



Research paper

Genetic variation in TLR pathway and the risk of pulmonary tuberculosis in a Moldavian population



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ARTICLE INFO

Keywords:

Pulmonary tuberculosis

Susceptibility

Host genetics

Toll-like receptors

Single nucleotide polymorphisms

Association study

ABSTRACT

Toll-like receptors (TLRs) play a critical role in initiating an immune response to infections. In this study, we examined whether single nucleotide polymorphisms (SNPs) in TLR pathway genes are associated with pulmonary tuberculosis (PTB) in a Moldavian population. Thirty-four SNPs in genes associated with the TLR pathway and two SNPs in *ASAP1* gene identified by GWAS were selected for genotyping in 272 patients and 251 community-matched healthy controls. Twenty-nine SNPs passed quality control and were statistically evaluated. SNPs *TLR9* rs352139, *TLR2* rs3804099 and *MYD88* rs4988453 were associated with PTB in females (OR = 0.49, $p = 0.0009$; OR = 0.51, $p = 0.0008$; OR = 0.33, $p = 0.027$; here and below log-additive model with minor alleles assumed as effect associated alleles), while SNP *TLR8* rs3764880 showed a significant association in males (OR = 0.44, $p = 0.0087$). Furthermore, SNPs *TLR9* rs352139 and *TLR8* rs3764880 were associated with PTB in the late-onset (≥ 39 -year-old) patient group (OR = 0.60, $p = 0.0029$ and OR = 0.70, $p = 0.021$, respectively) and SNPs *TLR2* rs3804099, *TLR4* rs4986790 and *TLR4* rs1927906 in the early-onset (≤ 38 -year-old) group (OR = 0.53, $p = 0.0012$; OR = 3.45, $p = 0.013$; OR = 2.31, $p = 0.044$, respectively). After correction for multiple testing, only SNPs *TLR9* rs352139 and *TLR2* rs3804099 in the female group and SNP *TLR2* rs3804099 in the early-onset group remained significant. In summary, we show an association of SNP *TLR8* rs3764880 with PTB in the Moldavian male population, providing support to previous studies conducted on other populations. Polymorphisms rs3804099 (*TLR2*) and rs352139 (*TLR9*) may also be associated with PTB risk in the Moldavian population but their effect is less consistent across different studies. Additional large-scale association studies along with functional tests are required to dissect the relevance of these associations.

1. Introduction

Tuberculosis (TB) is a serious life-threatening infectious disease primarily affecting the lungs, among other organs of the body (Lawn and Zumla 2011). Despite extensive treatment and immunization strategies, the incidence of TB and associated mortality rates remain high, particularly in developing countries (World Health Organization [WHO], 2016). *Mycobacterium tuberculosis* (*M.tb*) infection has been implicated as the key factor in TB etiology. The bacterium is hosted by approximately a third of the world's population, but a much smaller percentage actually develops clinical symptoms of the disease (Lawn

and Zumla 2011). Animal models, twin and family studies as well as numerous case-control association studies indicate that host genetics is important in determining susceptibility or resistance to TB (Orlova and Schurr 2017).

Genetic variation in genes involved in innate immunity is thought to be the most probable factor predisposing individuals to the development of TB (Casanova et al. 2011). Toll like receptors (TLRs) are the key components of the innate immune system which recognize conserved sequences on the surface of pathogens and trigger a host defense response (Akira et al. 2006). Furthermore, the co-receptor CD14 is necessary for efficient microbial recognition by TLR2 and TLR4

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<https://doi.org/10.1016/j.meegid.2018.12.005>

Received 2 October 2018; Received in revised form 20 November 2018; Accepted 4 December 2018

Available online 05 December 2018

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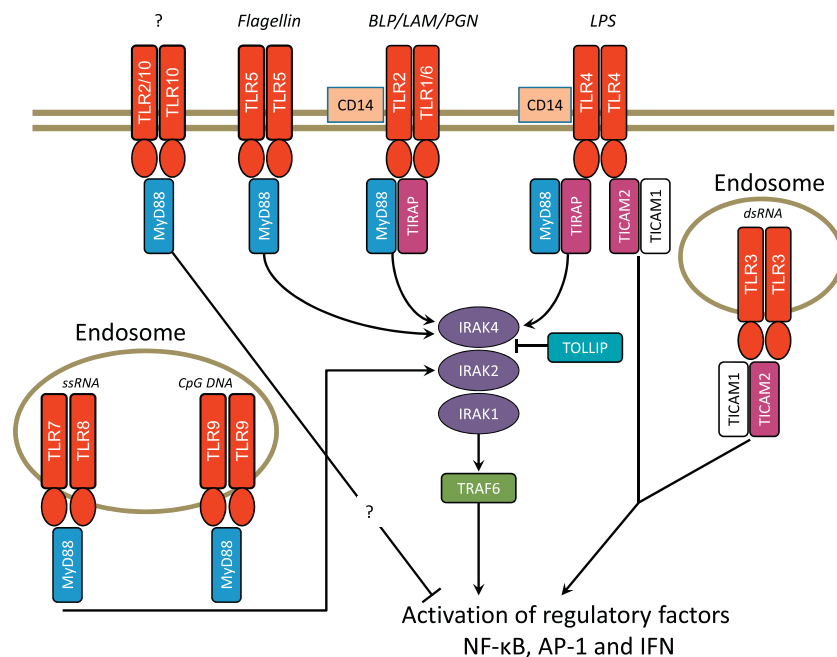


Fig. 1. Schematic representation of the genes involved in the TLR related pathway analyzed in this study (except TICAM1). Positive interactions are indicated with a sharp arrow, negative with a blunt-pointed arrow. Question marks indicate unknown interacting partners. Figure adapted from Khakpour et al. 2015 and Oosting et al. 2014.

(Triantafilou and Triantafilou 2002). Following ligand binding, TLRs form a dimer and recruit cytoplasmic adaptor proteins MyD88, TRIF, TIRAP (MAL), TOLLIP and TICAM-2 (TRAM). Subsequently, the activated adaptor proteins associate with the IL-1 receptor-associated kinases (IRAK-1, IRAK-2, IRAK-4) and tumor necrosis factor receptor associated factor 6 (TRAF6) to initiate the downstream cascades. These pathways result in the activation of transcription factors NF-κB, AP-1 and the IFN transcription factor family, and stimulate the production of pro-inflammatory and host-defense genes (Kawai and Akira 2010). The interactions in TLR signaling pathway are schematically presented on Fig. 1. The importance of the TLR pathway in the control of *M.tb* is supported by the observation that mice lacking the respective molecular components exhibit increased vulnerability to a number of pathogens, including *M.tb* (Abel et al. 2002; Drennan et al. 2004; Scanga et al. 2004).

There is evidence that polymorphisms in genes coding for proteins involved in the TLR pathway are associated with infectious diseases, including TB. For example, single nucleotide polymorphisms (SNPs) *TLR1* rs4833095 (Dittrich et al. 2015), *TLR2* rs3804099 (Xue et al. 2017), *TLR4* rs11536889 (Xue et al. 2017) and rs1927911 (Zaki et al. 2012), *TLR8* rs3764880 (Salie et al. 2015), *TLR9* rs352139 (Kobayashi et al. 2012; Torres-García et al. 2013), *CD14* rs2915863 (Xue et al. 2012), *MYD88* rs4988453 (Aggelou et al. 2016), *MYD88* rs6853 (Capparelli et al. 2013), and *TIRAP* rs8177374 (Khor et al. 2007; Capparelli et al. 2013) have been shown to be associated with TB in specific populations. However, in view of the inter-ethnic heterogeneity of genetic factors (e.g., allele frequencies) and environmental factors, these results cannot be generalized to other populations. Furthermore, other core genes involved in TLR signaling (e.g., *TLR7*, *TOLLIP*, *IRAK1*, *IRAK2*, *IRAK4*, and *TRAF6*) are hardly investigated for their association with TB.

For these reasons, the present case-control study was designed to evaluate possible associations between common variants in the TLR signaling genes and pulmonary tuberculosis (PTB) in Moldavian population. The Republic of Moldova exhibits the highest TB incidence in Europe (152 cases/100,000/year), i.e., over four times higher than the average in the European region (WHO, 2016). The results obtained from this study could be extended to the neighboring populations from Eastern and South-Eastern Europe, which also exhibit high incidence of tuberculosis and share genetic and cultural similarities with

Moldavians.

2. Materials and methods

2.1. Samples

This study was approved by the Ethics Committee of the Institute of Phthisiopneumology (Republic of Moldova), and adhered to the tenets of the Declaration of Helsinki. Study participants provided written informed consent for their participation in the study. The case group consisted of 272 unrelated patients (120 women and 152 men; mean age at recruitment = 40.7 ± 12.7 years; mean age at diagnosis = 39.1 ± 12.2 years) with infiltrative pulmonary TB. Diagnosis was based on clinical symptoms and chest radiographic findings followed by confirmation by bacteriological assessment. Patients known to be immunodeficient (e.g., due to HIV infection, diabetes, cancer, or administered immunosuppressive therapy) were excluded from the study. All patients were of European, and predominantly, Moldavian (91.5%) descent.

The control group comprised of 251 unrelated and ethnically matched (Moldavians, 87%) healthy individuals (150 women and 101 men; mean age at recruitment = 47.6 ± 14.1 years), who lived in TB-affected communities. Control subjects were excluded if they had a history of prior anti-TB therapy, signs or symptoms suggestive of active TB, and/or presented infiltrates on chest X-ray.

Venous blood samples (EDTA anticoagulant added) were collected from all study participants, and genomic DNA was extracted from peripheral blood leukocytes using a standard salting-out method (Varzari et al. 2015). All participants in the study were BCG vaccinated.

2.2. SNP selection

A candidate gene approach was used to select potentially relevant genes and SNPs based on the following criteria: (i) relevance to the TLR signaling pathway, (ii) previously described associations with infectious diseases and/or (iii) suggested or proven functionality on a molecular level, (iv) high prevalence in European population (minor allele frequency > 0.05 according to the 1000 genomes project database), and (v) low or moderate correlation with other SNPs ($r^2 < 0.80$). A total of 34 SNPs from *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR7*, *TLR8*, *TLR9*, *TLR10*,

CD14, *MyD88*, *IRAK2*, *TICAM-2*, *IRAK4*, *TIRAP*, *TOLLIP*, and *TRAF6* genes were selected for genotyping (Fig. 1). In addition, two SNPs (rs4733781 and rs10956514 in *ASAP1* gene) identified in a Russian genome-wide association study (GWAS) on PTB were included in the genotyping panel as a positive control (Curtis et al. 2015). The selected SNPs are described in Supplementary Table S1.

2.3. Genotyping and quality control

SNPs were genotyped with Agena iPLEX assays using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass array spectrometer (Agena, San Diego, CA) following manufacturer's recommendations. Primers were designed using the Assay Design Suite v2.0 (<https://agenacx.com>). Monoallelic SNPs or those that showed significant deviation from Hardy-Weinberg equilibrium (HWE) (p -value < 0.05) in the control group were excluded from further analysis.

2.4. Statistical analysis

Student's t -tests and Pearson's χ^2 tests were used to compare demographic data between TB cases and controls. Deviations from HWE were examined using the PLINK software package (version 1.9, <http://pngu.mgh.harvard.edu/purcell/plink/>) (Chang et al. 2015). Logistic regression was used to assess the log-additive association of each SNP with the risk of PTB. Minor alleles were assumed as effect associated alleles in all tests. Odds ratios (ORs), 95% confidence intervals (CIs) and p -values were calculated using PLINK. Linkage disequilibrium (LD) pairwise values (r^2 and D'), haplotype structure, and haplotype frequencies were determined using Haploview version 4.2 (Barrett et al. 2005). Differences in haplotype frequencies were tested using χ^2 test. Haplotypes with a frequency below 1% were excluded from the analyses. The significance level for all statistical tests was set at $p < 0.05$. Bonferroni correction (0.05/number of tests) was used to correct for multiple testing.

Power analysis was performed using CaTS with a log-additive genetic model, assuming significance (type I error) of 0.05 and the reported TB disease prevalence of 0.0025 (WHO, 2016). The study provides sufficient power (> 80%) to detect minimum ORs of 2.0 and 1.5 for high-risk allele frequencies of > 0.05 and > 0.20, respectively. The power was smaller in the sex- and age-stratified analyses.

3. Results

3.1. Characterization of the data set

The demographic characteristics are shown in Table 1. To account for possible bias due to statistically significant difference in age and sex distributions between case and control groups ($p < 0.0001$ and $p = 0.0004$, respectively), the variables age and sex were included as covariates in logistic regression analysis.

Table 1
Sex and age distribution of study population.

Characteristic	Controls (n = 251)	Cases (n = 272)	p -value
Sex	m: 101; f: 150	m: 152; f: 120	0.0004 ^a
Age at referral (years; mean $\pm$ SD)	47.6 $\pm$ 14.1	40.7 $\pm$ 12.7	< 0.0001 ^b
Age range	16–80	18–74	
Age at onset (mean $\pm$ SD)	NA	39.1 $\pm$ 12.2	
Age at onset (median)	NA	38	

^a Pearson's χ^2 test.

^b Student's t -test.

3.2. Single SNP analysis

Out of 36 SNPs selected for genotyping, rs5743854 failed at assay design; rs3138078 was monoallelic and rs11096957, rs5743611, rs4251552, rs4251545, and rs1059703 showed significant deviation from HWE in controls (p -value < 0.05) (Supplementary Table S2). These markers were excluded from the study and data from 29 SNPs were selected for further downstream statistical analyses (Table 2). The MAFs of these SNPs in healthy controls were similar to those in the populations of European descent from the 1000 Genomes Project database (Supplementary Table S1).

The European GWAS-identified risk SNPs rs4733781 and rs10956514 in the *ASAP1* gene were not associated with PTB in the Moldavian data set, which may be due to the limited power of the current study to detect associations with OR close to one. Nevertheless, both SNPs displayed similar ORs to those from the GWAS (0.88 vs 0.84 and 0.93 vs 0.85, respectively for the minor alleles of rs4733781 and rs10956514), providing support for further results reported here.

A significant association was observed for rs352139 (T > C) (p -value = 0.0048) in *TLR9* and rs3804099 (c.597 T > C) in *TLR2* (p -value = 0.0084). However, after Bonferroni's correction for multiple testing (29 tests; p -value < 0.05/29 = 0.0017) both associations lost statistical significance (Table 2). No other significant association between SNPs and PTB could be identified in this dataset.

3.3. Stratification by sex and age-of-onset

Sex-stratified analyses showed that rs3804099 (*TLR2*), rs352139 (*TLR9*) and rs4988453 (*MYD88*) are associated with PTB in females (OR = 0.51, 95% CI = 0.34–0.76, p -value = 0.00084; OR = 0.49, 95% CI = 0.32–0.74, p -value = 0.00092; OR = 0.33, 95% CI = 0.12–0.88, p -value = 0.027, respectively), but not in males (Table 3 and Supplementary Tables S3, S4). The associations of rs352139 (*TLR9*) and rs3804099 (*TLR2*) remained significant after correction for multiple testing (p -value < 0.0017). The X-chromosome linked SNP *TLR8* rs3764880 (A > G) showed association with PTB risk in the male group (OR = 0.44, 95% CI = 0.23–0.81, p -value = 0.0087; Table 3 and Supplementary Table S3), without reaching significance after correction for multiple testing.

To elucidate the effects of SNPs in TLR pathway genes on the age at disease onset, PTB patients were divided into two equal subgroups: early-onset (≤ 38 year old, $n = 137$) and late-onset (≥ 39 year old, $n = 135$). Each group was independently compared with the healthy controls. Strong evidence for an association was found between rs3804099 (*TLR2*) and PTB in the early-onset group (OR = 0.53, 95% CI = 0.36–0.78, p -value = 0.0012; Table 4 and Supplementary Table S5). In addition, nominal significance (p -value < 0.05) was detected for rs4986790 and rs1927906 (both in *TLR4*) in the early-onset group and for rs352139 (*TLR9*) and rs3764880 (*TLR8*) in the late-onset group (Table 4 and Supplementary Tables S5, S6).

3.4. Haplotype analysis

With the set of SNPs genotyped, *TLR4* formed five common haplotypes ($\geq 1\%$), *TOLLIP* and the gene-cluster *TLR1*–*TLR6*–*TLR10* formed four common haplotypes, and each of other genes (*TLR9*, *MYD88*, *IRAK2*, *CD14* and *ASAP1*) displayed three common haplotypes (LD blocks are presented on Supplementary Fig. S1). In general, haplotype analysis did not provide stronger associations over single SNPs (Supplementary Table S7). Consistent with the single-locus results (rs352139 within *TLR9*), the only significant associations were in the *TLR9* gene (p -value = 0.0021 and p -value = 0.0065 for haplotypes CA and TA, respectively). Likewise, these were lost after Bonferroni correction for multiple testing (28 tests, p -value < 0.05/28 = 0.0018).

Table 2
SNP association results.

Gene	SNP	Major/minor allele	MAF in controls	MAF in patients	OR (95% CI) ^a	p-value ^a
<i>ASAP1</i>	rs4733781	A/C	0.323	0.309	0.88 (0.67–1.16)	0.3574
<i>ASAP1</i>	rs10956514	A/G	0.395	0.385	0.93 (0.71–1.22)	0.6131
<i>TLR1</i>	rs4833095	T/C	0.322	0.328	1.00 (0.75–1.32)	0.9857
<i>TLR2</i>	rs3804099	T/C	0.451	0.397	0.69 (0.53–0.91)	0.00837
<i>TLR4</i>	rs4986790	A/G	0.031	0.044	1.62 (0.82–3.20)	0.1654
<i>TLR4</i>	rs1927906	T/C	0.052	0.061	1.37 (0.79–2.39)	0.2673
<i>TLR4</i>	rs7873784	G/C	0.157	0.133	0.85 (0.58–1.24)	0.3999
<i>TLR4</i>	rs11536889	G/C	0.151	0.133	0.84 (0.59–1.20)	0.3422
<i>TLR4</i>	rs1927911	G/A	0.259	0.237	0.98 (0.72–1.32)	0.8797
<i>TLR6</i>	rs5743810	G/A	0.267	0.317	1.30 (0.96–1.74)	0.08602
<i>TLR7</i>	rs179008	A/T	0.242	0.199	0.89 (0.69–1.15)	0.37
<i>TLR8</i>	rs3764880	A/G	0.246	0.217	0.79 (0.61–1.02)	0.065
<i>TLR9</i>	rs352139	C/T	0.470	0.381	0.67 (0.51–0.88)	0.00476
<i>TLR9</i>	rs5743836	A/G	0.128	0.115	1.11 (0.73–1.68)	0.6355
<i>TLR10</i>	rs11466657	A/G	0.031	0.043	1.54 (0.76–3.11)	0.231
<i>CD14</i>	rs2915863	T/C	0.418	0.424	1.05 (0.81–1.36)	0.7321
<i>CD14</i>	rs2569190	G/A	0.482	0.489	1.03 (0.80–1.33)	0.7952
<i>MyD88</i>	rs4988453	C/A	0.055	0.035	0.64 (0.34–1.21)	0.1715
<i>MyD88</i>	rs6853	A/G	0.133	0.135	0.96 (0.66–1.41)	0.8477
<i>TIRAP</i>	rs8177374	C/T	0.117	0.091	0.72 (0.47–1.11)	0.1391
<i>IRAK2</i>	rs35060588	C/G	0.076	0.085	1.09 (0.68–1.74)	0.7268
<i>IRAK2</i>	rs3844283	C/G	0.417	0.398	0.87 (0.66–1.14)	0.3171
<i>IRAK2</i>	rs708035	A/T	0.324	0.353	1.22 (0.92–1.61)	0.1604
<i>TOLLIP</i>	rs3750920	C/T	0.496	0.478	0.96 (0.74–1.24)	0.7358
<i>TOLLIP</i>	rs5743899	T/C	0.190	0.183	0.88 (0.62–1.24)	0.4632
<i>TOLLIP</i>	rs3793964	C/T	0.388	0.351	0.90 (0.69–1.18)	0.4425
<i>TOLLIP</i>	rs4963067	T/C	0.078	0.075	0.92 (0.56–1.52)	0.7575
<i>TICAM2</i>	rs746566	C/T	0.407	0.423	1.04 (0.79–1.35)	0.8012
<i>TRAF6</i>	rs4755453	G/C	0.191	0.181	0.80 (0.57–1.13)	0.2038

p-values below 0.05 are highlighted in bold.

^a Log-additive model adjusted for sex and age at recruitment.

4. Discussion

This study examined associations between 29 SNPs in TLR pathway genes and PTB in a Moldavian population and we report significant sex-dependent and age-of-onset-specific associations of SNPs in *TLR2*, *TLR8* and *TLR9* genes. These findings highlight the importance of genetic variation in the TLR pathway in the development of TB and require special consideration in the light of the previously published data on functional and genetic association studies.

The minor 'G' allele of the non-synonymous SNP rs3764880 (A > G, p.Met1Val), located in exon 1 of the *TLR8* gene, was found in this study to have a protective effect on PTB in males. Although the association has only nominal significance, this finding is in accordance with most previous studies (Davila et al. 2008; Dalgic et al. 2011; Salie

et al. 2015). In particular, a well-powered survey conducted in a genetically related Russian population reports a significant protective effect of the same allele in males (Davila et al. 2008). Furthermore, a recent study in an Indian population demonstrated that this allele confers protection only in subjects who had previously received the BCG vaccine against TB (Ugolini et al. 2018). In this regard, it should be mentioned that all research subjects in the present study were BCG vaccinated. The SNP rs3764880 eliminates a wild-type start codon of *TLR8*, resulting in peptide truncation by three amino acids (Oh et al. 2008). According to in silico predictions, this leads to a significant change in the secondary structure and may increase binding potential of *TLR8* (Ugolini et al. 2018). Consistent with this, in vitro gene expression assays demonstrated up-regulation of NF-κB in cells expressing the 'G' allele in response to stimulation by *TLR8*-specific ligands (Ugolini

Table 3
Results of genetic association analysis by sex.

SNP	Major (A)/minor (a) allele	Group	Genotype/allele distribution, n (%)					OR (95% CI) ^a	p-value ^a
			AA	Aa	aa	A	a		
Females									
TLR2 rs3804099	T/C	Controls	40 (27.6)	76 (52.4)	29 (20)	156 (53.8)	134 (46.2)	0.51 (0.34–0.76)	0.00084 [*]
		Cases	54 (47)	49 (42.6)	12 (10.4)	157 (68.3)	73 (31.7)		
TLR9 rs352139	C/T	Controls	32 (22.7)	80 (56.7)	29 (20.6)	144 (51.1)	138 (48.9)	0.49 (0.32–0.74)	0.00092 [*]
		Cases	42 (37.5)	58 (51.8)	12 (10.7)	142 (63.4)	82 (36.6)		
MYD88 rs4988453	C/A	Controls	129 (87.2)	19 (12.8)	0 (0)	277 (93.6)	19 (6.4)	0.33 (0.12–0.88)	0.0272
		Cases	114 (95)	6 (5)	0 (0)	234 (97.5)	6 (2.5)		
Males									
TLR8 rs3764880	A/G	Controls	^b	–	–	68 (68.7)	31 (31.3)	0.44 (0.23–0.81)	0.00871
		Cases	–	–	–	124 (83.2)	25 (16.8)		

Note: Only SNPs with nominal p-values < 0.05 are included. For full list of SNPs see Supplementary Tables S3 and S4.

^a Log-additive regression model adjusted for age at recruitment.

^b This SNP locates on X chromosome.

* The result remained significant after controlling for type I error by using Bonferroni correction ($\alpha = 0.05/29$).

Table 4
Results of genetic association analysis by age-at-onset.

SNP	Major (A)/minor (a) allele	Group	Genotype/allele distribution, n (%)					OR (95% CI) ^a	p-value ^a
			AA	Aa	aa	A	a		
18–38 years old patients									
<i>TLR2</i> rs3804099	T/C	Controls	72 (29.6)	123 (50.6)	48 (19.8)	267 (54.9)	219 (45.1)	0.53 (0.36–0.78)	0.0012*
		Cases	49 (38)	61 (47.3)	19 (14.7)	159 (61.6)	99 (38.4)		
<i>TLR4</i> rs4986790	A/G	Controls	228 (93.8)	15 (6.2)	0 (0)	471 (96.9)	15 (3.1)	3.45 (1.26–9.39)	0.013
		Cases	121 (91)	11 (8.3)	1 (0.8)	253 (95.1)	13 (4.9)		
<i>TLR4</i> rs1927906	T/C	Controls	218 (90.1)	23 (9.5)	1 (0.4)	459 (94.8)	25 (5.2)	2.31 (1.02–5.25)	0.044
		Cases	114 (87.7)	15 (11.5)	1 (0.8)	243 (93.5)	17 (6.5)		
39–73 years old patients									
<i>TLR9</i> rs352139	C/T	Controls	61 (26.1)	126 (53.9)	47 (20.1)	248 (53)	220 (47)	0.60 (0.43–0.84)	0.0029
		Cases	49 (37.7)	69 (53.1)	12 (9.2)	167 (64.2)	93 (35.8)		
<i>TLR8</i> rs3764880	A/G	Controls	157 (64.5)	52 (21.1)	38 (15.4)	366 (75.4)	128 (24.6)	0.70 (0.52–0.95)	0.021
		Cases	102 (76.7)	15 (11.3)	16 (12)	219 (82.3)	47 (17.7)		

Note: Only SNPs with nominal p-values < 0.05 are included. For full list of SNPs see Supplementary Tables S5 and S6.

^a Log-additive regression model adjusted for sex and age at recruitment.

^{*} The result remained significant after controlling for type I error by using Bonferroni correction ($\alpha = 0.05/29$).

et al. 2018). Furthermore, a significant increase in expression of NF- κ B dependent cytokines (TNF α , IL-8 and IL-12) by immune cells carrying the ‘G’ allele has been reported (Oh et al. 2008; Gu et al. 2016; Ugolini et al. 2018). Sex-specific association of rs3764880 with TB in males found in this and previous studies can be explained by the location of *TLR8* on X chromosome: being hemizygous, rs3764880 might have a more pronounced effect in males than in females. Besides the above mentioned functional effect of rs3764880 on *TLR8* peptide truncation, it needs to be regarded that substantial LD exists to polymorphisms in the upstream gene region (data not shown). These SNPs might have additional regulatory effects on *TLR8* gene expression.

Polymorphism *TLR9* rs352139, located in the first intron of *TLR9*, showed a significant association with PTB in this study, particularly in females, with the minor ‘T’ allele conferring protection against PTB. Interestingly, the same allele was significantly associated with reduced TB risk in Indonesian females previously (Kobayashi et al. 2012), whereas an association in opposite direction (risk effect) was observed in a group of indigenous females from Mexico (Torres-García et al. 2013). Additionally, a strong protective effect of the ‘T’ allele was seen in African Americans, but not in European Americans and African patients from Guinea-Bissau (Velez et al. 2010). Such diverse results might be explained by the involvement and interaction of other genetic and environmental factors like ethnicity, genetic backgrounds, vaccination history and bacterial characteristics of *M.tb* strains. Functionally this polymorphism was found to affect the gene transcription, such that the ‘T’ allele was associated with increased expression of *TLR9* (Tao et al. 2007). The mechanism for this regulation remains unclear and the intronic variant rs352139 might either directly affect transcription of the gene or be linked to another yet-to-be-identified functional variant, as considerable LD exists to other SNPs in this region.

The SNP rs3804099, located in exon 3 of *TLR2*, introduces a synonymous amino acid exchange (p.Asn199=) and shows no significant LD to other polymorphisms in the locus. Similar to *TLR9* rs352139, the association between this SNP and PTB was restricted to the female population in this study. Specifically, the minor allele ‘C’ showed a protective effect. These sex-specific protective associations of *TLR2* rs3804099 and *TLR9* rs352139 SNPs with PTB in females are in a general agreement with epidemiologic observations (female sex is associated with decreased risk of TB development) and might be explained by complex interactions between genes involved in innate immunity and sex hormones (Torcia et al. 2012; Seillet et al. 2013; Jaillon et al. 2017). Several studies examined the relationship between *TLR2* rs3804099 and TB but yielded contradictory results. Consistent with the current study, increased PTB risk was found to be associated with the ‘T’ allele in Hispanic Americans (Ma et al. 2007) and Tibetans (Xue et al.

2017). In contrast, the same allele was found to be significantly associated with PTB resistance in an Iranian population (Naderi et al. 2013). No effect was found in Colombian (Sanchez et al., 2014), Vietnamese (Thuong et al. 2007), West Chinese (Zhao et al. 2015), and Croatian (Etokebe et al. 2010) populations, as well as in African and European Americans (Velez et al. 2010). As mentioned above, these contradictory results might be the result of population-specific genetic and environmental effects or due to a lack of power to detect effects. The functional effect of rs3804099, which shows no considerable LD with any other SNP in the *TLR2* gene region, is unknown. However, in vitro studies have shown that the ‘C’ allele was correlated with an increased production of cytokines (i.e., IL-10, IL-8 and TNF- α) by peripheral blood leukocytes in response to LPS stimuli (Chen et al. 2011). It is therefore possible that the *TLR2* SNP rs3804099 influences susceptibility to TB via affecting the production of cytokines.

This study observed age-dependent associations with TB for SNP *TLR2* rs3804099 at a study-wide significance level, and at a nominal significance level for *TLR8* rs3764880, *TLR9* rs352139, *TLR4* rs4986790 and *TLR4* rs1927906. To the best of our knowledge, this is the first report describing such interactions for these loci, and it could be the basis for replication studies in independent populations. Age-dependent associations with TB have been described previously for other genetic loci by several reports (Mahasirimongkol et al. 2012; Grant et al. 2013; Varzari et al. 2018), but the underlying phenomena remain to be elucidated.

We explored possible effects of rs3764880, rs352139 and rs3804099 on gene expression using the tissue specific data from the Genotype-Tissue Expression Project (<https://gtexportal.org> on Nov. 15th 2018). The most prominent effect was found for rs3764880 with a lower expression of *TLR8* in presence of the protective minor allele ‘G’ in whole blood samples. This underlines the possibility that not only the peptide truncating effect of rs3764880, but also genetically linked polymorphisms in the potential promoter of the gene might be functionally relevant. Another interesting observation could be made for rs352139 as the expression of several other genes within 600 kb of the *TLR9* locus (e.g. *GLYCTK*, *NT5DC2*, *PBRM1*, *GNL3*, *NEK4*, *ITIH4*) correlated with presence of rs352139 alleles. This implies a broad regulatory influence of rs352139 (or a SNP in LD) on genes in this locus and should be investigated in more detail in the future. However, this SNP had no observable effect on *TLR9* expression itself, which might be due to the very low *TLR9* expression in all investigated tissues. The polymorphism rs3804099, which introduces a synonymous amino acid exchange in *TLR2* and shows no LD to other SNPs in the locus, showed an effect on *TLR2* expression in fibroblasts. However, this effect needs to be considered with caution, because *TLR2* expression was extremely low in

these cells.

No other SNPs in this study showed meaningful associations with PTB, suggesting that the investigated SNPs may have no effect on PTB in the Moldavian population. Alternatively, our calculations suggest that the sample size used in the present study might also have insufficient statistical power to detect marginal associations potentially present in the TLR variants studied. Remarkably, no association was observed between SNPs *MYD88* rs6853 and *TIRAP* rs8177374 and PTB, although strong associations for these SNPs were reported for Italians (Napoli), Romanians and Ukrainians (Capparelli et al. 2013). Such inconsistency was unexpected, as Moldavians are genetically closely related with Romanians and Ukrainians (Varzari et al. 2007; Varzari et al. 2013). The epidemiologic situation of TB in the Republic of Moldova is also very similar to the neighboring Romania and Ukraine. Moreover, the samples are comparable with respect to sample sizes and statistical power. The differences between our and the above study are primarily in the reported allele frequencies of *MYD88* rs6853 in the groups of healthy subjects. In the samples from Italy, Romania and Ukraine the MAF was strikingly higher (MAF = 0.27, 0.29 and 0.23, respectively) than in our study (MAF = 0.13), as well as in CEU USA (MAF = 0.11), UK (MAF = 0.08), Spain (MAF = 0.15), Toscana Italy (MAF = 0.18) (1000 Genomes Project database) and Napoli Italy (MAF = 0.21) (Fulgione et al. 2016). Further studies in European and other populations are needed to resolve the conflicting results regarding the effect of *MYD88* rs6853 on risk of PTB. Regarding the *TIRAP* rs8177374 SNP, absence of association with PTB in the Moldavian cohort is in line with an also negative finding in a large case-control study in a Russian population (Nejentsev et al. 2008).

Our study had a few limitations. Firstly, the number of participants was not sufficient to detect small effect sizes. Secondly, healthy controls were not evaluated for latent *M.tb* infection, and therefore it was not possible to distinguish between infected and virtually uninfected individuals. However, as mentioned, all individuals were recruited from TB-communities (e.g., household contacts) and were expected to be infected. Third, the number of SNPs in the TLR pathway genes analyzed was limited. Given the key role of TLR signaling in TB pathologies, additional risk loci and haplotypes in this pathway may possibly exist.

5. Conclusions

We report an association between *TLR8* rs3764880 SNP and PTB in Moldavian males, providing further support for the role of TLR8 in the pathogenesis of PTB. Furthermore, the study shows sex-specific effects on PTB for *TLR9* rs352139 and *TLR2* rs3804099 SNPs in females. Finally, the age-at-onset of PTB appears to be influenced by the *TLR2* SNP rs3804099. Further large-scale replication studies along with functional tests are warranted to validate these findings. This knowledge may then be valuable in the development of new therapeutic anti-TB strategies including the use of specific adjuvants.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2018.12.005>.

Authors' contributions

AV conceived the study design, participated in the statistical analyses, wrote the manuscript, and funded the study. IVD performed the statistical analyses and participated in writing the manuscript. IV participated in the collection of clinical data and subject recruitment. HG designed primers and conducted genotyping. MS assisted in study design and writing the final version of the manuscript. ET participated in the study design, subject recruitment and funded the study. TI participated in the study design and funded the study. All of the authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

Funding

The study was supported by the Academy of Sciences of Moldova (grant no. 13.817.09.01A) and Hannover Unified Biobank. Alexander Varzari was sponsored by the Alexander von Humboldt Foundation.

Acknowledgements

We thank Ina Suruceanu for assistance with DNA isolation and all the patients and control subjects for participating in this study and providing blood samples. We would like to thank Editage (www.editage.com) for English language editing.

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