulated in several cell types through the recognition of molecular patterns from external or internal sources, such as PAMPs or DAMPs (pathogen-associated molecular patterns and damage-associated molecular patterns, respectively) [7–11].

Toll-like receptors (TLRs) are the most well-characterized pattern recognition receptors (PRRs) and are a transmembrane protein coded by the *toll* genes family [7]. TLRs are expressed in different cell types including immune and nonimmune cells; they play a crucial role not only in the detection of many PAMPs and DAMPs but also in the activation and steering of the adaptive immune system by the upregulation of costimulatory molecules of the antigen-presenting cells [4, 7–10].

The endogenous TLR ligand-mediated signaling has an important role in autoimmune disorders [8]. The activation of some TLRs through the interaction of DAMPs can facilitate the repair of damaged tissues and the elimination of cell debris; on the other hand, this same interaction was associated with the chronic inflammatory process involved in RD [12]. Therefore, activation of TLR by DAMPS seems to play a role in the self-sustained inflammatory cycle and the progression of these chronic diseases [7].

The SpA can be considered as multifactorial diseases that have a pathogenesis involving interactions between the environment and genes [2]. The exact mechanisms of SpA immunopathogenesis have not yet been fully elucidated, and probably that other genes outside the MHC also contribute to this complex process.

There is still insufficient data to prove that polymorphisms in the *TLRs* genes are involved in autoimmunity processes and the development of RD [11]. However, some polymorphisms have already been associated with the pathogenesis of RD [4].

In light of this, the purpose of this study was to analyze the influence of important polymorphisms in *TLR2*, *TLR6*, and *TLR9* genes in the immunopathogenesis of the two most common SpA, whereas no study involving these polymorphisms in SpA has been performed in the Brazilian population.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the Human Research Ethics Committee of the State University of Maringá—CEP-UEM 687.222/2014, and all volunteer participants signed the informed consent term.

2.2. Clinical Characterization. In this case-control study were included 529 subjects living in the south of Brazil. The patient group is composed of 149 unrelated subjects diagnosed with SpA by a single rheumatologist using the ASAS criteria [13], and in patients with PsA we also used the CASPAR criteria [14] to complement the ASAS criteria and thus provide greater security in the data obtained. The patients were attended at the rheumatology outpatient clinic of the University Hospital of Maringá (PR, Brazil), who presented one of the two most frequent clinical forms of SpA in the population (AS or PsA). All patients had magnetic resonance imaging of the sacroiliac joints and were evaluated for the presence of *HLA-B27*.

The control group consists of 380 healthy subjects, and the inclusion criteria in this group were absences of SpA or autoimmune and/or rheumatic disease, unrelated to subjects from the same group or patient group, and residence in the same geographical area as the patients. Both groups were age and sex matched.

2.3. Genotyping of TLR Genes. Based on previous published data on the association of *TLR* genes with autoimmune and inflammatory diseases, four SNPs were selected from three *TLR* genes. For the *TLR2* gene (ENSG00000137462), the SNP *rs5743708*: G>A (2258G>A, Arg753Gln); for *TLR6* gene (ENSG00000174130), the SNP *rs5743810*: C>T (745C>T, Ser249Pro); and for *TLR9* gene (ENSG00000239732), the *rs5743836*: T>C (-1237T>C) and *rs187084*: T>C (-1486T>C) SNPs, both located in the promoter region of the *TLR9*. The primer design was according to Folwaczny et al. [15] for the *TLR2* and to Selvaraj et al. [16] for *TLR6* and *TLR9* [15, 16].

The DNA was extracted from the buffy coat by using the salting-out technique [17]. The genotyping of the *TLR* SNPs was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The PCR was carried out with 100 ng/μL DNA, 200 μM of each dNTP, 0.1 μM of each primer, 1.68 mM of MgCl2, 3 μL 10x PCR buffer, and 1.5 U Taq DNA polymerase (GoTaq® DNA Polymerase, Promega, USA), in a final volume of 15 μL. PCR products were digested using restriction enzymes, temperature, and time of digestion specifically for each SNP (see Table 1).

*2.4. Genotyping of HLA-B*27.* Genotyping of the *HLA-B*27* was performed by PCR-SSP according to Oliveira et al. [18], and a genetic association analysis was performed between the presence or absence of *HLA-B*27* and the *TLR* polymorphisms in both clinical forms [18].

2.5. Analysis of Disease Activity. Disease activity was assessed by BASDAI (Bath Ankylosing Spondylitis Disease Activity Index), considering patients with high disease activity when BASDAI ≥ 4 [1, 5]. The association analysis was performed among BASDAI and *TLR* polymorphisms.

2.6. Ethnic Classification. The ethnic composition of the Brazilian population is influenced by some races [19, 20], which is due to the high ethnic diversity, and considering the Paraná population according to Probst et al. [19], which is of predominantly European origin (80.5%) and has a small but significant contribution of African (12.5%) and Amerindian (7.0%) genes [19, 20].

In this study, the population was considered a mixed ethnic group. Thus, the risk of population stratification bias due to genetic differences presented by different ethnic groups was minimized with a comparison between patients and controls of the same ethnic origin.

Table 1: Primer sequences, restriction enzymes used, and restriction digestion patterns for genotyping of *TLRs* genes.

Gene	SNP	Primer sequences	Restriction enzymes	Restriction temperature (°C) (time)	Length of the restriction fragments
TLR2	rs5743708	F: 5′-CATTCCCCAGCGCTTCTGCAAGCTCC-3′ R: 5′-GGAACCTAGGACTTTATCGCAGCTC-3′	Msp I	37°C (overnight)	Allele G—104 bp + 25 bp Allele A—210 bp
TLR6	rs5743810	F: 5′-GCATTTCCAAGTCGTTTCTATGT- 3′ R: 5′-GCAAAAACCCTTCACCTTGTT- 3′	Ava II	37°C (3 hours)	Allele C—50 bp + 160 bp Allele T—210 bp
TLR9	rs5743836	F: 5′-CTGCTTGCAGTTGACTGTGT -3′ R: 5′-ATGGGAGCAGAGACATAATGGA-3′	Mva I	37°C (3 hours)	Allele C—27 bp + 48 bp + 60 bp Allele T—34 bp + 111 bp
	rs187084	F: 5′-TATCGTCTTATTCCCCTGCTGGAATGT-3′ R: 5′-TGCCCAGAGCTGACTGCTGG-3′	Afl II	37°C (1 hour)	Allele T—34 bp + 111 bp Allele C—145 bp

F: forward standard; R: reverse standard; TLR: toll-like receptor; °C: degrees Celsius; bp: base pair.

Table 2: Demographic data in control and SpA patients for the analyzed SNPs.

	TLR2 rs5743708		*TLR6 rs5743810*		*TLR9 rs5743836*		*TLR9 rs187084*	
	Controls $n = 380$	Patients $n = 149$	Controls $n = 221$	Patients $n = 149$	Controls $n = 380$	Patients $n = 149$	Controls $n = 221$	Patients $n = 149$
Age (years) ± mean	58.4 ± 15.4	49.5 ± 15	48.9 ± 13.7	49.5 ± 15	58.4 ± 15.4	49.5 ± 15	48.9 ± 13.7	49.5 ± 15
Gender (n as %)								
Female	200 (53)	78 (53)	113 (51.3)	78 (53)	200 (53)	78 (53)	113 (51.3)	78 (53)
Male	180 (47)	71 (47)	108 (48.7)	71 (47)	180 (47)	71 (47)	108 (48.7)	71 (47)

Table 3: Clinical characteristics of patient groups.

	SpA $n = 149$	AS $n = 95$	PsA $n = 54$
Female/male (n as %)	79/70 (53.0/47.0)	51/44 (52.7/47.3)	28/26 (51.8/48.1)
Family history SpA (n as %)	39 (58.1)	9 (13.4)	30 (44.7)
BASDAI ≥ 4 (n as %)	104 (69.8)	69 (73.4)	35 (65.5)
Disease duration-years (mean ± SD)	11.6 ± 8.6	10.8 ± 8	13.2 ± 10
Treatment time-years (mean ± SD)	4 ± 3.7	7.8 ± 7.9	6.3 ± 5.9
HLA-B*27 (n as %)			
Present	83 (55.7)	53 (55.8)	30 (55.5)
Absent	66 (44.3)	42 (44.2)	24 (44.5)

SpA: patients with spondyloarthritis; AS: ankylosing spondylitis; PsA: psoriatic arthritis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index.

2.7. Statistical Analysis. The allele and genotype frequencies were estimated and compared by chi-square distribution tables with Fisher's corrections, and the Hardy–Weinberg equilibrium was tested using the Arlequin and SNPStats software [21].

The logistic regression analysis was performed, and the choice of the best inheritance model was performed using the Akaike information (AIC) in order to minimize the expected entropy. Analysis of the linkage disequilibrium (Δ') between the *TLR* SNPs was calculated by the SNPStats software. The EM algorithm or the Markov chain Monte Carlo method was used for the estimation of haplotypes and allelic groups. All tests were performed at a significance level of 5%.

3. Results

The mean age for the patient group was 49.5 ± 15 years, composed of 47% men and 53% women (see Table 2). The patients analyzed presented two clinical forms of SpA: AS (95/63.8%) and PsA (54/36.2%) (see Table 3).

The genotypic frequency distribution of all SNPs was in the Hardy–Weinberg equilibrium in both analyzed groups. The codominant model was selected as best inheritance model to analyze the association between SNPs and the risk of developing SpA.

No association was observed among *HLA-B*27* and *TLR* polymorphisms ($p = 0.72$), nor among BASDAI and *TLR* polymorphisms ($p = 0.85$). The allele and genotype frequencies of the polymorphisms analyzed in patients and controls (including the two clinical forms) are shown in Table 4.

The variables analyzed in the logistic regression test were gender and age between controls and patients; these results are described below.

The presence of the *rs5743708*G*/A for the *TLR2* gene was a susceptibility factor to SpA for males (OR = 12.27, CI = 3.95 – 38.12) and females (OR = 3.56, CI = 1.29 – 9.86), but the risk is higher in men with SpA. However, the polymorphism of the *TLR6* gene was not significantly related to the susceptibility to SpA.

The *rs5743836*C* for the *TLR9* gene was associated with susceptibility to SpA (OR = 1.62, CI = 1.20 – 2.21), and the *rs5743836*T/C* presented risk for males (OR = 1.84, CI = 1.30 – 3.28), but the *rs5743836*C/C* presented risk for females (OR = 9.75, CI = 1.88 – 15.43). Both genotypes associated with risk of SpA development present the *rs5743836*C*; however, two copies of this allele seem to only affect women SpA.

The *TLR9 rs187084*T/C* was associated with susceptibility to develop SpA only in male patients (OR = 2.51, CI = 1.12 – 5.65).

3.1. Association between Haplotypes and Allelic Group in the Development of SpA. We analyzed the haplotypes with frequency ≥ 1% for the *TLR9* gene. The allelic group analyzed in this study was constructed through multiple SNP analyses that included the four SNPs of the investigated *TLR* genes.

The three *TLR9* haplotypes (*rs5743836_ rs187084*) were associated with risk to SpA: the *CC* haplotype (OR = 4.65; CI = 2.44-8.89; $p < 0.0001$); the *CT* (OR = 3.02; CI = 1.62 -5.63; $p = 0.000006$); and the *TC* (OR = 1.67; CI = 1.20-2.75, $p = 0.043$), and *CC* haplotype showed linkage disequilibrium

Table 4: Allelic and genotypic frequencies for the *TLR2* gene *rs5743708*, the *TLR6 rs5743810*, and *TLR9 rs5743836* and *rs187084* among the control and patient groups.

	Controls	Patients					
		SpA	OR (95% CI)*[a]	AS (%)	OR (95% CI)*[b]	PsA (%)	OR (95% CI)*[c]
TLR2 rs5743708	$n = 380$	$n = 149$		$n = 95$		$n = 54$	
Genotype							
G/G	369 (97.1)	112 (75.2)		72 (76)		40 (74)	
G/A	11 (2.9)	31 (20.8)	9.28 (4.52-19.07)	19 (20)	10.06 (4.17-24.28)	12 (22)	8.99 (4.09-19.73)
A/A	0	6 (4)	19.68 (2.86-166)	4 (4)	20.73 (2.41-179.4)	2 (4)	25.42 (3.46-223.2)
Allele							
G	374 (99)	127 (85.2)	0.098 (0.03-0.24)	80 (84.3)	0.099 (0.034-0.27)	46 (85)	0.093 (0.031-0.27)
A	6 (1)	22 (14.8)	10.52 (4.07-31.83)	13 (13.7)	10.05 (3.76-29.5)	8 (15)	10.73 (3.6-32.63)
TLR6 rs5743810	$n = 221$	$n = 149$		$n = 95$		$n = 54$	
Genotype							
C/C	135 (61.1)	93 (62.4)	n.s	56 (59)	n.s	36 (67.7)	n.s
C/T	77 (34.8)	46 (30.9)	n.s	32 (33.7)	n.s	14 (26)	n.s
T/T	9 (4.1)	10 (6.7)	n.s	7 (7.3)	n.s	4 (7.3)	n.s
Allele							
C	174 (79)	115 (77)	n.s	73 (76.8)	n.s	43 (79.7)	n.s
T	46 (21)	34 (23)	n.s	22 (23.2)	n.s	11 (20.3)	n.s
TLR9 rs5743836	$n = 380$	$n = 149$		$n = 95$		$n = 54$	
Genotype							
T/T	244 (64.2)	73 (49)		52 (54.7)		23 (42.6)	
T/C	128 (33.7)	68 (45.6)	1.77 (1.19-2.62)	39 (41.1)	n.s	27 (50)	2.33 (1.28-4.25)
C/C	8 (2.1)	8 (5.4)	3.37 (1.22-9.31)	4 (4.2)	1.60 (1.01-2.54)	4 (7.4)	5.52 (1.54-19.81)
Allele							
T	308 (81.1)	107 (71.8)	0.59 (0.38-0.92)	70 (73.7)	n.s	37 (68.5)	n.s
C	72 (18.9)	42 (28.2)	1.69 (1.62-2.62)	25 (26.3)	n.s	17 (31.5)	n.s
TLR9 rs187084	$n = 221$	$n = 149$		$n = 95$		$n = 54$	
Genotype							
T/T	86 (39)	64 (42.9)	n.s	46 (48.4)	n.s	18 (33.4)	n.s
T/C	103 (46.5)	68 (45.7)	n.s	41 (43.2)	n.s	26 (48.1)	n.s
C/C	32 (14.5)	17 (11.4)	n.s	8 (8.4)	n.s	10 (18.5)	n.s
Allele							
T	137 (62)	98 (65.8)	n.s	68 (71.6)	n.s	31 (57.4)	n.s
C	84 (38)	51 (34.2)	n.s	27 (28.4)	n.s	23 (42.6)	n.s

n: number of individuals; SpA: patients with spondyloarthritis; AS: ankylosing spondylitis; PsA: psoriatic arthritis; OR: odds ratio; CI: confidence interval with $p < 0.05$; n.s: not significant. *Calculated using the chi-square test; [a]SpA *vs* controls; [b]AS *vs* controls; [c]PsA *vs* controls.

($\Delta' = 0.897$, $p < 0.05$). However, the analysis of allelic groups (*rs5743836_ rs187084_rs5743708_rs5743810*) did not show linkage disequilibrium ($\Delta' = 0.1223$, $p > 0.05$).

4. Discussion

To the best of our knowledge, this is the first study evaluating *TLR2*, *TLR6*, and *TLR9* gene polymorphisms in the immunopathogenesis of SpA. The genetic variability of *TLRs* was featured with an involvement in the susceptibility in inflammatory diseases [7, 22].

Our findings suggest that the polymorphisms analyzed for *Toll-like receptor* genes may contribute to the development of the immunopathogenesis of SpA, without influence of the presence of antigen HLA-B27 or disease activity. The association of other genes in the development of SpA without presence of HLA-B27 antigen was described in the literature by our group, which may indicate that other genetic markers may be involved in SpA [23].

In this present study, the *TLR2* gene *rs5743708*A* polymorphism increased the chance of developing SpA by 10-fold. Furthermore, the presence of homozygote (*rs5743708*A/A*) was not observed among the controls, confirming our findings that the presence of the altered allele (*A*) may influence the development of SpA. This polymorphism has a very low frequency in the population (<1%) and this allele has been implicated in the risk

phenotype [24]. In this way, our data confirm that this polymorphism is a susceptibility factor, also among the spondyloarthritis, in both clinical forms analyzed.

The *TLR2 rs5743708* is a missense variant which affects the structure of the TLR-2 protein in the intracellular region and generates a nonfunctional protein, due to a replacement of an amino acid arginine for glycine at position 753 of the protein (Arg753Gln) [25–27]. This change in TLR-2 reduces the activation of NF-κB pathway and compromises the intracellular signaling cascade [25, 26]. The recognition of PAMPs by these nonfunctional TLR-2 is impaired, leading to failures in the recognition mechanism of the extracellular pathogens, such as the Gram negative/positive bacteria [25, 26]. However, the consequences of this nonfunctional protein are not restricted only to the recognition of pathogens, since TLR-2 activates inflammation through canonical and noncanonical NF-κB pathway [28].

The NF-κB pathway is an important cellular pathway of innate and adaptive immune response, and influences the expression of many genes involved in the regulation of the major processes of activation of the immune response [28]. And the expression of this factor is a major regulator of inflammation and can be active by TLRs (mainly TLR-2 and TLR-9) [28]. There are increasing evidences that suggest a role of NF-κB signaling in the development of various RD; the main genes associated with RD by genome-wide association studies (GWAS) show a correlation with this transcription factor and the production of proinflammatory cytokines [11].

Niebuhr et al. [29] demonstrated a change in the cytokine profile produced by monocytes in patients with the polymorphism *rs5743708* [29]. Levels of IL-12 and IL-6 cytokines were significantly increased, which could explain the increased inflammation in the skin of patients with atopic dermatitis who presented this *TLR2* mutation [29, 30]. Thus, this polymorphism may also affect the profile of the IL-12 cytokine profile in patients with SpA, since high levels of IL- 12 are found in psoriatic lesions and synovial involvement, and the Th17 inflammatory cascade is supported by high levels of IL-12 and IL-6 [31].

Another pattern of cytokines that is modified by the presence of *rs5743708* is the IL-8 profile; as discussed by Nedoszytko and Renke [25], the Th2 response appears to be enhanced by this polymorphism, impairing IL-8 production and may also affect neutrophil adhesion by an increase in IgE levels [25].

The TLR6 polymorphisms suggest that this variation does not present a direct involvement in the development of SpA. However, the frequencies observed for the rs5743810 in this study for patients and controls (21% and 23%) were higher than the world population frequency of 12% [24].

The two analyzed polymorphisms in the *TLR9* gene promoter region have been linked to autoimmunity and gene transcription rate [25, 32–34]. These polymorphisms are regulatory region variants of the *TLR9* gene; the *C* allele of both rs5743836 and *rs187084* polymorphisms is located in the regions of CTF-binding sites and sites of several transcription factors (TF) [24].

The *rs5743836*C* creates new NF-κB sites in the promoter region of TLR9 gene [25], and in silico, it has been observed that this allele generates new multiple binding sites for different transcription factors [34]. Furthermore, there is an increase in the expression rate of TLR9 mRNA by stimulation of IL-6, creating a positive feedback loop that amplifies TLR9 signaling through IL-6 in the presence of the *rs5743836*T/C* genotype and also interfering in proliferation rate of B cells [34].

Our data suggest that *TLR9 rs5743836*C* is a susceptibility factor regardless of gender or age, increasing by 1.69-fold the susceptibility to SpA. In addition, *rs5743836*T/C* and *rs5743836*C/C* genotypes are an even greater risk factor for patients with PsA. Although there are lacking studies confirming the role of *TLR9 rs5743836* in SpA, there are studies that have previously associated the *rs5743836*C* to the development of rheumatoid arthritis (RA) in women [28]. These data corroborate to ours, once that women with SpA, carrier of *rs5743836*C*, are at a higher risk than men with *rs5743836*C*.

The *rs187084* is associated with increased production of INF-γ and TNF-α in rs187084**T/T* subjects, and the *rs187084*C* increases the transcriptional rates of *TLR9* [28]. This polymorphism has been associated with susceptibility to diseases, and in the present study a susceptibility was observed only for men with *rs187084*T/C*, according to the predisposition already described for men to the development of SpA [28, 35, 36].

Gebura et al. [28] discussed the association among *rs187084*T* and increased IFN-γ and TNF-α productions in RA patients compared to healthy subjects; however, in RA patients, this allele presented a less favorable response to therapies with TNF-α inhibitors [28]. Likewise, patients with PsA do not respond to anti-TNF treatment [37]; this polymorphism in the *TLR9* promoter could be influencing cellular and molecular mechanisms in this disease.

In this study, the rs*187084*, *rs5743836*, and *rs5743708* polymorphisms were associated with the risk of SpA development, wherein, according to the literature, these mutations may lead to significant changes in the innate and adaptive immune response profile, as well as in the maintenance of the regulation of immunological mechanisms. In light of these, we may be inferring that these polymorphisms contribute to potentiate the Th1, Th2, and Th17 immune response seen in SpA, which may confer to individuals carrying the polymorphisms a predisposition to the development of SpA.

5. Conclusions

Our findings suggest that the polymorphisms analyzed for *Toll-like receptor* genes may contribute to the development of the immunopathogenesis of SpA, independently of the presence of antigen HLA-B27.

The polymorphisms analyzed for *Toll-like receptors* genes may contribute to the development of the immunopathogenesis of ankylosing spondylitis and psoriatic arthritis. The polymorphisms *rs5743708* for the *TLR2* and the *rs187084_rs5743836 TLR9* haplotypes appear to be involved in the

development of clinical forms of PsA and can be a possible therapeutic target for the rheumatic diseases. However, further studies are needed to more clearly understand the influence of the immunogenetics of these polymorphisms in the development of SpA, and these observations should be interpreted with caution due to limitations found in this study, such as the relatively small sample size and the fact that there were no analyses of the expression of the *TLR* genes nor of the cytokines involved in the SpA development process.

Data Availability

The authors declare that all the data that support the results of this study are available in the article.

Disclosure

Preliminary data was presented, in part, at the 2016 and 2017 ID Week and 2016 International Society for Disease Surveillance Annual Conferences.

Conflicts of Interest

All authors state that potential conflicts do not exist.

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References

[1] J. Sieper, M. Rudwaleit, M. A. Khan, and J. Braun, "Concepts and epidemiology of spondyloarthritis," *Best Practice & Research Clinical Rheumatology*, vol. 20, no. 3, pp. 401–417, 2006.

[2] S. Lipton and A. Deodhar, "The new ASAS classification criteria for axial and peripheral spondyloarthritis: promises and pitfalls," *International Journal of Clinical Rheumatology*, vol. 7, no. 6, pp. 675–682, 2012.

[3] J. D. Taurog, A. Chhabra, and R. A. Colbert, "Ankylosing spondylitis and axial spondyloarthritis," *The New England Journal of Medicine*, vol. 375, no. 13, article 1303, 2016.

[4] F. Brentano, D. Kyburz, O. Schorr, R. Gay, and S. Gay, "The role of toll-like receptor signalling in the pathogenesis of arthritis," *Cellular Immunology*, vol. 233, no. 2, pp. 90–96, 2005.

[5] T. L. Skare, A. B. Bortoluzzo, C. R. Gonçalves et al., "Ethnic influence in clinical and functional measures of Brazilian patients with spondyloarthritis," *The Journal of Rheumatology*, vol. 39, no. 1, pp. 141–147, 2012.

[6] P. D. Sampaio-barros, V. F. Azevedo, R. Bonfiglioli, and W. R. Campos, "Consenso Brasileiro de Espondiloartropatias : Outras Espondiloartropatias Diagnóstico e Tratamento – Primeira Revisão First update on the Brazilian Consensus for the Diagnosis and Treatment of Spondyloarthropathies : other spondyloarthropathies," *Revista Brasileira de Reumatologia*, vol. 47, no. 4, pp. 243–250, 2007.

[7] L. A. B. Joosten, S. Abdollahi-roodsaz, C. A. Dinarello, L. O. Neill, and M. G. Netea, "Toll-like receptors and chronic inflammation in rheumatic diseases: new developments," *Nature Reviews Rheumatology*, vol. 12, no. 6, pp. 344–357, 2016.

[8] C. Ropert, "How toll-like receptors reveal monocyte plasticity: the cutting edge of antiinflammatory therapy," *Cellular and Molecular Life Sciences*, vol. 76, no. 4, pp. 745–755, 2019.

[9] M. K. Vidya, V. G. Kumar, V. Sejian, M. Bagath, G. Krishnan, and R. Bhatta, "Toll-like receptors: significance, ligands, signaling pathways, and functions in mammals," *International Reviews of Immunology*, vol. 37, no. 1, pp. 20–36, 2018.

[10] K. Vijay, "Toll-like receptors in immunity and inflammatory diseases: Past, present, and future," *International Immunopharmacology*, vol. 59, pp. 391–412, 2018.

[11] M. Kato, "The role of genetics and epigenetics in rheumatic diseases: are they really a target to be aimed at?," *Rheumatology International*, vol. 38, no. 8, pp. 1333–1338, 2018.

[12] A. Arida, A. D. Protogerou, G. D. Kitas, and P. P. Sfikakis, "Systemic inflammatory response and atherosclerosis: the paradigm of chronic inflammatory rheumatic diseases," *International Journal of Molecular Sciences*, vol. 19, no. 7, article 1890, 2018.

[13] M. Rudwaleit, R. Landewé, D. van der Heijde et al., "The development of Assessment of SpondyloArthritis international Society classification criteria for axial spondyloarthritis (part I): classification of paper patients by expert opinion including uncertainty appraisal," *Annals of the Rheumatic Diseases*, vol. 68, no. 6, pp. 770–776, 2009.

[14] V. Chandran, C. T. Schentag, and D. D. Gladman, "Sensitivity and specificity of the CASPAR criteria for psoriatic arthritis in a family medicine clinic setting," *The Journal of Rheumatology*, vol. 35, no. 10, pp. 2069-2070, 2008.

[15] M. Folwaczny, J. Glas, H. Török, O. Limbersky, and C. F. Poliklinik, "Toll-like receptor (TLR) 2 and 4 mutations in periodontal disease," *Clinical and Experimental Immunology*, vol. 135, no. 2, pp. 330–335, 2004.

[16] P. Selvaraj, M. Harishankar, B. Singh, M. S. Jawahar, and V. V. Banurekha, "Toll-like receptor and TIRAP gene polymorphisms in pulmonary tuberculosis patients of South India," *Tuberculosis*, vol. 90, no. 5, pp. 306–310, 2010.

[17] D. M. Cardozo, G. A. Guelsin, S. L. Clementino et al., "Extração de DNA a partir de sangue humano coagulado para aplicação nas técnicas de genotipagem de antígenos leucocitários humanos e de receptores semelhantes à imunoglobulina DNA extraction from coagulated human blood for application in genotyping techniq," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 42, no. 6, pp. 651–656, 2009.

[18] G. C. Oliveira, E. Ambrosio-Albuqerque, and J. E. L. Visentainer, "Application of PCR-SSP method for *HLA-B*27* identification as an auxiliary tool for diagnosis of ankylosing spondylitis," *Jornal Brasileiro de Patologia e Medicina Laboratorial*, vol. 52, no. 4, pp. 217–222, 2016.

[19] A. C. M. Probst, E. P. Bompeixe, N. E. Pereira et al., "HLA polymorphism and evaluation of European, African, and Amerindian contribution to the White and Mulatto populations from Paraná, Brazil," *Human Biology*, vol. 72, pp. 597–617, 2014.

[20] F. C. Parra, R. C. Amado, J. R. Lambertucci, J. Rocha, C. M. Antunes, and S. D. Pena, "Color and genomic ancestry in

Brazilians," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 1, pp. 177–182, 2003.

[21] X. Solé, E. Guinó, J. Valls, R. Iniesta, and V. Moreno, "SNPStats: a web tool for the analysis of association studies," *Bioinformatics*, vol. 22, no. 15, pp. 1928-1929, 2006.

[22] M. G. Netea, C. Wijmenga, and L. A. J. O'Neill, "Genetic variation in Toll-like receptors and disease susceptibility," *Nature Immunology*, vol. 13, no. 6, pp. 535–542, 2012.

[23] M. A. R. Loures, L. C. Macedo, D. M. Reis et al., "Influence of *TNF* and *IL17* gene polymorphisms on the spondyloarthritis immunopathogenesis, regardless of HLA-B27, in a Brazilian population," *Mediators of Inflammation*, vol. 2018, Article ID 1395823, 7 pages, 2018.

[24] P. J. Kersey, J. E. Allen, A. Allot et al., "Ensembl Genomes 2018: an integrated omics infrastructure for non-vertebrate species," *Nucleic Acids Research*, vol. 46, no. D1, pp. D802–D808, 2018.

[25] B. Nedoszytko, M. Lange, J. Renke et al., "The possible role of gene variant coding nonfunctional toll-like receptor 2 in the pathogenesis of Mastocytosis," *International Archives of Allergy and Immunology*, vol. 177, no. 1, pp. 80–86, 2018.

[26] Y. Xiong, C. Song, G. A. Snyder, E. J. Sundberg, and A. E. Medvedev, "R753Q polymorphism inhibits Toll-like receptor (TLR) 2 tyrosine phosphorylation, dimerization with TLR6, and recruitment of myeloid differentiation primary response protein 88," *The Journal of Biological Chemistry*, vol. 287, no. 45, pp. 38327–38337, 2012.

[27] Y. Gao, H. Xiao, Y. Wang, and F. Xu, "Association of single-nucleotide polymorphisms in toll-like receptor 2 gene with asthma susceptibility," *Medicine*, vol. 96, no. 20, article e6822, 2017.

[28] K. Gębura, J. Świerkot, B. Wysoczańska et al., "Polymorphisms within genes involved in regulation of the nf-κb pathway in patients with rheumatoid arthritis," *International Journal of Molecular Sciences*, vol. 18, no. 7, article E1432, 2017.

[29] M. Niebuhr, J. Langnickel, C. Draing, H. Renz, A. Kapp, and T. Werfel, "Dysregulation of toll-like receptor-2 (TLR-2)-induced effects in monocytes from patients with atopic dermatitis: impact of the TLR-2 R753Q polymorphism," *Allergy*, vol. 63, no. 6, pp. 728–734, 2008.

[30] S. Mrabet-Dahbi, A. H. Dalpke, M. Niebuhr et al., "The Toll-like receptor 2 R753Q mutation modifies cytokine production and Toll- like receptor expression in atopic dermatitis," *The Journal of Allergy and Clinical Immunology*, vol. 121, no. 4, pp. 1013–1019, 2008.

[31] R. J. Thibodaux, M. W. Triche, and L. R. Espinoza, "Ustekinumab for the treatment of psoriasis and psoriatic arthritis: a drug evaluation and literature review," *Expert Opinion on Biological Therapy*, vol. 18, no. 7, pp. 821–827, 2018.

[32] R. Lazarus, W. T. Klimecki, B. A. Raby et al., "Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (*TLR9*): frequencies, pairwise linkage disequilibrium, and haplotypes in three U. S. ethnic groups and exploratory case – control disease association studies," *Genomics*, vol. 81, no. 1, pp. 85–91, 2003.

[33] E. Onalan, E. Halit, and E. Salih, "The investigation of toll-like receptor 3, 9 and 10 gene polymorphisms in Turkish rheumatoid arthritis patients," *Rheumatology International*, vol. 31, no. 10, pp. 1369–1374, 2011.

[34] A. Carvalho, N. S. Osório, M. Saraiva et al., "The C allele of rs5743836 polymorphism in the human TLR9 promoter links IL-6 and TLR9 up-regulation and confers increased B-cell proliferation," *PLoS One*, vol. 6, no. 11, article e28256, 2011.

[35] C. Pray, N. I. Irene, and N. N. Haroon, "Bone mineral density and fracture risk in ankylosing spondylitis: a meta-analysis," *Calcified Tissue International*, vol. 101, no. 2, pp. 182–192, 2017.

[36] H. Lin and Y. Gong, "Association of HLA-B27 with ankylosing spondylitis and clinical features of the HLA-B27-associated ankylosing spondylitis: a meta-analysis," *Rheumatology International*, vol. 37, no. 8, pp. 1267–1280, 2017.

[37] D. Wu, J. Yue, and L. Tam, "Efficacy and safety of biologics targeting interleukin-6, -12/23 and -17 pathways for peripheral psoriatic arthritis: a network meta-analysis," *Rheumatology*, vol. 57, no. 3, pp. 563–571, 2018.

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Research Article

Cross-Disease Innate Gene Signature: Emerging Diversity and Abundance in RA Comparing to SLE and SSc

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Overactivation of the innate immune system together with the impaired downstream pathway of type I interferon-responding genes is a hallmark of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc). To date, limited data on the cross-disease innate gene signature exists among those diseases. We compared therefore an innate gene signature of Toll-like receptors (TLRs), seven key members of the interleukin (IL)1/IL1R family, and CXCL8/IL8 in peripheral blood mononuclear cells from well-defined patients with active stages of RA ($n = 36$, DAS28 ≥ 3.2), SLE ($n = 28$, SLEDAI > 6), and SSc ($n = 22$, revised EUSTAR index > 2.25). Emerging diversity and abundance of the innate signature in RA patients were detected: RA was characterized by the upregulation of *TLR3*, *TLR5*, *IL1RAP*/*IL1R3*, *IL18R1*, and *SIGIRR*/*IL1R8* when compared to SSc ($P_{corr} < 0.02$) and of *TLR2*, *TLR5*, and *SIGIRR*/*IL1R8* when compared to SLE ($P_{corr} < 0.02$). Applying the association rule analysis, six rules (combinations and expression of genes describing disease) were identified for RA (most frequently included high *TLR3* and/or *IL1RAP*/*IL1R3*) and three rules for SLE (low *IL1RN* and *IL18R1*) and SSc (low *TLR5* and *IL18R1*). This first cross-disease study identified emerging heterogeneity in the innate signature of RA patients with many upregulated innate genes compared to that of SLE and SSc.

1. Introduction

Rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc) are systemic autoimmune diseases characterized by overactivation of the innate immune system together with impaired downstream pathway of type I interferon- (IFN-) responding genes (IFN signature). Nevertheless, a certain heterogeneity in the IFN signature among those diseases has been recognized, and some patients even lack its presence [1–4].

Although the emerging role of the innate immunity in the pathogenesis of RA, SLE, and SSc has been demonstrated, there is no data on the cross-disease innate gene signature as well as its heterogeneity among those diseases yet. Numerous studies on individual innate immunity members in RA, SLE, and SSc showed the crucial role of Toll-like receptors (TLRs) and IL1 family [5, 6]. Notable examples of common innate pathways are (i) the involvement of the adapter protein MyD88 which is required for signal transduction by TLRs and receptors of the IL1 family, (ii) the activation of

Table 1: Demographic and clinical characteristics of enrolled patients.

	RA ($n = 36$)	SLE ($n = 28$)	SSc ($n = 22$)
Female/male	26/10	24/4	15/7
Age (years) mean (min-max)	57.5 (39-80)	40.1 (19-67)	58.0 (38-77)
Duration of the disease (years) mean (min-max)	18.1 (9-50)	10.0 (1-20)	5.4 (0-21)
Medications (% (n))			
Steroids	89 (32)	82 (23)	96 (21)
NSAIDs	78 (28)	14 (4)	0 (0)
Methotrexate	83 (30)	14 (4)	9 (2)
Other DMARDs*	36 (13)	100 (28)	73 (16)
Biologics	39 (14)	0 (0)	0 (0)
Relative white blood count (%)			
Lymphocytes (mean (95% CI))	24.9 (20.5-29.3)	22.9 (18.5-27.3)	21.4 (17.5-25.4)
Neutrophils (mean (95% CI))	62.9 (57.9-67.9)	67.1 (61.6-72.6)	67.3 (62.5-72.2)
Monocytes (mean (95% CI))	8.9 (7.9-9.9)	8.5 (7.1-9.9)	9.2 (7.9-10.4)

NSAIDs: nonsteroidal anti-inflammatory drugs; DMARDs: disease-modifying antirheumatic drugs; CI: confidence interval. *Other DMARDs taken were hydroxychloroquine (RA/SLE/SSc; $n = 3/26/0$), leflunomide (8/0/0), sulfasalazine (2/0/0), azathioprine (0/8/12), mycophenolate mofetil (0/6/0), cyclophosphamide (0/3/3), and cyclosporine (0/1/1).

the type I IFN, and (iii) the presence of endogenous TLR ligands [7]. Besides shared innate pathways, disease-specific molecular and cellular mechanisms exist. In SLE, recent evidence has suggested a close relationship between the endosomal TLR activation and the disease onset [8, 9] with an essential role of endosomal TLRs in the generation of antinuclear antibodies and type I IFNs [10]. In RA, abundant activation of individual members of TLR and IL1 families was already evidenced with a proposed role for exogenous TLR ligands in the disease onset (i.e., *Proteus* infection of urinary tract, Epstein-Barr virus, and parvovirus B19) and for endogenous ligands in self-sustaining of the inflammatory loop [5, 11]. In SSc, signaling via TLR is increasingly recognized as a key player driving the persistent fibrotic response and is linked to the activity of TGF-β; however, the pathological role of TLRs and their ligands in SSc still remains unclear [12].

We undertook this study to elucidate the underlying differences in the innate immunity signature across three major autoimmune disorders using multivariate analysis. This first cross-disease analysis of the innate gene expression signature of 10 *TLRs*, 7 key members of the *IL1/IL1R* family, and interleukin 8 (*CXCL8*) in peripheral blood mononuclear cells (PBMC) from patients with active SLE, RA, and SSc revealed emerging diversity and abundance in RA compared to SLE and SSc. Our study contributes to further understanding of the innate signature underlying the immunopathology of major autoimmune diseases, with special emphases to discriminate shared and disease-specific expression patterns.

2. Materials and Methods

2.1. Study Subjects. The study cohort consisted of 86 Caucasian patients with autoimmune diseases from a single rheumatology center in Olomouc, Moravia region of Czech Republic. All enrolled RA/SLE/SSc patients met the 2010 ACR/EULAR classification criteria for RA [13], the ACR classification criteria for SLE [14], and the 2013 ACR/EULAR classification criteria for SSc, respectively [15]. To exclude heterogeneity due to the activity and inactivity of the diseases, only cases with active phenotypes of the disease classified according to common activity scores (Disease Activity Score in 28 joints (DAS28), SLE Disease Activity Index (SLEDAI), and revised European Scleroderma Trials and Research group (EUSTAR) index) were included: RA ($n = 36$, DAS28 ≥ 3.2), SLE ($n = 28$, SLEDAI > 6), and SSc ($n = 22$, revised EUSTAR index > 2.25).

The demographic and clinical features, used medication, duration of disease, and relative white blood count are described in Table 1. Distribution of lymphocyte, neutrophil, and monocyte counts did not differ between studied patient's groups ($P > 0.05$). The healthy control cohort consisted of 77 subjects (mean age 51 yrs, min-max 24-90 yrs, female/male 58/19) out of which were formed three age-/gender-matched groups for each disease: 63 controls for RA (mean age 56 yrs, min-max 41-90 yrs, female/male 45/18), 33 controls for SLE (40, 24-50, 27/6, respectively), and 48 controls for SSc (58, 48-90, 34/14, respectively). In all healthy subjects, presence of inflammatory autoimmune diseases in first or second degree relatives, recent vaccination, infection, and usage of immunosuppressive drugs were excluded by questionnaire.

The patients and control subjects provided written informed consent about the usage of peripheral blood for the purpose of this study, which was approved by the ethics committee of the University Hospital and Palacký University Olomouc.

2.2. Sample Processing and Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The PBMC were isolated from the peripheral blood collected in K_3EDTA tubes by Ficoll density gradient centrifugation (Sigma-Aldrich, Germany) and stored in TRI Reagent (Sigma-Aldrich, Germany) at −80°C until analysis. Total RNA was extracted using a Direct-zol RNA kit (Zymo Research, USA) according to the manufacturer's recommendations. After reverse transcription with a Transcriptor First Strand cDNA Synthesis

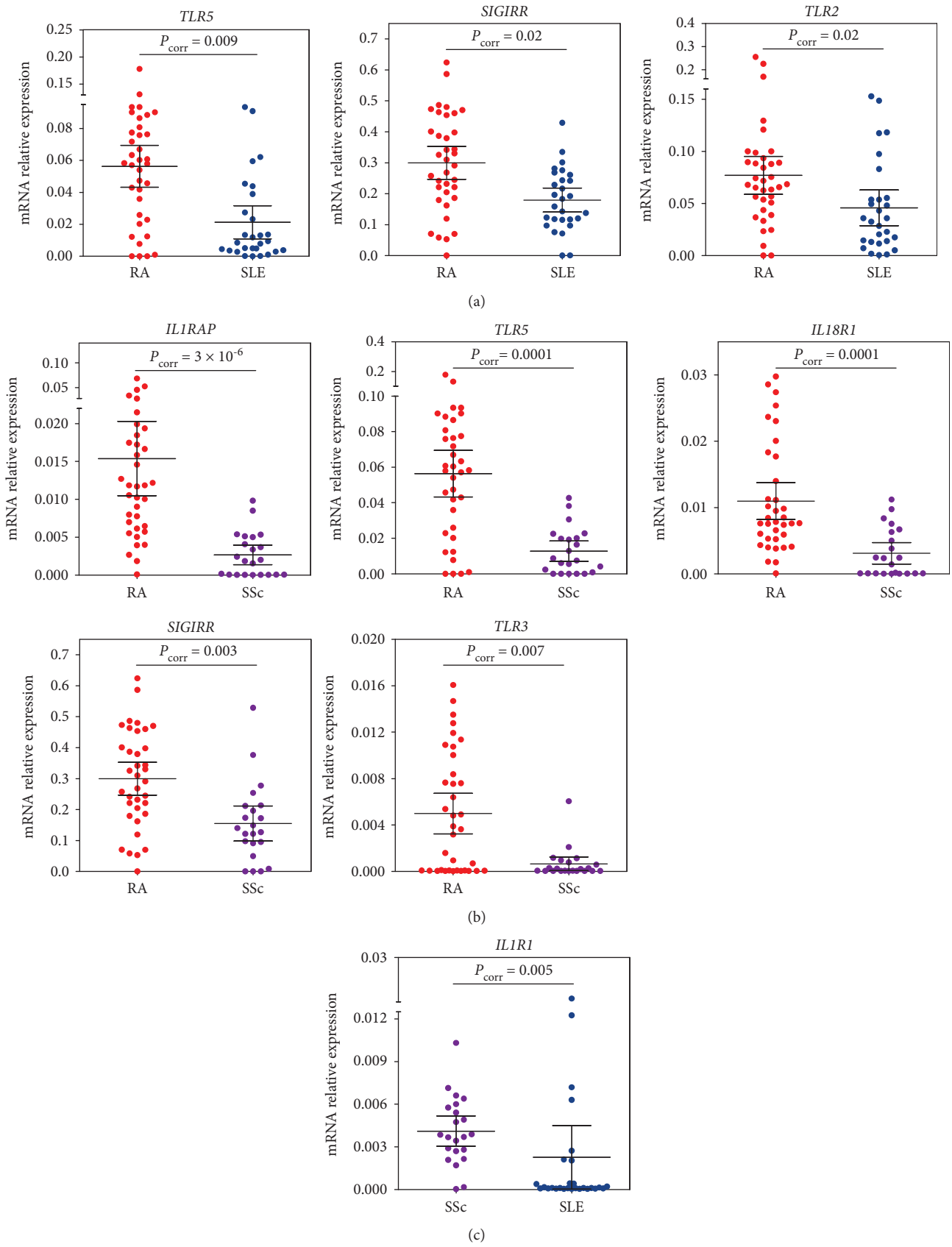

Figure 1: Relative mRNA expression levels of genes differentially expressed in (a) RA vs. SLE, (b) RA vs. SSc, and (c) SSc vs. SLE. Group means are indicated by horizontal bars; error bars indicate 95% CI.

Table 2: Relative mRNA expression levels of genes differentially expressed between (a) RA vs. SLE, (b) RA vs. SSc, (c) SSc vs. SLE.

(a) RA vs. SLE

Gene	Mean (95% CI) RA	Mean (95% CI) SLE	FC	P value	P_{corr}
TLR5	0.056 (0.043-0.070)	0.021 (0.011-0.032)	6.49	5.2×10^{-4}	9.3×10^{-3}
SIGIRR	0.300 (0.247-0.353)	0.179 (0.141-0.218)	1.76	2.0×10^{-3}	2.0×10^{-2}
TLR2	0.077 (0.059-0.095)	0.046 (0.029-0.063)	2.00	3.7×10^{-3}	2.2×10^{-2}

(b) RA vs. SSc

Gene	Mean (95% CI) RA	Mean (95% CI) SSc	FC	P value	P_{corr}
IL1RAP	0.015 (0.011-0.020)	0.003 (0.001-0.004)	6.08	1.7×10^{-7}	3.0×10^{-6}
TLR5	0.056 (0.043-0.070)	0.013 (0.007-0.019)	7.16	1.1×10^{-5}	9.8×10^{-5}
IL18R1	0.011 (0.008-0.014)	0.003 (0.002-0.005)	4.08	2.0×10^{-5}	1.2×10^{-4}
SIGIRR	0.300 (0.247-0.353)	0.155 (0.098-0.211)	2.26	5.9×10^{-4}	2.6×10^{-3}
TLR3	0.005 (0.003-0.007)	0.001 (6.1×10^{-5}-0.001)	28.5	1.8×10^{-3}	6.6×10^{-3}

(c) SSc vs. SLE

Gene	Mean (95% CI) SSc	Mean (95% CI) SLE	FC	P value	P_{corr}
IL1R1	0.004 (0.003-0.005)	0.002 (3.1×10^{-5}-0.004)	34.8	2.7×10^{-4}	4.8×10^{-3}

P_{corr} value corrected for multiple comparisons (Benjamini-Hochberg correction). FC (fold change) between group medians of relative mRNA expression levels.

Kit (Roche, Switzerland), qPCR was performed in a 100 nl reaction volume containing a LightCycler 480 SYBR Green I Master mix (Roche, Switzerland) using a high-throughput SmartChip Real-Time-qPCR System (WaferGen, USA) as reported previously [16, 17]. Primer sequences are listed in Table S1 (Integrated DNA Technologies, USA). The relative mRNA expression was calculated using phosphoglycerate kinase 1 as a reference gene [18].

In order to assess the innate immunity gene expression pattern, the expression of *TLR* (*TLR1-10*), *IL1*/*IL1R* family (21 members), and *CXCL8* was investigated in PBMC. Based on pilot evaluation of qPCR assays on a cohort of 20 RA, 20 SLE, and 20 SSc patients, 14 assays of *IL1*/*IL1R* family members (*IL1A*, *IL36RN*, *IL36A*, *IL36B*, *IL36G*, *IL37*, *IL38*, *IL33*, *IL1R2*, *IL18RAP*, *IL1RL1*, *IL1RL2*, *IL1RAPL1*, and *IL1RAPL2*) were below the limit of detection of the system and thus excluded from further analysis. The study continued therefore by expression profiling of 18 innate immunity genes: *TLR1-10*, 7 members of the *IL1*/*IL1R* family together with *CXCL8*.

2.3. Statistical Analysis and Data-Mining Methods. Statistical analysis (Mann–Whitney U test, Benjamini-Hochberg correction) of relative gene expression values was performed using Genex (MultiD Analyses AB, Sweden) and GraphPad Prism 5.01 (GraphPad Software, USA). P value < 0.05 was considered as significant.

In this study, a set of multivariate data-mining analyses to visualize and characterize the gene expression heterogeneity between and within the diseases was applied. For a flowchart of the analysis process used, see Figure S1.

First, correlation networks using the LRNet algorithm [19] and Spearman's rank correlation coefficient were constructed and visualized to investigate the relationships between expressions of individual studied genes within the innate gene signature and to nominate the most representative molecules for the particular disease.

Second, Andrews curve analysis was applied for visualization of the structure in multidimensional expression data [20–23]. The relative gene expression values of individual patients were transformed using Andrews' formula (Equation S1); all calculations were performed by package Andrews from the R library [24]. The Andrews curves were plotted to visualize the differences between particular diseases using a set of significantly deregulated genes and the whole set of studied genes. The difference is demonstrated by separation of the Andrews curve's amplitudes and phase shift [20, 22, 23]. The curves of similar relative gene expression overlap between studied groups (Figure S2), while separation of curves demonstrates the differences in expression profiles (Figure S3) [20, 22, 23]. More detailed description of the Andrews curve analysis is stated in Supplementary File.

Third, we applied association rule mining, a technique for finding frequent patterns, correlations, or associations among the given data set [25] to investigate the heterogeneity within the diseases themselves. Firstly, each gene data set was divided into low/high expression groups by arithmetic means of relative gene expressions within the whole data

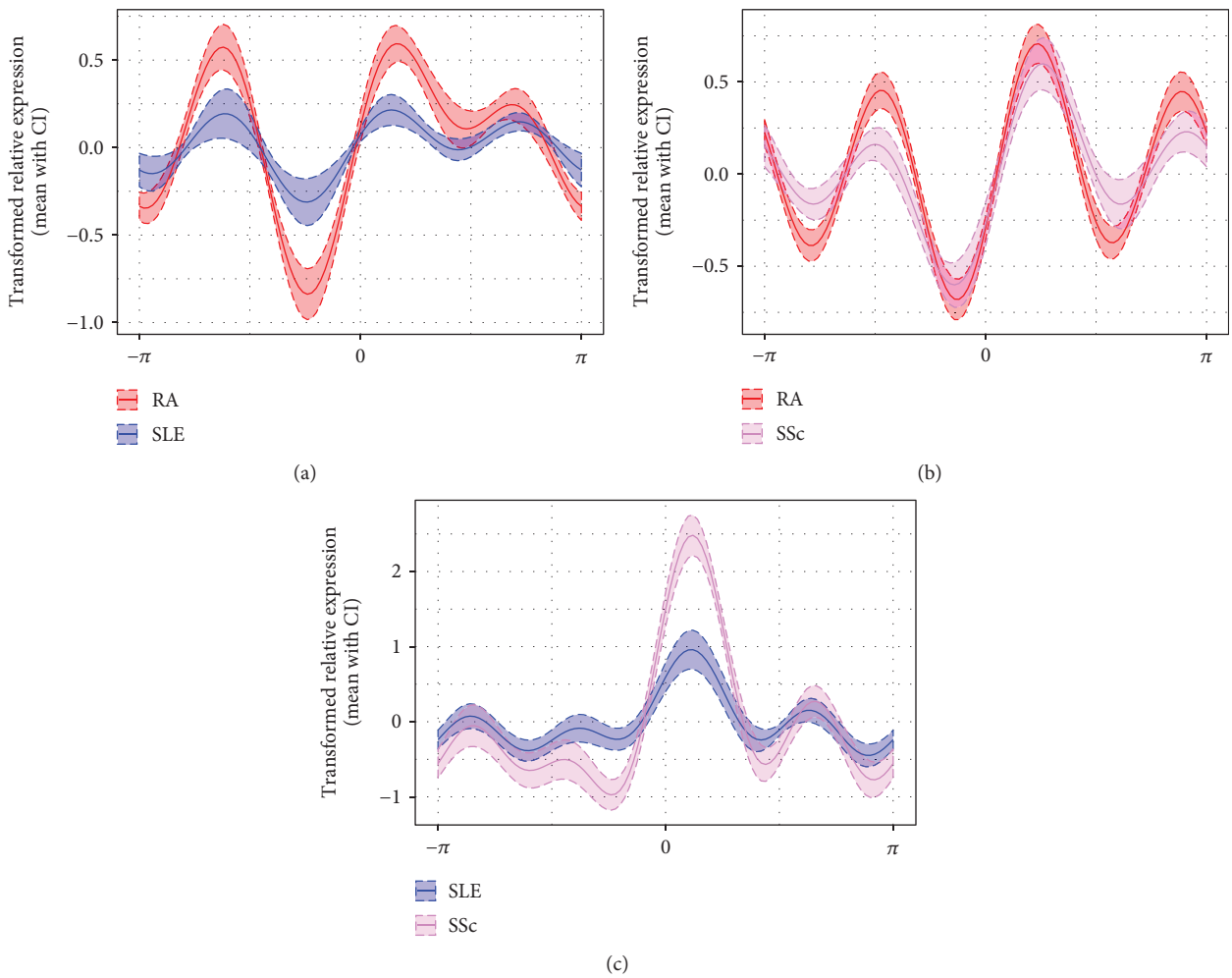

Figure 2: Differential innate gene expression analysis by Andrews curves between (a) RA vs. SLE, (b) RA vs. SSc, and (c) SLE vs. SSc—representative examples. The Andrews curves were calculated for various combinations of gene expression values from the whole set of studied genes. Examples show the results of the Andrews curve analysis for the combination of (a) *TLR3*, *TLR7*, *TLR8*, *IL1R1*, *IL1RN*, and *IL18R1*; (b) *TLR3*, *TLR4*, *TLR6*, *TLR10*, *IL1B*, *IL1R1*, and *SIGIRR*; and (c) *TLR4*, *TLR6*, *TLR7*, *TLR8*, *IL1R1*, *IL1RN*, and *IL18*. For those sets of genes, a good separation of diseases was observed as visualized by separation of the curve's amplitudes and phase shift. An example of combination of genes which does not discriminate between disease groups is shown in Figure S2. Full lines represent the mean values, the dashed lines 95% confidence intervals.

set. The applied package "arules" in the R system [26] was used to extract rules (combinations of genes and its expression levels associated with the particular disease). Only a minimum number of top ranked rules describing the particular disease with a good confidence (threshold 0.75) and support were used.

3. Results

3.1. Innate Immune Gene Expression Pattern of RA, SLE, and SSc. In order to characterize innate immune signature in studied diseases, the expression profiles of selected innate immune genes between patients and healthy controls in all diseases were compared.

To exclude the influence of age on the gene expression, the healthy controls were subdivided into age-matched subgroups despite no differences being observed in the expression profile of all investigated genes in the formed subgroups ($P_{corr} > 0.05$). RA differed from controls by the upregulated expression of *TLR2*, *TLR3*, *TLR5*, *TLR8*, *IL1B*, *IL18*, *IL18R1*, *IL1RN*, *IL1RAP*, and *SIGIRR*/*IL1R8* ($P_{corr} \leq 0.05$; Figure S4A, Table S2A). In patients treated with anti-TNF-α therapy, a trend to lower TLR5 levels in our RA patients was observed ($P = 0.07$). In SLE, downregulation of *TLR10* was observed when compared to healthy controls ($P = 0.02$); however, it did not reach significance after the correction for multiple comparisons (Figure S4B, Table S2B). SSc differed from controls by the upregulated expression of *IL1RN*, *IL18*, and *CXCL8* and downregulated expression of *IL1RAP* and *IL18R1* ($P_{corr} \leq 0.05$; Figure S4C, Table S2C).

3.2. Cross-Disease Analysis of Innate Pattern in RA, SLE, and SSc. To investigate the disease-specific innate immune gene expression pattern, we compared RA, SLE, and SSc patients to each other. RA differed from SLE and SSc by the

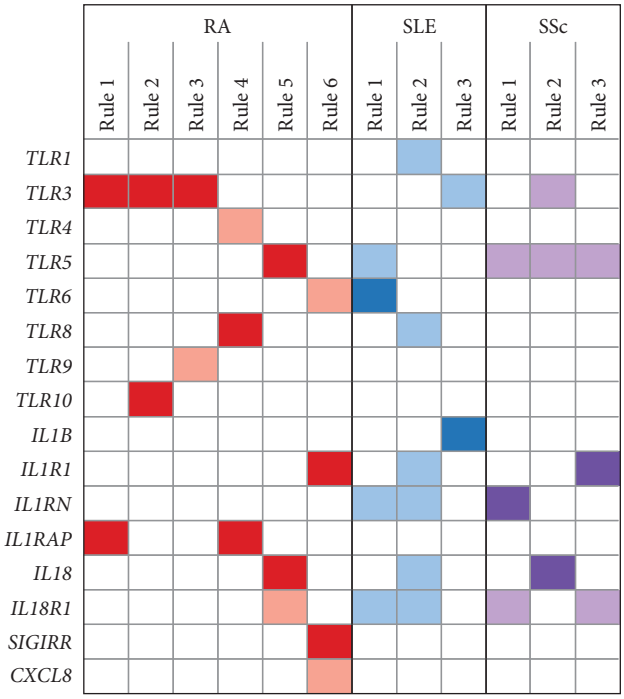

Figure 3: Association rules describing RA, SLE, and SSc. Association rule analysis revealed a minimum of six rules for RA, three rules for SLE, and three rules for SSc, able to discriminate among all studied diseases with the accuracy above 77%. Columns represent individual rules (combinations of genes and its expression levels characterizing the particular disease). Dark/light color means high/low gene expression levels (cut-off: mean gene expression of the whole data set).

upregulated expression of *TLR5* and *SIGIRR* ($P_{corr} < 0.02$; Figures 1(a) and 1(b), Tables 2(a) and 2(b), and Tables S3A and S3B). RA further differed from SLE by the upregulated expression of *TLR2* ($P_{corr} = 0.02$; Figure 1(a), Tables 2(a) and S3A) and from SSc by the upregulation of *TLR3*, *IL1RAP*, and *IL18R1* genes ($P_{corr} < 0.007$; Figure 1(b), Tables 2(b) and S3B). In SSc, the upregulated expression of *IL1R1* ($P_{corr} = 0.005$; Figure 1(c), Tables 2(c) and S3C) was observed when compared to SLE.

3.3. Visualization of Disease-Associated Gene Expression Pattern by Andrews Curves. To investigate the disease-associated gene expression pattern, Andrews curves were used to visualize the differences between particular diseases using a set of significantly deregulated genes and the whole set of studied genes. First, we assessed the differences in the innate expression pattern of genes revealed by classical statistics. Although a good separation of Andrews curves on the basis of significant genes was observed (Figure S3), better separation of the studied diseases was obtained when a whole set of studied genes was used (Figure 2).

3.4. Innate Pattern Characteristics of RA, SLE, and SSc. Next, we applied the association rule analysis to identify rules (set of genes including their expression levels) describing a certain disease within the three studied diseases. Based on the results from the Andrews curves, association rule analysis was performed using the whole gene set.

For RA, six rules were identified, thus showing high heterogeneity within this group of patients when compared to SLE and SSc (Figure 3), where for each of them, three rules were identified. In RA, a high level of *TLR3* and *IL1RAP* mRNA was identified in three and two rules, respectively. In SLE, low expression levels of *IL1RN* and *IL18R1* appeared in two rules, and in SSc, a low level of *TLR5* and *IL18R1* mRNA occurred in three and two rules, respectively. The obtained association rules and their support and confidence values deciphered for RA, SLE, and SSc patients are listed in Table 3. The accuracy of classification by using these rules for RA, SLE, and SSc was 83%, 78%, and 77%, respectively. Comparison of rules for each disease revealed that *TLR3*, *TLR5*, *IL18*, *IL18R1*, and *IL1R1* genes occurred in rules for all studied diseases, showing good discriminant power among studied autoimmune diseases as visualized by the Andrews curves (Figure S5).

4. Discussion

This study focused on the innate immunity gene signature among major autoimmune diseases: RA, SLE, and SSc, showing heterogeneity in the innate signature among and within these diseases. This first cross-disease study showed the highest diversity and abundance in the innate signature in RA when compared to SLE and SSc.

Innate immunity plays a key role in the pathogenesis of autoimmune rheumatic diseases as evidenced from numerous studies on individual members of innate immunity pathways [5, 6]. However, little is known about the similarities and differences in the innate signature at the molecular level between and within these diseases. Therefore, we investigated the differential expression of key innate genes in RA, SLE, and SSc. Importantly, our study was restricted only to the cases with active disease in order to exclude heterogeneity due to the activity and inactivity of the diseases. To obtain a more complex picture, the multivariate analysis was applied to assess the complexity of the differential innate signature having an advantage over classical statistical approaches due to taking into account the intrinsic characteristics of gene expression data and assessing the relationships between studied molecules.

Firstly, we applied Andrews curve analysis for assessment of differences and similarities in the gene innate signature between studied diseases, an approach particularly useful for visualization of the structure in multidimensional data [20, 21]. When using combination of genes reaching statistical significance as well as using the whole gene set, we confirmed the diversity among innate profiles in RA, SLE, and SSc by Andrews curve analysis. Upregulated expression of *TLR3*, *TLR5*, and *SIGIRR* was characteristic for RA when compared to both SLE and SSc. An intracellular receptor TLR3 recognizing dsRNA has been shown to be involved in the RA pathogenesis: necrotic synovial fluid cells release RNA that can activate TLR3 in RA synovial fibroblasts [27]. TLR5, a surface receptor highly upregulated in our RA patients, recognizes bacterial

Table 3: Association rules identified for (a) RA, (b) SLE, and (c) SSc.

No.	Rule	Support	Confidence	Number of patients identified
(a) RA				
1	*TLR3* high & *IL1RAP* high	0.13	1.00	11
2	*TLR3* high & *TLR10* high	0.12	1.00	10
3	*TLR3* high & *TLR9* low	0.12	1.00	10
4	*TLR4* low & *TLR8* high & *IL1RAP* high	0.14	1.00	12
5	*TLR5* high & *IL18* high & *IL18R1* low	0.14	1.00	12
6	*TLR6* low & *IL1R1* high & *SIGIRR* high & *CXCL8* low	0.12	0.91	10
(b) SLE				
1	*TLR5* low & *TLR6* high & *IL1RN* low & *IL18R1* low	0.10	0.90	9
2	*TLR1* low & *TLR8* low & *IL1R1* low & *IL1RN* low & *IL18* low & *IL18R1* low	0.13	0.85	11
3	*TLR3* low & *IL1B* high	0.10	0.82	9
(c) SSc				
1	*TLR5* low & *IL1RN* high & *IL18R1* low	0.10	1.00	9
2	*TLR5* low & *TLR3* low & *IL18* high	0.10	0.82	9
3	*TLR5* low & *IL1R1* high & *IL18R1* low	0.10	0.75	9

The data set for each gene was divided into low/high expression by means of a particular gene expression of the whole data set.

flagellin. However, their endogenous ligand(s) in synovial fluid able to activate TLR5 in RA is(are) still unknown [28, 29]. In line with our results, increased TLR5 in peripheral blood myeloid cells correlated with RA disease activity and TNF-alpha levels [30]. There is also evidence that anti-TNF-α therapy markedly suppress TLR5 expression in RA monocytes [31], a trend which was also observed in our study. Also, the next highly upregulated SIGIRR (IL1R8/TIR8), an orphan receptor required for the anti-inflammatory effects of IL37, has been reported in RA synovial tissue previously [32].

Also, other genes such as *TLR2*, *IL1RAP*, and *IL18R1* from the differential innate signature associated with RA revealed by our analysis were reported in autoimmune conditions previously. In line with our results, abundant TLR2 on monocyte subsets in active RA produced a spectrum of proinflammatory cytokines after stimulation [33]. TLR2 recognizes a wide range of conserved microbial products, probably due to its cooperation with TLR1 or TLR6, as well as its hypothetic ligand HMGB1 released from dying and activated cells [34]. Regarding *IL1RAP* and *IL18R1*, their upregulated expression in RA was reported recently [16] and their downregulation in SSc we report here for the first time. Finally, SSc was characterized by an increase in *IL1R1* in comparison to SLE. The first evidence about critical involvement of IL1R1, an essential mediator for proinflammatory IL1 signaling [35], in fibrotic processes has been already reported in a murine lung injury model [36]. Importantly, data from our cross-disease analysis are in line with previous studies on individual innate members and basic statistical analysis and further highlight the activation of innate immunity in RA when compared to SLE and SSc. The infectious agents and endogenous ligands activating innate receptors leading to a self-sustaining inflammatory loop responsible for chronic and destructive progression in RA need to be further elucidated.

Next, we investigated the differential innate signature among and within the studied diseases by association rule analysis, a method commonly used to uncover the most frequently purchased combinations of items in a market basket analysis. It has been shown that this analysis is highly convenient for gene expression datasets [37, 38] and gives additional information due to preservation of the causality between the gene expression level and phenotype [37]. For RA, six rules were identified, thus showing high heterogeneity within this group of patients when compared to SLE and SSc, where three rules were identified for each of them. In RA, the association rules most frequently included high expression of *TLR3* and/or *IL1RAP*/*IL1R3*, thus again highlighting activation of the innate system in active RA. In SLE, a low expression of *IL1RN* and *IL18R1* and in SSc, a low level of *TLR5* and *IL18R1* occurred ofen in the rules. Applying association rules (combinations of genes describing a particular disease), excellent confidence and accuracy above 77% was achieved for all investigated diseases.

Interestingly, about half of the patients in each disease were characterized by multiple rules, while others were typical by only one gene expression pattern rule. The existence of several innate profile subgroups within RA patients lets us suggest that the heterogeneity in the innate pattern in RA may contribute to various clinical disease manifestations [4, 16], thus deserving future investigation. We also hypothesize that observed heterogeneity in the innate signature may contribute to the heterogeneity in the IFN signature recently reported in RA [4]. Our data further highlighted the application of advanced multivariate data analysis especially for diseases such as SLE, where many clinical phenotypes exist. This may be reflected in the high variability in the expression pattern which might be underestimated by univariate statistics, especially in the case of low abundant genes. Finally, our data points out the involvement of various key innate molecules as

well as the different interplay between individual innate receptors in the studied diseases.

To gain a more complete picture of the innate signature in autoimmune diseases, we report also the differential profile of the innate signature in studied diseases compared to healthy controls. This comparison revealed the upregulation of four members of TLR (*TLR2*, *TLR3*, *TLR5*, and *TLR8*) and six members of the IL1/IL1R family (*IL1B*, *IL1RN*, *IL1RAP*, *IL18R1*, *IL18*, and *SIGIRR*) in RA when compared to healthy controls. In line with our results, deregulation of these genes or their protein products was already registered in RA [16, 30, 32, 39–44]. In SLE, this study showed for the first time downregulation of *TLR10*, a broad negative regulator of TLR signaling [45, 46]. The first evidence about the possible involvement of TLR10 in autoimmunity has been already observed: downregulated *TLR10* expression was reported in PBMC of patients with microscopic polyangiitis [47] as well as RA patients with active disease [16]. In contrast to the murine models of SLE [48], we did not observe increased *TLR7* and *TLR9* expression in our SLE patients. In SSc, our study revealed upregulation of *IL1RN*, *IL18*, and *CXCL8* and downregulation of *IL1RAP* and *IL18R1*. In line with our results, upregulated *IL1RN* mRNA [49], increased IL18 expression in skin biopsies [50], and elevated serum IL8 in patients with scleroderma [51] were reported. Here, we report for the first time downregulation of *IL1RAP* and *IL18R1* in SSc. IL1RAP (IL1R3) is a coreceptor of IL1R1 and is indispensable for the transmission of IL1 signaling [35]. Regarding *IL18R1*, it encodes the α subunit of the IL18 receptor responsible for IL18 binding. The activated receptor then initiates the same signaling pathway as IL1 to activate NF-κB [52]. How these proteins contribute to the SSc pathogenesis deserves future investigations.

The authors are aware of some limitations. The study was performed as a cross-sectional analysis in a real-world setting of patients in different stages of the disease; however, the authors restricted analysis only to patients in the active disease stage in order to obtain a more homogenous cohort. Due to the small number of patients in the subgroups with particular gene patterns revealed by association analysis, the subanalysis of their association with clinical parameters was not performed. Future studies on larger cohorts with well-defined patients would be advisable to further confirm our results.

5. Conclusions

To conclude, this first cross-disease study highlighted the heterogeneous nature among and within RA, SLE, and SSc, with the identification of RA having the highest diversity and abundance in the innate signature when compared to SLE and SSc. Moreover, the results from applied data mining approaches show the importance of a multiple multivariate analysis for better understanding of relationships between individual molecules, especially in highly heterogeneous diseases.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Consent

All subjects have given their written informed consent.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

A.P. and R.F. performed the measurements. M.Sk., A.S., and P.H. provided the samples and clinical data. A.P., M.R., and M.K. analyzed the data. A.P. and E.K. designed the study and wrote the paper. P.H. and F.M. helped with the discussion of the data and revising the paper. E.K. supervised the study. All authors were involved in reviewing the paper and had final approval of the submitted and published versions.

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Supplementary Materials

Methods: description of Andrews curves analysis. Correlation analysis of gene patterns in RA, SLE, and SSc. Table S1: investigated genes and primers used for qRT-PCR. Table S2: relative mRNA expression levels of genes differentially expressed between (A) RA vs. healthy controls, (B) SLE vs. healthy controls, and (C) SSc vs. healthy controls. Table S3: relative mRNA expression levels of genes differentially expressed between (A) RA vs. SLE, (B) RA vs. SSc, and (C) SSc vs. SLE. Figure S1: algorithm flow chart of statistics and advanced data-mining methods used in this study. Figure S2: Andrews curve analysis using a gene expression data which does not discriminate between diseases—representative example. Figure S3: Andrews curves using a set of significantly deregulated genes between (A) RA vs. SLE, (B) RA vs. SSc, and (C) SLE vs. SSc. Figure S4: relative mRNA expression levels of genes differentially expressed in (A) RA vs. healthy controls, (B) SLE vs. healthy controls, and (C) SSc vs. healthy controls. Figure S5: Andrews curves using a set of genes revealed by association rules for discrimination of RA, SLE, and SSc. Figure S6: correlation network for studied innate genes in (A) RA, (B) SLE, and (C) SSc. Equation S1: formula used for computing and plotting of Andrews curves. *(Supplementary Materials)*

References

[1] F. A. H. Cooles, A. E. Anderson, D. W. Lendrem et al., "The interferon gene signature is increased in patients with early treatment-naive rheumatoid arthritis and predicts a poorer response to initial therapy," *The Journal of Allergy and Clinical Immunology*, vol. 141, no. 1, pp. 445–448.e4, 2018.

[2] P. Laurent, V. Sisirak, E. Lazaro et al., "Innate immunity in systemic sclerosis fibrosis: recent advances," *Frontiers in Immunology*, vol. 9, p. 1702, 2018.

[3] K. M. Pollard, G. M. Escalante, H. Huang et al., "Induction of systemic autoimmunity by a xenobiotic requires endosomal TLR trafficking and signaling from the late endosome and endolysosome but not type I IFN," *Journal of Immunology*, vol. 199, no. 11, pp. 3739–3747, 2017.

[4] J. Rodríguez-Carrio, M. Alperi-López, P. López, F. J. Ballina-García, and A. Suárez, "Heterogeneity of the type I interferon signature in rheumatoid arthritis: a potential limitation for its use as a clinical biomarker," *Frontiers in Immunology*, vol. 8, p. 2007, 2018.

[5] L. A. B. Joosten, S. Abdollahi-Roodsaz, C. A. Dinarello, L. O'Neill, and M. G. Netea, "Toll-like receptors and chronic inflammation in rheumatic diseases: new developments," *Nature Reviews Rheumatology*, vol. 12, no. 6, pp. 344–357, 2016.

[6] C. A. Dinarello, "Overview of the IL-1 family in innate inflammation and acquired immunity," *Immunological Reviews*, vol. 281, no. 1, pp. 8–27, 2018.

[7] K. C. M. Santegoets, L. van Bon, W. B. van den Berg, M. H. Wenink, and T. R. D. J. Radstake, "Toll-like receptors in rheumatic diseases: are we paying a high price for our defense against bugs?," *FEBS Letters*, vol. 585, no. 23, pp. 3660–3666, 2011.

[8] T. Celhar and A. M. Fairhurst, "Toll-like receptors in systemic lupus erythematosus: potential for personalized treatment," *Frontiers in Pharmacology*, vol. 5, p. 265, 2014.

[9] Y. W. Wu, W. Tang, and J. P. Zuo, "Toll-like receptors: potential targets for lupus treatment," *Acta Pharmacologica Sinica*, vol. 36, no. 12, pp. 1395–1407, 2015.

[10] R. M. Clancy, A. J. Markham, and J. P. Buyon, "Endosomal Toll-like receptors in clinically overt and silent autoimmunity," *Immunological Reviews*, vol. 269, no. 1, pp. 76–84, 2016.

[11] E. Pretorius, O. O. Akeredolu, P. Soma, and D. B. Kell, "Major involvement of bacterial components in rheumatoid arthritis and its accompanying oxidative stress, systemic inflammation and hypercoagulability," *Experimental Biology and Medicine*, vol. 242, no. 4, pp. 355–373, 2017.

[12] S. Bhattacharyya and J. Varga, "Emerging roles of innate immune signaling and toll-like receptors in fibrosis and systemic sclerosis," *Current Rheumatology Reports*, vol. 17, no. 1, p. 474, 2015.

[13] D. Aletaha, T. Neogi, A. J. Silman et al., "2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative," *Annals of the Rheumatic Diseases*, vol. 69, no. 9, pp. 1580–1588, 2010.

[14] M. C. Hochberg, "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus," *Arthritis and Rheumatism*, vol. 40, no. 9, p. 1725, 1997.

[15] F. van den Hoogen, D. Khanna, J. Fransen et al., "2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative," *Annals of the Rheumatic Diseases*, vol. 72, no. 11, pp. 1747–1755, 2013.

[16] A. Petrackova, P. Horak, M. Radvansky et al., "Revealed heterogeneity in rheumatoid arthritis based on multivariate innate signature analysis," *Clinical and Experimental Rheumatology*, vol. 37, 2019(in press).

[17] T. Tomankova, E. Kriegova, R. Fillerova, P. Luzna, J. Ehrmann, and J. Gallo, "Comparison of periprosthetic tissues in knee and hip joints: differential expression of CCL3 and DC-STAMP in total knee and hip arthroplasty and similar cytokine profiles in primary knee and hip osteoarthritis," *Osteoarthritis and Cartilage*, vol. 22, no. 11, pp. 1851–1860, 2014.

[18] V. R. Falkenberg, T. Whistler, J. R. Murray, E. R. Unger, and M. S. Rajeevan, "Identification of phosphoglycerate kinase 1 (PGK1) as a reference gene for quantitative gene expression measurements in human blood RNA," *BMC Research Notes*, vol. 4, no. 1, p. 324, 2011.

[19] E. Ochodkova, S. Zehnalova, and M. Kudelka, "Graph construction based on local representativeness," in *Computing and Combinatorics. COCOON 2017. Lecture Notes in Computer Science, vol 10392*, Y. Cao and J. Chen, Eds., pp. 654–665, Springer, Cham, 2017.

[20] R. E. Moustafa, "Andrews curves," *WIREs Computational Statistics*, vol. 3, no. 4, pp. 373–382, 2011.

[21] P. Niedzielski, M. Mleczek, A. Budka et al., "A screening study of elemental composition in 12 marketable mushroom species accessible in Poland," *European Food Research and Technology*, vol. 243, no. 10, pp. 1759–1771, 2017.

[22] D. F. Andrews, "Plots of high-dimensional data," *Biometrics*, vol. 28, no. 1, pp. 125–136, 1972.

[23] C. García-Osorio and C. Fyfe, "Visualization of high-dimensional data via orthogonal curves," *Journal of Universal Computer Science*, vol. 11, pp. 1806–1819, 2005.

[24] J. Myslivec, "andrews: Andrews curves. R package version 1.0," 2012, http://CRAN.R-project.org/package=andrews.

[25] R. Agrawal, T. Imielinski, and A. N. Swami, "Mining association rules between sets of items in large databases," in *SIGMOD '93 Proceedings of the 1993 ACM SIGMOD international conference on Management of data*, pp. 207–216, Washington, DC, USA, May 1993.

[26] M. Hahsler, C. Buchta, B. Gruen, and K. Hornik, "arules: Mining Association Rules and Frequent Itemsets. R package version 1.6-1," 2018, https://CRAN.R-project.org/package=arules.

[27] F. Brentano, O. Schorr, R. E. Gay, S. Gay, and D. Kyburz, "RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3," *Arthritis and Rheumatism*, vol. 52, no. 9, pp. 2656–2665, 2005.

[28] S. J. Kim, Z. Chen, N. D. Chamberlain et al., "Angiogenesis in rheumatoid arthritis is fostered directly by Toll-like receptor 5 ligation and indirectly through interleukin-17 induction," *Arthritis and Rheumatism*, vol. 65, no. 8, pp. 2024–2036, 2013.

[29] H. A. Elshabrawy, A. E. Essani, Z. Szekanecz, D. A. Fox, and S. Shahrara, "TLRs, future potential therapeutic targets for RA," *Autoimmunity Reviews*, vol. 16, no. 2, pp. 103–113, 2017.

[30] N. D. Chamberlain, O. M. Vila, M. V. Volin et al., "TLR5, a novel and unidentified inflammatory mediator in rheumatoid arthritis that correlates with disease activity score and joint TNF-α levels," *Journal of Immunology*, vol. 189, no. 1, pp. 475–483, 2012.

[31] S. Kim, Z. Chen, N. D. Chamberlain et al., "Ligation of TLR5 promotes myeloid cell infiltration and differentiation into mature osteoclasts in rheumatoid arthritis and experimental

arthritis," *Journal of Immunology*, vol. 193, no. 8, pp. 3902–3913, 2014.

[32] G. Cavalli, M. Koenders, V. Kalabokis et al., "Treating experimental arthritis with the innate immune inhibitor interleukin-37 reduces joint and systemic inflammation," *Rheumatology*, vol. 55, no. 12, pp. 2220–2229, 2016.

[33] P. Lacerte, A. Brunet, B. Egarnes, B. Duchêne, J. P. Brown, and J. Gosselin, "Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists," *Arthritis Research & Therapy*, vol. 18, no. 1, p. 10, 2016.

[34] H. Aucott, A. Sowinska, H. E. Harris, and P. Lundback, "Ligation of free HMGB1 to TLR2 in the absence of ligand is negatively regulated by the C-terminal tail domain," *Molecular Medicine*, vol. 24, no. 1, p. 19, 2018.

[35] C. A. Dinarello, "Immunological and inflammatory functions of the interleukin-1 family," *Annual Review of Immunology*, vol. 27, no. 1, pp. 519–550, 2009.

[36] P. Gasse, C. Mary, I. Guenon et al., "IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice," *The Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3786–3799, 2007.

[37] S. C. Chen, T. H. Tsai, C. H. Chung, and W. H. Li, "Dynamic association rules for gene expression data analysis," *BMC Genomics*, vol. 16, no. 1, p. 786, 2015.

[38] S. Alagukumar and R. Lawrance, "A selective analysis of microarray data using association rule mining," *Procedia Computer Science*, vol. 47, pp. 3–12, 2015.

[39] M. Iwahashi, M. Yamamura, T. Aita et al., "Expression of Toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 50, no. 5, pp. 1457–1467, 2004.

[40] N. D. Chamberlain, S. J. Kim, O. M. Vila et al., "Ligation of TLR7 by rheumatoid arthritis synovial fluid single strand RNA induces transcription of TNFα in monocytes," *Annals of the Rheumatic Diseases*, vol. 72, no. 3, pp. 418–426, 2013.

[41] C. K. Edwards, J. S. Green, H. D. Volk et al., "Combined anti-tumor necrosis factor-α therapy and DMARD therapy in rheumatoid arthritis patients reduces inflammatory gene expression in whole blood compared to DMARD therapy alone," *Frontiers in Immunology*, vol. 3, p. 366, 2012.

[42] S. Ramírez-Pérez, U. de la Cruz-Mosso, J. Hernández-Bello et al., "High expression of interleukine-1 receptor antagonist in rheumatoid arthritis: association with *IL1RN*2/2* genotype," *Autoimmunity*, vol. 50, no. 8, pp. 468–475, 2017.

[43] X. T. Shao, L. Feng, L. J. Gu et al., "Expression of interleukin-18, IL-18BP, and IL-18R in serum, synovial fluid, and synovial tissue in patients with rheumatoid arthritis," *Clinical and Experimental Medicine*, vol. 9, no. 3, pp. 215–221, 2009.

[44] Q. Q. Huang, Y. Ma, A. Adebayo, and R. M. Pope, "Increased macrophage activation mediated through Toll-like receptors in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 56, no. 7, pp. 2192–2201, 2007.

[45] M. Oosting, S. C. Cheng, J. M. Bolscher et al., "Human TLR10 is an anti-inflammatory pattern-recognition receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 42, pp. E4478–E4484, 2014.

[46] S. Jiang, X. Li, N. J. Hess, Y. Guan, and R. I. Tapping, "TLR10 is a negative regulator of both MyD88-dependent and -independent TLR signaling," *Journal of Immunology*, vol. 196, no. 9, pp. 3834–3841, 2016.

[47] Y. Lai, C. Xue, Y. Liao et al., "Expression profiles of toll-like receptor signaling pathway related genes in microscopic polyangiitis in Chinese people," *International Journal of Clinical and Experimental Pathology*, vol. 9, pp. 5515–5524, 2016.

[48] T. Celhar, H. Yasuga, H. Y. Lee et al., "Toll-like receptor 9 deficiency breaks tolerance to RNA-associated antigens and up-regulates Toll-like receptor 7 protein in Sle1 mice," *Arthritis & Rhematology*, vol. 70, no. 10, pp. 1597–1609, 2018.

[49] F. K. Tan, X. Zhou, M. D. Mayes et al., "Signatures of differentially regulated interferon gene expression and vasculotrophism in the peripheral blood cells of systemic sclerosis patients," *Rheumatology*, vol. 45, no. 6, pp. 694–702, 2006.

[50] M. A. Martínez-Godínez, M. D. Cruz-Domínguez, L. J. Jara et al., "Expression of NLRP3 inflammasome, cytokines and vascular mediators in the skin of systemic sclerosis patients," *The Israel Medical Association Journal*, vol. 17, pp. 5–10, 2015.

[51] H. Ihn, S. Sato, M. Fujimoto, K. Kikuchi, and K. Takehara, "Demonstration of interleukin 8 in serum samples of patients with localized scleroderma," *Archives of Dermatology*, vol. 130, no. 10, pp. 1327-1328, 1994.

[52] G. Kaplanski, "Interleukin-18: biological properties and role in disease pathogenesis," *Immunological Reviews*, vol. 281, no. 1, pp. 138–153, 2018.

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Research Article

The Role of TLR4 Asp299Gly and TLR4 Thr399Ile Polymorphisms in the Pathogenesis of Urinary Tract Infections: First Evaluation in Infants and Children of Greek Origin

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Urinary tract infections are one of the most common and serious bacterial infections in a pediatric population. So far, they have mainly been related to age, gender, ethnicity, socioeconomic level, and the presence of underlying anatomical or functional, congenital, or acquired abnormalities. Recently, both innate and adaptive immunities and their interaction in the pathogenesis and the development of UTIs have been studied. The aim of this study was to assess the role and the effect of the two most frequent polymorphisms of TLR4 Asp299Gly and Thr399Ile on the development of UTIs in infants and children of Greek origin. We studied 51 infants and children with at least one episode of acute urinary tract infection and 109 healthy infants and children. We found that 27.5% of patients and 8.26% of healthy children carried the heterozygote genotype for TLR4 Asp299Gly. TLR4 Thr399Ile polymorphism was found to be higher in healthy children and lower in the patient group. No homozygosity for both studied polymorphisms was detected in our patients. In the group of healthy children, a homozygote genotype for TLR4 Asp299Gly (G/G) as well as for TLR4 Thr399Ile (T/T) was showed (1.84% and 0.92 respectively). These results indicate the role of TLR4 polymorphism as a genetic risk for the development of UTIs in infants and children of Greek origin.

1. Introduction

Urinary tract infections (UTIs) are one of the most common and potentially important and serious bacterial infections in a pediatric population affecting approximately 2.6-7.5% of febrile children annually [1]. Up to 11% of girls and 7% of boys will experience UTI by the age of 16 years, and the recurrence of the infection seems to happen very often (13–19%) [2, 3]. Although the prognosis of a single episode of febrile UTI is usually good, major concerns are related to the risk of permanent renal injury, which may predispose to hypertension and renal insufficiency in some patients [4, 5]. Identifying children at major risk could therefore allow for the implementation of preventive measures to preserve the final renal outcome in these children.

In the past, UTIs have mainly been related to age, gender, ethnicity, socioeconomic level, and the presence of underlying anatomical or functional, congenital, or acquired abnormalities. Recently, the function of innate immunity in the control of UTIs was studied. The need to clarify the role of the host (uroepithelium) response to recognize uropathogenic strains and the local immune response against their invasion has made the study of new risk factors imperative. The discovery of Pattern Recognition Receptors (PRRs) introduces the research of the role of innate immunity in relation to UTIs. PRRs are important

immunologic biosensors that detect pathogens within the host's cells and tissues by recognizing their structural components (PAMPS) [6, 7].

Toll-like receptors (TLRs) are the best studied PRRs. Among them, 13 TLR family members recognize products of a variety of invading antigens. The TLR4 is a 224 amino acid protein that is encoded by the TLR4 gene in humans (Gene ID = 7099; 9q33.1), which spans a genomic region of ~13.3 kb with three exons (NCBI; https://www.ncbi.nlm.nih.gov/). TLR4 is widely distributed on the cell surface of many cells, mainly monocytes, neutrophils, and epithelial cells such as those of uroepithelium. Several simple nucleotide polymorphisms (SNPs) have been identified in the TLR4 gene with some of them being strongly associated with an increased susceptibility to Gram-negative bacterial infections and an increased incidence of septicemia [8].

The two most studied SNPs of TLR4 in animal models and in vitro studies are TLR4 Asp299Gly and TLR4 Thr399Ile. Specifically, in the Asp299Gly polymorphism, we have an A to G transition (SNP ID = rs4986790), resulting in aspartate-glycine substitution at position 299 and, in the TLR4Thr399Ile polymorphism, a C to T transition (SNP ID = rs4986791), resulting in threonine-isoleucine substitution at position 399 [9, 10]. These two nonsynonymous SNPs have been described with population frequencies > 5%. They are identified in human populations more often compared to most of other SNPs with low frequencies (<1%) [11]. Therefore, the study of TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms is of enormous clinical and therapeutic interest, as they could provide new aspects and more efficient approaches in the treatment and prevention of UTIs in children. The aim of this study was to conduct a genetic study of the TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms in a population of infants and children of Greek origin and to assess their role and relation to UTIs. The secondary aim was to compare demographic, laboratory, and clinical parameters between patients, carriers, and noncarriers of the polymorphisms.

2. Materials and Methods

2.1. Study Design and Populations. This is a case-control study in a population of Greek origin. In total, 160 infants and children were included. We studied 51 infants and children (19 males, 32 females) with at least one episode of acute urinary tract infection (AUTI) in their records, with or without underlying anatomic genitourinary anomalies (average age at entry to the study is 3.27 ± 3.20). We also studied 109 phenotypically healthy infants and children (average age at entry to the study is 6.65 ± 4.57). The latter group of children had no history of severe or chronic illness and of UTI, no symptoms of acute infection, and negative urine culture at the time of sample collection.

All patients who participated in the study should meet the following criteria: (a) Those suffering from lower urinary tract infection (cystitis) should have significant bacteriuria of a single microorganism (10^5 CFU mL^{-1}), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) at normal levels (ESR < 20 mm/h and CRP < 0.8 mg/dL, respectively), symptoms of dysuria, polyuria and/or abdominal pain, and absence of fever [12, 13]. (b) Those suffering from upper urinary tract infection (pyelonephritis) should have a urine white blood cell count of ≥25 cells per μL (1+ with a dipstick) and significant bacteriuria of a single microorganism (10^5 CFU mL^{-1}), plus the presence of ≥2 of the following criteria: fever ≥ 38°C, high levels of ESR and/or CRP or neutrophil levels above normal values for age [10, 11].

Patients formed Group A which was subsequently divided in two subgroups: Group A1 (35/51 patients)—infants and children diagnosed with lower UTI, and Group A2 (16/51 patients)—infants and children diagnosed with upper UTI. All patients were hospitalized at the Pediatric Clinic, Fourth Department of Pediatrics, Faculty of Medicine, Papageorgiou General Hospital, Aristotle University of Thessaloniki. The control group (Group C) consisted of healthy infants and children who were followed up at the General Pediatrics Outpatient Unit of the Fourth Department of Pediatrics.

In all patients and 79/109 of Group C, personal history was recorded, a detailed clinical examination was conducted, and a blood sample was collected for the determination of biochemical and immunological parameters as well as a urine sample. They had complete blood count, measurement of urea and creatinine, ESR, CRP, urine culture, and urine microbiological analysis. A complete immunological profile was also evaluated in every child including immunoglobulins: IgG IgA, IgM, IgG subclasses (IgG_1, IgG_2, IgG_3, IgG_4), and complement components C3 and C4. In all patients, kidney ultrasound was performed. In all 160 children of the study, genetic testing was performed to determine the TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms. Before entering the study, an informed consent was obtained by parents or guardians of the children. The research protocol was declared at the service of ClinicalTrials.gov and approved by the Ethics Committee of the Faculty of Medicine of Aristotle University of Thessaloniki. The study was conducted according to the criteria of the Declaration of Helsinki.

2.2. TLR4 Asp299Gly and Thr399Ile Genotyping

2.2.1. Genomic Isolation of Deoxyribonucleic Acid (DNA). Whole blood was collected into a sterile vacutainer containing anticoagulant ethylenediaminetetraacetic acid (EDTA). Molecular genetic analysis was carried out on genomic DNA extracted from EDTA using according to the manufacturer's instructions.

2.2.2. Genomic DNA Isolation and Polymerase Chain Reaction (PCR). Genomic DNA was extracted from the whole blood using Invisorb Spin Blood Mini Kit (STRATEC Molecular GmbH, Germany) following the manufacturer's instructions. The 249 bp gene fragment carrying the TLR4 Asp299Gly mutation was amplified from whole genomic DNA using the 5′-GATTAGCATACTTAGACTACTACC TCCATG-3′ and 5′-GATCAACTTCTGAAAAGCATT CCCAC-3 forward and reverse primers, respectively, and

Table 1: Demographic data of the children of the study.

	Group A (patients)	Group A1	Group A2	Group C (controls)
n	51	35	16	109
Age (years) Mean ± SD	3.27 ± 3.20	3.73 ± 3.70	2.34 ± 2.12	6.65 ± 4.57
p				0.001
Males	19	17	2	55
Females	32	18	14	54
p				0.017

the 407 bp fragment carrying the TLR4 Thr399Ile mutation was amplified using the 5′-GGTTGCTGTTCTCAAAGTG ATTTTGGGAGAA-3′ and 5′-ACCTGAAGACTGGAGA GTGAGTTAAATGCT3′ forward and reverse primers, respectively. PCR primers were designed to allow a distinction of wild-type and mutant TLR4 alleles based on the presence of restriction enzyme recognition sites. PCR was performed as follows: 200-300 ng of genomic DNA, 1.5 mM $MgCl_2$, 400 mM dNTPs, 20 pM forward primer, 20 pM reverse primer, and 1.5 U Taq polymerase (AmpliTaq, Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 51°C and 45 seconds at 72°C, and final extension at 72°C for 10 minutes. All PCR reactions were carried out in a Prime Thermal Cycler (Techne, Stone, Staffordshire, UK). PCR products were visualized in a 2% agarose gel.

2.2.3. Restriction Fragment Length Polymorphism Method (RFLP). The presence of TLR4 gene mutations was determined by RFLP. The 249 bp PCR product was cut with NcoI to determine the presence of TLR4 Asp299Gly mutation, and the 407 bp PCR product was cut with HinfI to determine for the presence of Thr399Ile mutation, as described by Lorenz et al. [14]. Restriction digest products were visualized on a 3.5% agarose gel.

2.2.4. Electrophoresis. Subjects were subjected to electrophoresis on gels containing a mixture of 3% and 3.5% agarose gel.

2.3. Statistical Analysis. Data were analyzed using the IBM statistical program Statistical Package for Social Science software for Windows, version 22.0 (SPSS Inc., Chicago, Illinois, USA). Expected and observed frequencies of genotypes and alleles in patients and controls were compared in 2×3 and 2×2 tables, respectively, according to the Hardy-Weinberg equilibrium. The differences in frequencies of genotypes and alleles between patients and controls and between patient subgroups based on qualitative variables were analyzed by the χ^2 or Fisher exact test. Normal distribution of quantitative variables was examined using the Kolmogorov-Smirnov test. Normally distributed mean age at diagnosis was analyzed according to genotype analysis using the analysis of variance (ANOVA). The level of statistical significance was defined at $p < 0.05$.

Table 2: Clinical data of the patients.

	Group A (patients) Total ($n = 51$)	Gender (M/F) Females ($n = 32$)	Males ($n = 19$)
Lower UTI (Group A1)	35	18	17
Upper UTI (Group A2)	16	14	2
1 episode of UTI	41	28	13
≥2 episodes of UTI	10	4	6
Gram-/Gram+	51/0	32/0	19/0
US (+/-)	13/38	7/25	6/13

UTI: urinary tract infection; US: ultrasound.

3. Results

3.1. Demographic and Clinical Features of the Study Groups. The demographic and the clinical features of Group A (patients), Subgroups A1 and A2 (patients), and Group C (controls) are shown in Tables 1 and 2. Fifty-one patients were evaluated. Thirty-five patients were diagnosed with lower UTI and sixteen with upper UTI. For forty-one patients, that was their first episode of UTI while ten patients suffered from UTI twice or more. Girls developed UTI more frequently than boys. Most of the episodes of the UTIs concerned the lower urinary tract in equal proportion between boys and girls. Furthermore, girls developed upper UTI more often compared to boys.

Urine cultures of all patients showed Gram-negative bacteria with *E. coli* superiority in 43/51 patients (84.3%). The renal ultrasound revealed that 13/51 of the examined patients (25.5%) had pathological findings.

3.2. Laboratory Data of the Study Groups. Laboratory data are shown in Table 3. The total number of the leukocytes, the absolute count of the neutrophils, and the inflammatory markers (ESR, CRP) were indicatively increased in patients than in healthy children (79/109), as expected. Finally, from the analysis of the immunological parameters (immunoglobulins IgG, IgA, and IgM and subclasses of IgG), no primary immunodeficiency was detected in any of the study subjects.

3.3. Study of TLR4 Polymorphisms. In this study, the distribution of genotypes in both patients and healthy subjects did not differ significantly from the expected Hardy-Weinberg equilibrium ($p = 0.987$ and $p = 0.959$, respectively). The TLR4 Asp299Gly genotype was detected more

Table 3: Laboratory data of the study groups.

Parameters	Patients ($N = 51$) Mean ± SD	Controls ($N = 79$) Mean ± SD	p (t-test)
Leukocytes ($\times 10^3$/u)	14621 ± 5761	9308 ± 2719	<0.001
Neutrophils (%)	55.8 ± 16.38	49.9 ± 15.71	<0.001
Lymphocytes (%)	31.7 ± 14.73	37.54 ± 13.86	<0.001
Absolute neutrophil count	8270 ± 4598	4657 ± 2278	<0.001
IgG (g/L)	7.03 ± 3.38	10.07 ± 2.55	<0.001
IgA (g/L)	0.60 ± 0.50	1.11 ± 0.71	<0.001
IgM (g/L)	0.91 ± 0.47	1.15 ± 1.12	0.419
IgG1 (g/L)	4.91 ± 2.69	6.84 ± 1.91	<0.001
IgG2 (g/L)	1.30 ± 0.83	2.13 ± 1.32	<0.001
IgG3 (g/L)	0.29 ± 0.17	0.41 ± 0.18	<0.001
IgG4 (g/L)	0.40 ± 0.40	1.12 ± 0.96	<0.001
C3 (g/L)	1.42 ± 0.62	1.14 ± 0.24	<0.001
C4 (g/L)	0.23 ± 0.10	0.18 ± 0.50	0.004
CRP (mg/dL)	6.36 ± 6.26	0.50 ± 0.42	<0.001
ESR (mm)	45.75 ± 35.09	9.57 ± 6.41	<0.001
Serum urea (mg/dL)	22.29 ± 6.94	27.27 ± 7.18	<0.001
Serum creatinine (mg/dL)	0.47 ± 0.09	0.54 ± 0.099	<0.001

Table 4: The distribution of TLR4 Asp299Gly and Thr399Ile polymorphisms in patients and controls.

Frequency (%)	Patients		Controls	
	Group A ($N = 51$)	Group A1 ($N = 35$)	Group A2 ($N = 16$)	Group C ($N = 109$)
TLR4 Asp299Gly	Genotype			
AA	37 (72.5)	25 (71.4)	12 (75.0)	98 (89.9)
AG	14 (27.5)	10 (28.6)	4 (25.0)	9 (8.26)
GG	0 (0.00)	0 (0.00)	0 (0.00)	2 (1.84)
p	0.02446			
Allele				
A	88 (0.86)	60 (0.86)	28 (0.88)	205 (0.94)
G	14 (0.14)	10 (0.14)	4 (0.12)	13 (0.06)
p	0.06361			
TLR4 Thr399Ile	Genotype			
CC	50 (98.04)	34 (97.1)	16 (100)	96 (88.07)
CT	1 (1.96)	1 (2.9)	0 (0.00)	12 (11.01)
TT	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.92)
p	0.3465			
Allele				
C	101 (0.99)	69 (0.99)	32 (1)	(0.94)
T	1 (0.01)	1 (0.01)	0 (0.00)	(0.06)
p	0.542			

frequently in patients (27.5%) compared to controls (10.1%) at a statistically significant level ($p = 0.02$) (Table 4). The TLR4 Thr399Ile genotype was more frequent in the control group (11.93%) than in the patient group (1.96%), but at a nonstatistically significant level ($p = 0.34$) (Table 4). The copredominant model of inheritance emerged for the TLR4 Asp299Gly genotype and the predominant model for the TLR4 Thr399Ile genotype, as the most probable reaching a statistical significance ($p < 0.05$).

The distribution of TLR4 Asp299Gly and TLR4 Thr399Ile genotypes carrying the predisposing allele did not differ significantly between males and females in the patient group as well as compared with the control group (Table 5). No statistically significant association of the TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms as far as the number of the episodes of the UTIs in any of the subgroups of patients (A1, A2) was found (Table 6).

Although 13/51 (25.5%) of the examined patients had abnormal kidney ultrasound findings, only 3 patients heterozygous for the TLR4 Asp299Gly polymorphism were among them. Therefore, no statistically significant association of the TLR4 Asp299Gly polymorphism with abnormal findings in kidney ultrasound was found (Table 6). However, the limitation of this comparison is the small number of the patients' groups examined. No statistically significant association was found between the studied polymorphisms and the total number of leukocytes, the percentage of neutrophils and lymphocytes, the inflammatory markers (ESR, CRP), and the values of urea and creatinine; IgG, IgA, and IgM immunoglobulins; the subclasses of IgG; and the complements C3 and C4 (Tables 7 and 8).

4. Discussion

Urinary tract infection is one of the most usual bacterial infections in infancy and childhood that occurs in 8.4% of girls and 1.7% of boys under the age of 6 years [15]. The most commonly isolated organism in infants and children with urinary tract infections is by far *E. coli* with a high rate of 80-90% followed by *Enterococcus* species, *Enterobacter*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis*, and *Staphylococcus spp.* [16].

The urinary tract is continuously exposed to microorganisms and pathogens mainly coming from the intestinal tract. It resists by developing defense mechanisms that represent mainly functions of innate immunity. Uroepithelium arises barriers, especially when it encounters infections of the lower urinary tract. For this reason, the role of innate immunity is the best studied regarding the pathogenesis of urinary tract infections compared to that of adaptive immunity.

Over the last 20 years, researchers have studied both innate and adaptive immunities and their interaction in the pathogenesis and the development of UTIs. It is suggested that genetic factors are capable of interfering with any step during the process of bacteria invasion and regulate the inflammatory response before, during, and after UTI. These factors are different gene products such as cytokines, receptors, and adhesion molecules. TLRs are receptors expressed

Table 5: The distribution of TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms in patients and controls according to gender.

SNPs	Genotype	Gender n (%)							
		Group A ($N = 51$)		Group A1 ($N = 35$)		Group A2 ($N = 16$)		Group C ($N = 109$)	
		Males	Females	Males	Females	Males	Females	Males	Females
TLR4 Asp299Gly	AA	15 (79)	22 (69)	14 (82)	12 (67)	1 (50)	10 (71)	50 (90)	48 (89)
	AG + GG	4 (21)	10 (31)	3 (18)	6 (33)	1 (50)	4 (29)	5 (10)	6 (11)
	p	0.4301		0.2886		0.5408		0.7263	
TLR4 Thr399Ile	CC	18 (95)	32 (100)	16 (94)	18 (100)	2 (100)	14 (100)	51 (93)	45 (83)
	CT + TT	1 (5)	0	1 (6)	0	0	0	4 (7)	9 (17)
	p	0.19		0.2965		NA		0.1303	

Table 6: Analysis of the relation between genotypes and the number of urinary tract infections and findings of the ultrasound.

SNPs	Genotypes	Number of episodes					
		Group A ($N = 51$)		Group A1 ($N = 35$)		Group A2 ($N = 16$)	
		1	≥2	1	≥2	1	≥2
TLR4 Asp299Gly	AA	29 (70.7)	7 (70)	19 (73)	6 (66.7)	11 (73.3)	2 (100)
	AG + GG	12 (29.3)	3 (30)	7 (27)	3 (33.3)	4 (26.7)	0 (0)
	p	0.9637		0.713		0.4036	
TLR4 Thr399Ile	CC	40 (97.6)	10 (100)	25 (96.2)	9 (100)	15 (100)	1 (100)
	CT + TT	1 (2.4)	0 (0)	1 (3.8)	0 (0)	0 (0)	0 (0)
	p	0.6179		0.5505		NA	
		Findings of the ultrasound					
SNPs	Genotypes	Group A ($N = 51$)		Group A1 ($N = 35$)		Group A2 ($N = 16$)	
		Normal (%)	Pathological (%)	Normal (%)	Pathological (%)	Normal (%)	Pathological (%)
TLR4 Asp299Gly	AA	27 (71)	10 (77)	19 (73)	6 (66.7)	8 (66.7)	4 (100)
	AG + GG	11 (29)	3 (23)	7 (27)	3 (33.3)	4 (33.3)	0 (0)
	p	0.4961		0.7137		0.1824	
TLR4 Thr399Ile	CC	37 (97.3)	13 (100)	25 (96.1)	8 (100)	12 (100)	4 (100)
	CT+TT	1 (2.7)	0 (0)	1 (3.9)	0 (0)	0 (0)	0 (0)
	p	0.5547		0.5734		NA	

Table 7: Analysis of the relation between genotypes and WBC, neutrophils, lymphocytes, ESR, CRP, serum urea, and serum creatinine.

SNPs	Genotypes	Mean ± SD (n)						
		WBC	Neutrophils (%)	Lymphocytes (%)	ESR (mm)	CRP (mg/dL)	Serum urea (mg/dL)	Serum creatinine (mg/dL)
TLR4 Asp299Gly	AA	15165 ± 5986	54.84 ± 17.19	32.62 ± 15.13	48.97 ± 34.13	6.44 ± 5.94	21.95 ± 7.29	0.46 ± 0.10
	AG + GG	13180 ± 5035	58.49 ± 14.27	29.39 ± 13.85	34.33 ± 29.87	6.6 ± 6.13	23.21 ± 6.06	0.49 ± 0.09
	p	0.286*	0.506*	0.540*	0.165*	0.950*	0.325*	0.212*
TLR4 Thr399Ile	CC	14678 ± 5805	56.27 ± 16.25	31.25 ± 14.45	45.76 ± 33.45	7.85 ± 6.5	22.28 ± 7.01	0.47 ± 0.10
	CT + TT	11720	34.3	56.3	-	0.39	23	0.53
	p	0.634*	0.154*	0.135*	N/A	0.248*	0.785*	0.377*

*Kruskal-Wallis Test. WBC: white blood cells; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

either on the surface of many cells in the blood or on the surface of epithelial cells, such as uroepithelium. They play a pivotal role in the identification of infectious factors and the rapid activation of signaling pathways for the elimination of microbial pathogens or the mobilization of adaptive immunity. It is perceived that TLRs are acting as a connecting bridge between innate and adaptive immunities. In the last 10 years, researchers have focused on polymorphisms or polymorphism combinations of genes coding for TLRs [7, 17].

Table 8: Analysis of the relation between genotypes and immunoglobulins, IgG subclasses, C3, and C4.

SNPs	Genotypes	Mean ± SD (*n*)								
		IgG (g/L)	IgA (g/L)	IgM (g/L)	IgG1 (g/L)	IgG2 (g/L)	IgG3 (g/L)	IgG4 (g/L)	C3 (g/L)	C4 (g/L)
TLR4 Asp299Gly	AA	6.72 ± 3.00	0.54 ± 0.50	0.91 ± 0.44	4.82 ± 2.43	1.23 ± 0.70	0.27 ± 0.12	0.51 ± 0.40	1.43 ± 0.45	0.23 ± 0.07
	AG + GG	7.89 ± 3.51	0.76 ± 0.48	0.89 ± 0.48	5.14 ± 2.29	1.45 ± 0.96	0.35 ± 0.22	0.39 ± 0.34	1.52 ± 0.44	0.22 ± 0.05
	p	0.347*	0.101*	0.709*	0.634*	0.542*	0.277*	0.283*	0.488*	0.888*
TLR4 Thr399Ile	CC	7.07 ± 3.17	0.60 ± 0.51	0.90 ± 0.45	4.93 ± 2.39	1.31 ± 0.78	0.29 ± 0.16	0.47 ± 0.40	1.42 ± 0.45	0.22 ± 0.06
	CT + TT	5.210	0.530	1.050	4.020	0.860	0.340	0.180	1.540	0.329
	p	0.572*	0.944*	0.572*	0.735*	0.735*	0.598*	0.970*	0.590*	0.190*

*Kruskal-Wallis Test.

The aim of this study was to assess the role and the effect of the two most frequent polymorphisms of TLR4 Asp299Gly and Thr399Ile on the development of UTIs in infants and children of Greek origin. Identifying the possible role of genetic variants in UTIs would help clinicians understand the pathogenesis of the inflammation and distinguish which children have a greater risk of developing UTI, based on their genetics.

The association of TLR4 Asp299Gly or Thr399Ile polymorphisms with a faulty response to the lipopolysaccharide (LPS) of Gram-negative bacteria in mice was first reported by Arbour et al. in 2000. They suggested that these mutations of the TLR gene may affect the TLR structure or expression and therefore have a negative effect on the response to bacterial endotoxins. This was later confirmed by many other studies [11, 18–23].

Case-control studies, so far, have established associations of TLR4 Asp299Gly or Thr399Ile polymorphisms with the development of a variety of diseases. Most of the studies were conducted on adult populations.

The first reference to the relationship between TLR4 and UTIs is found in 2003 by Schilling et al., who demonstrated that TLR4 on stromal and hematopoietic cells, in mice, mediates innate resistance to uropathogenic *E. coli*. This study is one of the first ones to reveal that bladder epithelial cells play a critical role in TLR4-mediated innate immunity *in vivo* during a mucosal bacterial infection [24].

Since 2006, however, numerous and continuously growing studies on human populations have been done dealing with TLR4 polymorphisms and their association with UTIs [7, 11, 25–31]. As far as the study of all TLR polymorphisms and especially of TLR4 polymorphisms in pediatric populations is concerned, the international literature can present only a limited number of articles. Moreover, these studies are heterogeneous regarding the ethnicity of the population being studied and thus difficult to compare with each other and come to a general agreement. What is worth mentioning is that the relationship of TLR4 Asp299Gly and TLR4 Thr399Gly polymorphisms to UTIs in infants and children in Greece has never been studied so far.

Reviewing the literature, we found that the first to study a possible relation of TLR4 polymorphisms with UTIs were Karoly et al. [32], in Hungary. In this study, an attempt was made to assess among other parameters the role of TLR4 A(896)G polymorphisms using allele-specific PCR in 103 children with recurrent UTI and to compare the allelic prevalence with reference values of 235 healthy children. TLR4 299AG genotype and TLR4 299G alleles were observed with an increased frequency statistically significant in children with recurrent episodes of UTI compared to the control group. It was also found that the TLR4 299AG genotype and the TLR4 299G alleles were also observed more frequently in children with recurrent UTI without vesicoureteral reflux (VUR) than in children with VUR. This finding is in accordance with our finding that notes that TLR4 A/G genotype is a risk factor for recurrent UTI independent of urinary anomalies [32].

In our results, we found that 27.5% of patients and 8026% of healthy children carried the heterozygote genotype for TLR4 Asp299Gly, a difference which was statistically significant. On the opposite, TLR4 Thr399Ile polymorphism was higher in healthy children (11.01%) and lower in the patient group (1.96%). No homozygosity for both studied polymorphisms was detected in our patients. However, in the group of healthy children in our study, 1.84% showed a homozygote genotype for TLR4 Asp299Gly (G/G) and 0.92% showed a homozygote genotype for TLR4 Thr399Ile (T/T).

Mutlubas et al. [33] tried to investigate the distribution of TLR4 gene polymorphisms among 69 pediatric renal-transplanted patients in relation to chronic allograft nephropathy and 115 healthy controls. Neither renal recipients nor healthy controls showed a homozygote genotype for Asp299Gly and Thr399Ile polymorphisms [33]. According to Mutlubas et al., as far as the heterozygote genotype for TLR4 Asp299Gly is concerned, 6 healthy children were found to carry it (5.3%). This percentage is lower compared to our finding (8.26%). As far as the TLR4 Thr399Gly polymorphism is concerned, a percentage (4.3%) lower than that of our study (11,01%) in 5 of the healthy children was found. Our findings regarding the percentage of both the genotype of the TLR4 Asp299Gly polymorphism and its alleles and the part of the urinary tract infected were in agreement with the study of Yin et al. which showed that the gene prevalence of TLR4c.896A<G and TLR4 896 G alleles in adult patients with lower UTI (urethritis, cystitis) was higher than that in the control group [28]. On the contrary, Hawn et al. [34] showed an opposite effect compared to our study suggesting that TLR4c.896A<G polymorphism is associated with a protective role against recurrent UTI and cystitis, but not for pyelonephritis

(PN) in a study population of adult women [34]. In the study of Ertan et al. [35], in children with rUTI, TLR4 gene Thr399Ile polymorphism was not observed in any child, a finding that comes in disagreement with our study where TLR4 gene Thr399Ile polymorphism was found in 1.96% of our patients and 11.01% of the healthy children (in heterozygosity). Genotype distribution and allele frequency of Asp299Gly polymorphism were similar both in the children with rUTI and in healthy controls; thus, this study could not reveal a significant role of this gene in the pathogenesis of UTI [35]. Akil et al. [13] found out that TLR4 Asp299Gly polymorphism and TLR-4 (896) G allele frequency were not different in children with UTI in comparison with healthy children (genotype frequency 12.5 vs. 15.1% and allele frequency 7.1 vs. 6.9%, respectively). Our study however showed a statistical significant difference between the genotype frequencies of the TLR4 Asp299Gly polymorphism which was found higher in patients than in healthy children. This study also pointed out a possible association between genetic factors and renal scar formation. Although no significant difference in TLR4 Asp299Gly polymorphism was found between scar-positive and scar-negative pyelonephritis patients, it was nearly two times more in scar-positive pyelonephritis [13]. In Turkey, Bayram et al. [17] tried to determine the relation of TLR-4 Asp299Gly and Thr399Ile polymorphisms to febrile UTI and renal scar development in children. In the group of children with scars, the incidence rate of TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms was found at 3.5% and at 14% while in the group of children without scars at 7% and at 12.2% respectively. These results contradict ours, since we found TLR4 Thr399Gly polymorphism is rare in children with UTI, whereas TLR4 Asp299Gly polymorphism is more common [17]. Recently, Harshman et al. [36] investigated the prevalence of the TLR4 polymorphism (Asp299Gly, Gly299Gly) among children with chronic pyelonephritis and healthy children. In the majority of healthy children (96.8%), the normal distribution of alleles AA of TLR4 gene was detected. AG heterozygous genotype was detected in a nonsignificant percentage of 3.2% and mutant genotype GG was not detected at all. These findings differ from ours as we found, in the group of healthy children, a higher percentage of the TLR4 Asp299Gly polymorphism in heterozygosity (8.26%) as well as in homozygosity. In children with chronic pyelonephritis, AG heterozygous genotype was detected at a 86.7% frequency and mutant genotype GG at a 11.7% frequency respectively, and it reliably exceeded the parameters of the control group. These results differ once more with those of our study where in the patient group, no child with mutant GG genotype was found and a frequency of 27.5% was detected for AG heterozygous genotype [36]. In the study of Hussein et al. [37], it was showed that 88.9% of the children with UTIs and 94% of the healthy children did not carry the TLR4 Asp299Gly polymorphism at all. 10% of the patients' group and 6% of the healthy group carry the AG heterozygous genotype for the TLR4 Asp299Gly polymorphism. Only 4 patients (1.1%) carried the mutant genotype G. In addition, they showed for the TLR4 Thr399Ile polymorphism that the genotype and the allele frequency did not differ significantly between the patients and the control group. The causative role of the TLR4 Asp299Gly was suggested in the occurrence and progression of UTIS to involve renal parenchyma, which is in total agreement with the results of our study. Moreover, the percentage of TLR4 Asp299Gly (A/G) polymorphism in our patients was nearly three times higher than that reported by Hussein et al. (27.5% vs. 10%, respectively) which reinforces the hypothesis that TLR4 Asp299Gly polymorphism is related to the risk of developing UTI [37].

In this study, we made an attempt to record the frequency of TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms in healthy children and in children with one or more episodes of UTI. Moreover, we tried to look into a possible relation between the presence of these polymorphisms with parameters from their personal history such as gender, number of UTI episodes, renal function, kidney ultrasound findings, and laboratory data. What differentiates our study is that the percentage of the heterozygous for the TLR4 Asp299Gly polymorphism patients was much higher than any other percentage recorded in similar studies so far. TLR4 Thr399Ile polymorphism was also found to be more frequent in healthy children. Our results suggest a causative role of the TLR4 Asp299Gly polymorphism in the occurrence and progression of UTIs in children of Greek origin while, on the contrary, a protective role of the TLR4 Thr399Ile polymorphism against urinary tract infections. These findings could be attributed to the fact that the distribution of many SNPs differs among different populations. It should also be taken into consideration the fact that these results may be applicable in the Greek children and not in the general population. More and larger studies need to be conducted to confirm this statement.

5. Conclusions

Our data is the first to be published in Greece, addressing the role of TLR4 Asp299Gly and TLR4 Thr399Gly polymorphisms as a genetic risk for the development of UTIs in infants and children of Greek origin. We identify the possible role of the TLR4 genetic variants in the pathogenesis of UTI that would help to early recognition of the children at a greater risk and could serve as a useful tool to personalized management of UTIs in children. Our study contributes to growing evidence of the role of TLRs to UTI, however indicating the need for a larger sample and more multicenter studies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This research was performed as part of the employment of the authors.

Conflicts of Interest

No conflict of interests has been declared by the authors.

References

[1] A. Sood, F. J. Penna, S. Eleswarapu et al., "Incidence, admission rates, and economic burden of pediatric emergency department visits for urinary tract infection: data from the nationwide emergency department sample, 2006 to 2011," *Journal of Pediatric Urology*, vol. 11, no. 5, pp. 246.e1–246.e8, 2015.

[2] J. Larcombe, "Urinary tract infection in children: recurrent infections," *BMJ Clinical Evidence*, vol. 2015, article 0306, 2015.

[3] D. J. Desai, B. Gilbert, and C. A. McBride, "Paediatric urinary tract infections : diagnosis and treatment," *Australian Family Physician*, vol. 45, no. 8, pp. 558–564, 2016.

[4] N. Hooman, R. Isa-Tafreshi, S. H. Mostafavi, F. Hallaji, A. Tavasoli, and H. Otukesh, "The prevalence of hypertension in children with renal scars," *Minerva Pediatrica*, vol. 69, no. 3, pp. 200–205, 2017.

[5] P. D. Olson, L. K. McLellan, A. Liu et al., "Renal scar formation and kidney function following antibiotic-treated murine pyelonephritis," *Disease Models & Mechanisms*, vol. 10, no. 11, pp. 1371–1379, 2017.

[6] T. Eleftheriadis, G. Pissas, V. Liakopoulos, I. Stefanidis, and B. R. Lawson, "Toll-like receptors and their role in renal pathologies," *Inflammation & Allergy-Drug Targets*, vol. 11, no. 6, pp. 464–477, 2012.

[7] E. Behzadi and P. Behzadi, "The role of Toll-Like receptors (TLRs) in urinary tract infections (UTIs)," *Central European Journal of Urology*, vol. 69, no. 4, pp. 404–410, 2016.

[8] E. D. Papadimitraki, G. K. Bertsias, and D. T. Boumpas, "Toll like receptors and autoimmunity: a critical appraisal," *Journal of Autoimmunity*, vol. 29, no. 4, pp. 310–318, 2007.

[9] K. Takeda, T. Kaisho, and S. Akira, "Toll-like receptors," *Annual Review of Immunology*, vol. 21, no. 1, pp. 335–376, 2003.

[10] B. A. Iwalokun, A. Oluwadun, S. O. Iwalokun, and P. Agomo, "Toll-like receptor (*TLR4*) Asp299Gly and Thr399Ile polymorphisms in relation to clinical falciparum malaria among Nigerian children: a multisite cross-sectional immunogenetic study in Lagos," *Genes and Environment*, vol. 37, no. 1, pp. 1–9, 2015.

[11] B. Ferwerda, M. B. B. McCall, K. Verheijen et al., "Functional consequences of Toll-like receptor 4 polymorphisms," *Molecular Medicine*, vol. 14, no. 5–6, pp. 346–352, 2008.

[12] I. K. Hewitt, P. Zucchetta, L. Rigon, F. Maschio, P. P. Molinari, L. Tomasi et al., "Early treatment of acute pyelonephritis in children fails to reduce renal scarring: data from the Italian Renal Infection Study Trials: Editorial comment," *International Brazilian Journal of Urology*, vol. 34, no. 6, p. 795, 2008.

[13] I. Akil, F. Ozkinay, H. Onay, E. Canda, G. Gumuser, and S. Kavukcu, "Assessment of toll-like receptor-4 gene polymorphism on pyelonephritis and renal scar," *International Journal of Immunogenetics*, vol. 39, no. 4, pp. 303–307, 2012.

[14] E. Lorenz, K. L. Frees, and D. A. Schwartz, "Determination of the TLR4 genotype using allele-specific PCR," *Biotechniques*, vol. 31, no. 1, pp. 22–24, 2001.

[15] K. Y. Bin, C. L. Tang, and J. W. Koo, "Is vaginal reflux associated with urinary tract infection in female children under the age of 36 months?," *Korean Journal of Pediatrics*, vol. 61, no. 1, pp. 17–23, 2018.

[16] G. Pouladfar, M. Basiratnia, M. Anvarinejad, P. Abbasi, F. Amirmoezi, and S. Zare, "The antibiotic susceptibility patterns of uropathogens among children with urinary tract infection in Shiraz," *Medicine*, vol. 96, no. 37, article e7834, 2017.

[17] M. T. Bayram, A. Soylu, H. Ateş, S. Kizildağ, and S. Kavukçu, "TLR-4 polymorphisms and leukocyte TLR-4 expression in febrile UTI and renal scarring," *Pediatric Nephrology*, vol. 28, no. 9, pp. 1827–1835, 2013.

[18] N. C. Arbour, E. Lorenz, B. C. Schutte et al., "TLR4 mutations are associated with endotoxin hyporesponsiveness in humans," *Nature Genetics*, vol. 25, no. 2, pp. 187–191, 2000.

[19] X. Du, A. Poltorak, M. Silva, and B. Beutler, "Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor," *Blood Cells, Molecules, and Diseases*, vol. 25, no. 6, pp. 328–338, 1999.

[20] F. Bihl, L. Lariviere, S. T. Qureshi, L. Flaherty, and D. Malo, "LPS-hyporesponsiveness of mnd mice is associated with a mutation in Toll-like receptor 4," *Genes & Immunity*, vol. 2, no. 1, pp. 56–59, 2001.

[21] C. Schmitt, A. Humeny, C. M. Becker, K. Brune, and A. Pahl, "Polymorphisms of *TLR4*: Rapid genotyping and reduced response to lipopolysaccharide of *TLR4* mutant alleles," *Clinical Chemistry*, vol. 48, no. 10, pp. 1661–1667, 2002.

[22] I. Smirnova, M. T. Hamblin, C. McBride, B. Beutler, and A. di Rienzo, "Excess of rare amino acid polymorphisms in the toll-like receptor 4 in humans," *Genetics*, vol. 158, no. 4, pp. 1657–1664, 2001.

[23] C. Erridge, J. Stewart, and I. R. Poxton, "Monocytes heterozygous for the Asp299Gly and Thr399Ile mutations in the Toll-like receptor 4 gene show no deficit in lipopolysaccharide signalling," *Journal of Experimental Medicine*, vol. 197, no. 12, pp. 1787–1791, 2003.

[24] J. D. Schilling, S. M. Martin, C. S. Hung, R. G. Lorenz, and S. J. Hultgren, "Toll-like receptor 4 on stromal and hematopoietic cells mediates innate resistance to uropathogenic Escherichia coli," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 4203–4208, 2003.

[25] X. Zikou and K. Siamopoulos, "Role of Toll-like receptors in the pathogenesis of kidney disease," *Hellenic Nephrology*, vol. 21, no. 1, pp. 25–33, 2009.

[26] S. Batsford, U. Duermueller, C. Seemayer, C. Mueller, H. Hopfer, and M. Mihatsch, "Protein level expression of Toll-like receptors 2, 4 and 9 in renal disease," *Nephrology Dialysis Transplantation*, vol. 26, no. 4, pp. 1413–1416, 2011.

[27] M. Zaffanello, G. Malerba, L. Cataldi et al., "Genetic risk for recurrent urinary tract infections in humans: a systematic review," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 321082, 9 pages, 2010.

[28] X. Yin, T. Hou, Y. Liu et al., "Association of Toll-like receptor 4 gene polymorphism and expression with urinary tract infection types in adults," *PLoS One*, vol. 5, no. 12, article e14223, 2010.

[29] D. W. Hilbert, K. E. Pascal, E. K. Libby, E. Mordechai, M. E. Adelson, and J. P. Trama, "Uropathogenic *Escherichia coli* dominantly suppress the innate immune response of bladder epithelial cells by a lipopolysaccharide- and Toll-like receptor 4-independent pathway," *Microbes and Infection*, vol. 10, no. 2, pp. 114–121, 2008.

[30] J. Song and S. N. Abraham, "TLR-mediated immune responses in the urinary tract," *Current Opinion in Microbiology*, vol. 11, no. 1, pp. 66–73, 2008.

[31] F. Bäckhed and M. Hornef, "Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat?," *Microbes and Infection*, vol. 5, no. 11, pp. 951–959, 2003.

[32] E. Karoly, A. Fekete, N. F. Banki et al., "Heat shock protein 72 (HSPA1B) gene polymorphism and toll-like receptor (TLR) 4 mutation are associated with increased risk of urinary tract infection in children," *Pediatric Research*, vol. 61, no. 3, pp. 371–374, 2007.

[33] F. Mutlubas, S. Mir, A. Berdeli, N. Ozkayin, and B. Sozeri, "Association between Toll-like receptors 4 and 2 gene polymorphisms with chronic allograft nephropathy in Turkish children," *Transplantation Proceedings*, vol. 41, no. 5, pp. 1589–1593, 2009.

[34] T. R. Hawn, D. Scholes, S. S. Li et al., "Toll-like receptor polymorphisms and susceptibility to urinary tract infections in adult women," *PLoS One*, vol. 4, no. 6, article e5990, 2009.

[35] P. Ertan, A. Berdeli, O. Yilmaz, D. A. Gonulal, and H. Yuksel, "LY96, UPKIB mutations and TLR4, CD14, MBL polymorphisms in children with urinary tract infection," *The Indian Journal of Pediatrics*, vol. 78, no. 10, pp. 1229–1233, 2011.

[36] V. P. Harshman, T. O. Kryuchko, I. O. Kolenko, T. V. Kushnereva, and O. Y. Tkachenko, "Role of genetic mutations in development of immunological and clinical disorders in children with chronic pyelonephritis," *Wiadomości Lekarskie*, vol. 70, no. 1, pp. 47–51, 2017.

[37] A. Hussein, K. Saad, E. Askar et al., "Functional variants in intercellular adhesion molecule-1 and toll-like receptor-4 genes are more frequent in children with febrile urinary tract infection with renal parenchymal involvement," *Acta Paediatrica*, vol. 107, no. 2, pp. 339–346, 2018.