Synergistic effect of genetic polymorphisms in TLR6 and TLR10 genes on the risk of pulmonary tuberculosis in Moldavian population

Alexander M. Varzari (alexander.varzari@mail.ru)
Chiril Draganiuc Institute of Phthisiopneumology https://orcid.org/0000-0003-1855-3095
Igor V. Deyneko
Laboratory of Functional Genomics, Timiryazev Institute of Plant Physiology Russian Academy of Sciences, Moscow, Russia
Elena Tudor
Laboratory of Human Genetics, Chiril Draganiuc Institute of Phthisiopneumology, Kishinev, Republic of Moldova
Harald Grallert
Research Unit of Molecular Epidemiology, Institute of Epidemiology, Helmholtz Zentrum München Research Center for Environmental Health, Neuherberg, Germany
Thomas Illig
Hannover Unified Biobank, Hannover Medical School, Hannover, Germany. Department of Human Genetics, Hannover Medical School, Hannover, Germany

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Abstract

BACKGROUND

Host immunity is essential for efficient recognition and clearance of *M. tuberculosis* infection. Polymorphisms in genes that regulate immune response have been reported to influence the susceptibility/resistance to pulmonary tuberculosis (TB). Here we evaluated associations between 14 polymorphisms in 12 core genes involved in immune responses and pulmonary TB in Moldavian population, and investigated whether interactions between these and previously analyzed polymorphisms could exist and modulate the risk of pulmonary TB.

METHODS

Polymorphisms *VDR* rs7975232, *VDR* rs1544410, *VDR* rs2228570, *MR1* rs1052632, *TLR1* rs5743618, *TLR2* rs111200466, *TLR10* rs11096957, *SLC11A1* rs2276631, *IL1B* rs1143643, *IL10* rs1800896, *IFNG* rs2430561, *TNF* rs1800629, *IRAK1* rs1059703, and *FOXP3* rs2232365 were genotyped in 271 Moldavian pulmonary TB cases and 251 community-matched healthy controls. Associations were tested using Fisher test and logistic regression. Complemented with the data from our previous study (PMID: 30529560), investigation of gene-gene interactions was performed for a total of 43 loci. Significance level was adjusted by the Bonferroni correction.

RESULTS

Single polymorphism analysis revealed a nominal association between *TNF* rs1800629 and pulmonary TB (Fisher exact test *p*-value = 0.01843). Marginal differences between cases and controls were observed for haplotypes in the gene cluster *TLR1-TLR6-TLR10* and gene *TLR2*. In the pairwise interaction analysis, the combination of genotypes *TLR6* rs5743810 GA and *TLR10* rs11096957 GT was significantly associated with an increased genetic risk of pulmonary TB (OR = 2.48, 95% CI = 1.62–3.85; Fisher exact test *p*-value = 1.5×10^{-5}, significant after Bonferroni correction).

CONCLUSION

The *TLR6* rs5743810 and *TLR10* rs11096957 two-locus interaction confers a significantly higher risk for pulmonary TB and has potential as a novel biomarker for predicting TB susceptibility.

Introduction

Tuberculosis (TB), caused by the bacillus *Mycobacterium tuberculosis* (M.tb), is a major cause of morbidity and mortality in many developing countries and a significant threat to health in the developed world. One-third of the world's population is infected with M.tb, but only a minor fraction of those (~ 5–10%) develops the active form of the disease [1]. Although several environmental and clinical conditions, such as diabetes mellitus, malnutrition, alcohol abuse, smoking, age, AIDS and immunosuppressive therapies, are known to promote the development of the disease, many TB patients have no above mentioned or other obvious risk factors. Evidence from animal and human studies indicates the importance of host genetic factors in the development of TB [2].

To date a considerable number of genetic candidates for TB susceptibility have been detected and characterized across populations by means of candidate gene approaches and genome wide association studies [3, 4]. Particular interest was focused on genetic factors of the immunoinflammatory response modulating infectivity and progression of infection, including Toll-like receptors (*TLR1-TLR10*), the natural resistance–associated macrophage protein (*NRAMP1*; also called as solute carrier 11a1, *SLC11A1*), vitamin-D receptor (*VDR*), cytokines tumor necrosis factor alpha (*TNF*), interleukin 1 (*IL1B*), interferon-γ (*IFNG*), and interleukin 10 (*IL10*). Polymorphisms in these and other genes involved in immune response have been reported to be associated with TB in different populations, although results were inconsistent [3, 4, 5].

We have earlier evaluated the effect of common genetic variations in TLR pathway on the risk of pulmonary TB in Moldavian population and identified variants in *TLR2*, *TLR8* and *TLR9* as being associated with TB [6]. In the present work, we extended the analysis to 14 additional polymorphisms from 12 TB immune response candidate genes and investigated all pairwise genetic interactions for these and the previous genetic variants (altogether 43 polymorphisms). Selected polymorphisms for this study have previously been shown to change the level or function of corresponding gene products and to influence susceptibility/resistance to infections (Table 1).

Table 1
Candidate genes and polymorphisms that are of interest in the present study.

Gene	Locus	Protein	Gene class	Protein function	Polymorphism	Nucleotide substitution	Location	Frequency [a]	Ref.
TLR1	4p14	Toll-like receptor 1	Pattern recognition receptor	Recognizes bacterial lipopeptides, activates macrophages and initiates immune response	rs5743618	C > A	Missense (p.Ser602Ile)	0.172-0.500	[7, 8] Uciechov 2011; Naderi et 2016
TLR2	4q31.3	Toll-like receptor 2	Pattern recognition receptor	Recognizes bacterial lipopeptides, activates macrophages and initiates immune response	rs111200466	23 bp del	5' UTR	0.116–0.172	[9] Velez al. 2010
TLR10	4p14	Toll-like receptor 10	Pattern recognition receptor	Recognizes bacterial ligands, activates macrophages and initiates immune response	rs11096957	T > G	Missense (p.Asn241His)	0.324–0.486	[10, 11] Oosting e al. 2014; Bulat-Kardum e al. 2015
IRAK1	Xq28	Interleukin-1 receptor-associated kinase 1	Serine/ threonine kinase	Regulates TLR and IL-1R mediated signaling	rs1059703	G > A	Missense (p.Ser532Leu)	0.088–0.211	[12, 13] Sampath al. 2013; et al. 201
IFNG	12q15	Interferon-γ (IFN-γ)	Cytokine	Activates macrophages to eliminate intracellular pathogens, including Mtb	rs2430561	A > T	Intron	0.354–0.528	[14, 15] Pravica e al. 2000; Wei et al. 2017
TNF	6p21.3	Tumor necrosis factor alpha (TNF-α)	Cytokine	Activates macrophages to eliminate intracellular pathogens, attracts immunity cells to the site of infection	rs1800629	G > A	Promoter	0.094–0.187	[16, 17, 1 Elahi et a 2009; Correa et 2005; de Arellano al. 2020
IL1B	2q14.1	Interleukin 1 beta (IL-1β)	Cytokine	Activates T-cells and promotes the production of IFN-γ	rs1143643	C > T	Intron	0.286–0.388	[19] Hall al. 2015
IL10	1q32.1	Interleukin 10 (IL-10)	Cytokine	Inhibits IFN-γ production and MHC class II expression on macrophages	rs1800896	A > G	Promoter	0.456–0.601	[20, 21] Turner et 1997; Areeshi e al. 2017
SLC11A1	2q35	Natural Resistance-Associated Macrophage Protein 1	Metal ion transporter	Influences Mtb survival by regulating cation levels in the macrophage	rs2276631	C > T	Synonymous	0.202–0.332	[22, 23] Freïdin et 2006; Yu et al. 201

[a] Allele frequency range in European populations (CEU, FIN, GBR, IBS, TSI) according to 1000 Genomes (http://www.1000genomes.org); minor allele in CEU w used as the reference. Minor allele in CEU population is underlined.

SNP rs2276631 in SLC11A1 is non-functionmal (synonymous) variant, which is in strong LD with the nearby TB associated SNP rs3731865 (INT4) (D' = 0.97 and r^2 = 0.9479 in European populations).

Gene	Locus	Protein	Gene class	Protein function	Polymorphism	Nucleotide substitution	Location	Frequency [a]	Ref.
FOXP3	Xp11.23	Forkhead box protein P3 (FoxP3)	Transcriptional regulator	Inhibits cytokine production (IFN-γ) and T-cell effector function	rs2232365	T > C	Intron	0.338–0.450	[24] Beiranva et al. 201
VDR	12q13.11	Vitamin D receptor	Ligand activated transcription factor	Regulates expression of a number of genes involved in killing of Mtb	rs7975232	C > A	Intron	0.404–0.539	[25, 26] Uitterlind et al. 200 Xu and Shen 20
					rs1544410	C > T	Intron	0.338–0.470	[25, 26, 2 Uitterlind et al. 200 Chen et a 2013; Xu and Sher 2019
					rs2228570	A > G	Missense (p.Met1Thr)	0.327–0.429	[25, 26, 2 Uitterlind et al. 200 Chen et a 2013; Xu and Sher 2019
MR1	1q25.3	Major histocompatibility complex class I-related gene protein	Antigen-presenting molecule	Presents metabolites of microbial vitamin B to MAITs	rs1052632	G > A	Intron	0.266–0.303	[28] Seshadri al. 2017

[a] Allele frequency range in European populations (CEU, FIN, GBR, IBS, TSI) according to 1000 Genomes (http://www.1000genomes.org); minor allele in CEU w used as the reference. Minor allele in CEU population is underlined.

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The Republic of Moldova is a country with an unfavorable epidemiological situation regarding TB, ranking first for TB incidence (86/100,000 in 2018) in the European region [29]. The identification of host genetic factors may provide a biological and theoretical basis for better understanding the high prevalence of TB within Moldavian population and ultimately for the development of effective TB prevention and control strategies.

Materials And Methods

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 suggesting an 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 [30]. All participants in the study were BCG vaccinated.

Polymorphisms selection and genotyping

The following polymorphisms, *VDR* rs7975232, *VDR* rs1544410, *VDR* rs2228570, *MR1* rs1052632, *TLR1* rs5743618, *TLR2* rs111200466, *TLR10* rs11096957, *SLC11A1* rs2276631, *IL1B* rs1143643, *IL10* rs1800896, *IFNG* rs2430561, *TNF* rs1800629, *IRAK1* rs1059703, *FOXP3* rs2232365 were investigated. Polymorphisms were selected based on previously published associations with TB, thus increasing the chance of selecting polymorphisms with phenotyping effects (Table 1). All polymorphisms except the insertion/deletion variant rs111200466 in *TLR2* (-196 to -174 ins/del) were single nucleotide polymorphisms (SNPs).

Genotypes of the 12 SNPs (*VDR* rs7975232, *VDR* rs1544410, *VDR* rs2228570, *MR1* rs1052632, *TLR10* rs11096957, *SLC11A1* rs2276631, *IL1B* rs1143643, *IL10* rs1800896, *IFNG* rs2430561, *TNF* rs1800629, *IRAK1* rs1059703, *FOXP3* rs2232365) were determined 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). Two of the SNPs were not in Hardy-Weinberg equilibrium (HWE) in the control group –

rs11096957 in *TLR10* ($p = 0.0071$) and rs2430561 in *IFNG* (p = 0.013). Genotyping errors were excluded by random re-genotyping of the respective SNPs. Confirmatory genotyping of *TLR10* rs11096957 was performed in 52 control samples (21%) using polymerase chain reaction – restriction fragment length polymorphism (PCR–RFLP). PCR products were digested with the restriction endonuclease *Nla*III (New England Biolabs; Arundel, Australia) and subjected to 2% agarose gel electrophoresis. Confirmatory genotyping of *IFNG* rs2430561 was carried out by PCR with sequence-specific primers (SSPs) in 105 control samples (42%); amplified SNP products were electrophoresed on 2% agarose gel. All results were 100% consistent with those generated by MALDI-TOF.

Two polymorphisms *TLR1* rs5743618 and *TLR2* rs111200466 could not be included in the Agena iPlex assay, and therefore, were genotyped by PCR and PCR - RFLP. Genotyping of rs5743618 was performed using PCR–RFLP. PCR products were digested with the restriction endonuclease *Alu*I (New England Biolabs; Arundel, Australia) and subjected to 2% agarose gel electrophoresis. Genotyping of rs111200466 polymorphism was conducted by standard PCR and agarose (2%) gel electrophoresis.

The primer sequences, PCR conditions and restriction enzymes for genotyping of *TLR*2 rs111200466, *IFNG* rs2430561 and *TLR1* rs5743618 and *TLR10* rs11096957 are outlined in Additional file1: Table S1. The methodology used for the genotyping of *TLR2* rs111200466, *IFNG* rs2430561 and *TLR1* rs5743618 polymorphisms has been described elsewhere [14, 31, 32].

Statistical analysis

Hardy–Weinberg equilibrium (HWE) tests were performed in controls and cases by Fisher exact test. Fisher exact tests were also used for comparison of differences in allele frequencies between groups. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to measure associations between SNPs and the risk of pulmonary TB. Minor alleles were assumed as effect associated alleles in all tests. In addition, logistic regression was used to assess association of each SNP with the risk of pulmonary TB under log-additive genetic model. 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. All genetic tests described above are based on single SNP tests and were performed using PLINK software package (version 1.9, http:// pngu.mgh.harvard.edu/purcell/plink/) [33]. For *IRAK1* rs1059703 and *FoxP3* rs2232365 SNPs located on X chromosome, HWE *p*-values were calculated in females only and association tests were performed separately in males and females; therefore 16 tests were conducted in total. To correct significance level for multiple testing, Bonferroni correction was applied, and a significance level for all statistical tests was set at $p < 0.003125$ (0.05/16).

Haplotype analysis was performed for five polymorphisms in the *TLR1-TLR6-TLR10* gene cluster, two polymorphisms in the *TLR2* gene and three *VDR* gene polymorphisms using Haploview version 4.2 software [34]. SNPs *TLR1* rs4833095, *TLR2* rs3804099, *TLR6* rs5743810 and *TLR10* rs11466657 were taken from the previous study conducted on the same cohort of TB patients and healthy controls. The Solid spine of the linkage disequilibrium (LD) algorithm was applied to define the haplotype blocks. Differences in haplotype frequencies were tested using $\chi 2$ test. Haplotypes with a frequency below 1% were excluded from the analyses. Multiple comparisons of haplotype frequencies were corrected by generating empirical p-values via 10,000 permutations.

Pairwise epistatic interactions associated with susceptibility to TB were investigated in two-step approach. In the first step, logistic regression adjusted for sex and age under additive genetic model (implemented in software package SNPstats) was applied to all possible pairwise combinations of the 43 genetic markers from this and our previous study [35]. In total, 1065 (741 common and 2 × 162 sex-specific) comparisons were tested. In the second step, all genotype combinations for all significant marker pairs ($p < 0.05$) identified at the first step were analyzed using Fisher exact test and sex and age adjusted logistic regression [36, 37]. To account for multiple testing, we used Bonferroni correction and counted all tests of the first and second steps as independent, yielding a multiplication factor of 1/ (1065 + 543) = 0.000622.

Power analysis was performed using CaTS [38] with a log-additive genetic model, assuming significance (type I error) of 0.05 and the reported TB disease prevalence of 0.0025 [29]. The present 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.

Results

Single polymorphism analysis

Distributions of alleles of the investigated SNPs were in accordance with HWE in both groups except for *TLR10* rs11096957 (Additional file 2: Table S2), which showed some deviation from HWE in controls ($p = 0.0078$). The MAFs of all the polymorphisms in our controls were similar to those in the populations of European descent from the 1000 Genomes Project database (Table 2). Association analysis between individual SNPs and TB revealed an evidence for moderate association between rs1800629 in *TNF* and pulmonary TB risk (Fisher exact test: OR = 0.63, *p*-value = 0.01843; logistic regression: OR = 0.64, *p*-value = 0.03643) (Table 1), which did not remain significant after Bonferroni correction for multiple testing (*p*-value > 0.003125). None of the other studied loci showed associations with pulmonary TB risk (Table 2).

Table 2
Association analysis of individual polymorphisms with TB risk. For genotype data, see Supplementary Table 2.

Polymorphism	Major/Minor allele	MAF in controls	MAF in patients	Fisher exact test		Logistic regression [a]	
				p-value	OR (95% CI)	*p*-value	OR (95% CI)
Both sexes							
VDR rs7975232	C/A	0.481	0.509	0.3797	1.12 (0.87–1.43)	0.1819	1.20 (0.92–1.57)
VDR rs1544410	C/T	0.379	0.374	0.8977	0.98 (0.76–1.26)	0.9695	0.99 (0.76–1.30)
VDR rs2228570	G/A	0.438	0.400	0.2262	0.85 (0.66–1.10)	0.3169	0.88 (0.67–1.14)
MR1 rs1052632	G/A	0.271	0.259	0.7118	0.94 (0.70–1.26)	0.7854	0.96 (0.71–1.30)
TLR1 rs5743618	G/T	0.402	0.407	0.8991	1.02 (0.80–1.31)	0.9359	1.01 (0.78–1.32)
TLR2 rs111200466	23 bp ins/del	0.175	0.165	0.6813	0.93 (0.68–1.29)	0.5575	0.90 (0.65–1.27)
TLR10 rs11096957	T/G	0.474	0.472	0.9504	0.99 (0.78–1.27)	0.7115	1.05 (0.82–1.35)
SLC11A1 rs2276631	C/T	0.242	0.249	0.8266	1.04 (0.78–1.39)	0.9478	0.99 (0.73–1.35)
IL1B rs1143643	C/T	0.363	0.343	0.5127	0.92 (0.71–1.18)	0.2367	0.85 (0.65–1.11)
IL10 rs1800896	T/C	0.447	0.447	1.0000	1.00 (0.77–1.31)	0.9803	1.00 (0.75–1.32)
IFNG rs2430561	T/A	0.464	0.442	0.4925	0.92 (0.72–1.17)	0.8409	1.02 (0.79–1.32)
TNF rs1800629	G/A	0.138	0.091	**0.01843**	**0.63 (0.42–0.92)**	**0.03643**	**0.64 (0.43–0.97)**
Males							
IRAK1 rs1059703	A/G	0.192	0.242	0.4356	1.34 (0.72–2.51)	0.3563	1.34 (0.72–2.51)
FoxP3 rs2232365	C/T	0.404	0.456	0.4353	1.24 (0.74–2.07)	0.4156	1.24 (0.74–2.07)
Females							
IRAK1 rs1059703	A/G	0.250	0.233	0.686	0.91 (0.61–1.36)	0.6544	0.91 (0.61–1.36)
FoxP3 rs2232365	C/T	0.463	0.413	0.2562	0.81 (0.58–1.15)	0.2431	0.81 (0.58–1.15)

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

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

Bold: significant associations (nominal *p*-value < 0.05).

Haplotype analysis

Nine common haplotypes in the gene cluster *TLR1-TLR6-TLR10*, four in the gene *TLR2* and three in the gene *VDR* were identified at a frequency ≥ 1% by LD analysis (Fig. 1). The rs11466657-rs11096957-rs5743618-rs4833095-rs5743810 haplotype A-T-G-T-A of the gene cluster *TLR1-TLR6-TLR10* was significantly higher in the TB group compared to the controls ($p = 0.0485$; Table 3). However, after 10 000-fold permutation testing this haplotype lost its significance ($p = 0.4463$). In addition, marginal differences between cases and controls were observed for the haplotype A-T-G-T-G (rs11466657-rs11096957-rs5743618-rs4833095-rs5743810) in the gene cluster *TLR1-TLR6-TLR10* (p = 0.0688) and haplotypes ins-T and ins-C (rs111200466-rs3804099) in *TLR2* ($p = 0.0732$ and $p = 0.0987$, respectively). None of the remaining haplotypes were associated with the risk of pulmonary TB in this study (Table 3).

Table 3
Haplotype frequencies and associations with pulmonary TB.

Haplotypes [a]	Frequency in Patients [b]	Frequency in Controls	*p*-value [c]	Permutation *p*-value
TLR2				
insT	0.591	0.536	0.0732	0.6576
insC	0.244	0.289	0.0987	0.7665
delC	0.154	0.160	0.7951	1
delT	0.012	0.016	0.5744	1
TLR1-TLR6-TLR10				
ATGTA	0.298	0.243	**0.0485**	0.4463
AGTCG	0.255	0.263	0.7784	1
ATGTG	0.170	0.215	0.0688	0.6201
AGGTG	0.110	0.121	0.5933	1
AGTTG	0.045	0.041	0.7844	1
GGTCG	0.044	0.028	0.1705	0.9125
ATTTG	0.030	0.033	0.7762	1
ATTCG	0.023	0.034	0.2712	0.9818
AGGTA	0.010	0.018	0.27	0.9816
VDR				
CC	0.492	0.515	0.4556	1
AT	0.375	0.371	0.8939	1
AC	0.133	0.113	0.3484	0.9917

[a] Haplotypes: *TLR2* [rs111200466, rs3804099]; *TLR1-TLR6-TLR10* [rs11466657, rs11096957, rs5743618, rs4833095, rs5743810]; *VDR* [rs7975232, rs1544410]

[b] Only haplotypes with a frequency > 0.01 were included in this table.

[C] *p*-values were calculated using χ^2 test.

Bold: significant differences (nominal *p*-value < 0.05) in haplotype distribution.

Gene-gene interaction analysis

Analysis of SNP-SNP combinations by logistic regression model revealed 59 significant (p-value < 0.05) genetic interactions (Additional file 3: Table S3), with the smallest *p*-values observed for the marker pairs *TOLLIP* rs3793964 - *IRAK2* rs3844283 ($p = 0.0025$), *TLR2* rs111200466 - *TOLLIP* rs5743899 (p = 0.0044), and *TLR10* rs11096957 - *TLR6* rs5743810 ($p = 0.0052$). Although none of these associations exceeded the conservative Bonferroni-corrected threshold of *p*-value = 4.69484E-05 (0.05/1065), they suggest a potential role in conferring TB risk and therefore were pipelined into in depth genotype combination analysis. This resulted in 62 nominally significant associations (p-value < 0.05 for either logistic regression or Fisher exact test) out of 543 possible genotype combinations (Additional file 4: Table S4). The strongest association was observed between TB and the combination of *TLR6* rs5743810 GA and *TLR10* rs11096957 GT genotypes (Fisher exact test *p*-value = 1.5×10^{-5}; logistic regression *p*-value = 1.9×10^{-5}; Table 4). This association remained significant after Bonferroni correction (*p*-value = 0.05/ (1065 + 543) = 3.11×10^{-5}). Remarkably, the effect of this genotype combination on TB risk was much greater compared to the effects of individual genotypes (heterozygous model, OR = 1.72, $p = 0.0042$ and OR = 1.33, $p = 0.13$ for rs5743810 and rs11096957, respectively), implying strong synergistic interaction between them. None of the other genotype combinations passed Bonferroni correction.

Table 4
Association of combined genotypes *TLR6* rs5743810 and *TLR10* rs11096957 with pulmonary TB.

Genotype		Counts, *n* (%)		Association (*p*-value)		OR (95% CI) [a]
TLR6 rs5743810	*TLR10* rs11096957	Controls	Patients	Fisher test	Logistic regression [a]	
GG	TT	58 (23.8)	56 (21.1)	0.56	0.83	0.94 (0.60–1.47)
GG	GT	57 (23.4)	43 (16.2)	0.059	0.033	0.61 (0.38–0.96)
GG	GG	19 (7.8)	16 (6.0)	0.55	0.59	0.80 (0.37–1.72)
GA	TT	7 (2.9)	2 (0.8)	0.14	0.094	0.25 (0.025–1.36)
GA	GT	42 (17.2)	91 (34.3)	**1.5×10^{-5}**	**1.9×10^{-5}**	**2.48 (1.62–3.85)**
GA	GG	42 (17.2)	38 (14.3)	0.45	0.34	0.79 (0.48–1.30)
AA	TT	17 (7.0)	18 (6.8)	0.94	0.86	0.91 (0.43–1.91)
AA	GT	2 (0.8)	1 (0.4)	0.94	0.60	0.45 (0.0077–8.86)

[a] Adjusted for sex and age at recruitment.

Bold: significant association after Bonferroni correction (*p*-value < 3.10945×10^{-5}).

Discussion

In the present study, we applied a candidate-gene approach and tested the association of 14 polymorphisms in genes *VDR*, *MR1*, *TLR1*, *TLR2*, *TLR10*, *SLC11A1*, *IL1B*, *IL10*, *IFNG*, *TNF*, *IRAK1*, and *FOXP3* with the risk of pulmonary TB in Moldavian population. Although these genes are critical components of human immunity and their polymorphisms have been implicated in susceptibility/resistance to TB, we could not find convincing statistical evidence for their association with the risk of pulmonary TB in this study. A borderline association was revealed for polymorphism rs1800629 in the *TNF* gene only, but this did not survive corrections for multiple testing by Bonferroni method. Overall, the results presented here do not support a major role of the analyzed common variants in conferring susceptibility/resistance to pulmonary TB in Moldavian population.

The inability to reach significance level after Bonferroni correction may be explained by a fairly small sample size and inadequate statistical power to produce convincing associations for polymorphisms with low and moderate effects (with OR < 1.5). For this reason, we cannot completely rule out the possibility of true functional effects for variants with nominal associations, in particular for *TNF* rs1800629 polymorphism. The TNF gene encodes a multifunctional proinflammatory cytokine TNF-α which is mainly produced by activated monocytes, macrophages and T-lymphocytes when stimulated by mycobacterial antigens. TNF-α acts synergistically with IFN-γ to induce macrophages killing of M.tb [39]. In addition, TNF-α is involved in the recruitment of leukocytes to the site of infection and contributes to the formation of TB granuloma, preventing the spread of infection [39]. It is reported that treatment with TNF-α inhibitors leads to reactivation of latent TB infection, indicating TNF-α as a key cytokine towards resistance to M.tb [40]. SNP rs1800629 (– 308G > A) is located within regulatory hotspot region and thus influences transcription critically [41, 42]. The minor allele A of rs1800629 has been associated with increased expression of *TNF* and higher plasma levels of TNF-α [17]. In agreement with published functional studies, our results demonstrated a higher frequency of allele A in controls than in cases, suggesting its protective role against TB (OR = 0.63). Similarly, allele A was protectively associated with TB in Colombian [16] and Mexican [18] populations. However, other genetic epidemiologic studies involving patient cohorts from various population groups, including Malawi [43], Iran [44], Indian [45], Cambodian [46], Chinese Han and Tibetan [47], did not confirm the above findings. The disparity in results across studies may be explained by certain factors such as inadequate sample sizes and differences in environmental, demographic, cultural, host genetic and bacterial characteristics of M.tb strains.

Additionally, a nominal yet suggestive association was demonstrated for haplotype A-T-G-T-A of the block rs11466657-rs11096957-rs5743618-rs4833095-rs5743810 in the gene cluster *TLR1-TLR6-TLR10*. Genes *TLR1*, *TLR6*, and *TLR*10 are located in a 54-kb genomic region on chromosome 4p14 and encode proteins that share a high degree of homology in their amino acid sequences. All three genes belong to the TLR2 subfamily of TLRs, which plays a critical role in the early recognition of M.tb and subsequent activation of immune responses [48]. Individual polymorphisms and haplotypes within the *TLR10-TLR1-TLR6* locus have been associated with altered susceptibility to infectious disease, including mycobacterial infections of leprosy and TB [11, 49, 50, 51, 52]. Unfortunately, different sets of SNPs used in this and in other studies complicate direct comparisons of the results. Even so, the identified haplotype A-T-G-T-A and, more generally, variations in genes *TLR10*, *TLR1* and *TLR6* could be a promising replication target for future studies in larger cohorts.

Genetic interactions are thought to underlie susceptibility/resistance to TB [5, 53], so they could explain some of the missing heritability in this study. Therefore, we also analyzed the impact of allele combinations on TB risk. The strongest evidence for interaction in our data was between SNPs rs5743810 and rs11096957 located in genes *TLR6* and *TLR10*, respectively. Interestingly, the two SNPs showed no or only weak effect on TB susceptibility when evaluated alone, indicating a synergetic mechanism of *TLR6* rs5743810 and *TLR10* rs11096957 in conferring risk for pulmonary TB.

Interaction between these SNPs is biologically plausible. Firstly, TLRs are the key players in the host defense against infections. Specifically, TLR6 functionally interacts with TLR2 to mediate the cellular response to bacterial lipoproteins and activate the NF-κB pathway and inflammatory events through MyD88 dependent signaling [10, 48, 52]. TLR10 has also the ability to form heterodimers with TLR2, but its specific ligands have not yet been identified and its downstream signaling is not fully understood. It is thought to act through both MyD88 dependent and independent signaling pathways with mainly inhibitory effects on inflammation [10]. The genetic interaction between *TLR6* and *TLR10* observed in this study may reflect their mutual functional contribution to M.tb

recognition and subsequent downstream signaling (Fig. 2). Secondly, the investigated SNPs in *TLR6* and *TLR10* genes had been shown before to be of functional significance. In fact, the two SNPs are non-synonymous variants located in the extracellular (leucine-rich repeat) domains of the encoded proteins. Both *ex vivo* and *in vitro* experiments showed that SNPs rs5743810 (Ser249Pro) and rs11096957 (Asn241His) may influence pro-inflammatory cytokine production in humans [10, 54, 55]. In addition, polymorphism rs5743810 was observed to affect NF-κB signaling activity, thereby modulating inflammatory responses [56]. Furthermore, the two polymorphisms have been associated with several immune-related pathologic conditions and infectious diseases, including TB [11, 51, 52, 57]. These data support the relevance of additive interaction between SNPs rs5743810 and rs11096957 and suggest a molecular mechanism by which genetic variations in *TLR6* and *TLR10* genes might increase susceptibility to TB (Fig. 2).

The present study is the first to identify an interaction between *TLR6* rs5743810 and *TLR10* rs11096957 gene variants in TB risk. Further larger case–control studies followed by functional tests are warranted to validate this initial finding and eventually translate it into clinical practice. Particularly, given the high spread of the combined *TLR6* rs5743810 GA - *TLR10* rs11096957 GT genotype in European population (~ 15–20%), it might be used as a novel predictive biomarker for identification of individuals at high risk for active TB disease.

Some limitations of our study deserve consideration. Firstly, it was limited in power to detect the weak association signals, so our negative results should be treated with caution. 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 polymorphisms in the candidate genes analyzed was limited. Given their key role in TB pathogenesis, additional TB risk variants, haplotypes and allele combinations may possibly exist.

A potential limitation of this study is a significant deviation from HWE of the interacting SNP *TLR10* rs11096957 (Asn241His) in the controls. Such deviations can result from genotyping errors, recruiting biases, natural selection or be simply a chance. We excluded genotyping errors by random re-genotyping of *TLR10* rs11096957 in 21% of samples. Also, our study design prevented the recruitment of any relatives. The natural selection could be the reason for the observed deviation, which may be supported by the evidence of similar heterozygosity deficiency in Toscani in Italy, TSI (HWE *p*-value = 0.02; 1000 Genomes Project data) and considerable intra-population variation of SNP *TLR10* rs11096957 within Europe (the allele G frequency range: 32.4% in British, GBR − 48.6% in Iberian population, IBS; 1000 Genomes Project data). Moreover, the recruitment of healthy controls from TB communities used in this project may have a similar kind of impact for the locus *TLR10* like natural selection, contributing to the deficiency of rs11096957 heterozygotes. Taken together, these arguments justify the inclusion of *TLR10* rs11096957 in association tests.

Conclusion

In the current study, we replicated a significant association between rs1800629 in the *TNF* gene and pulmonary TB. Also, haplotypes in the gene cluster *TLR1-TLR6-TLR10* showed a weak yet suggestive association. Further, we provide convincing statistical evidence for a synergistic effect between polymorphic variants in the *TLR6* and *TLR10* genes on TB risk, which further supports the importance of TLR signaling in the genetic basis of TB and the concept of genetic interactions accounting for missing heritability. Further studies are warranted to validate the interaction between *TLR6* and *TLR10* and to elucidate its role in TB risk, which would be beneficial for human health.

Abbreviations

TB
tuberculosis
M.tb
Mycobacterium tuberculosis
AIDS
acquired immune deficiency syndrome
TLR1
toll like receptor 1 (gene)
TLR2
toll like receptor 2 (gene)
TLR6
toll like receptor 6 (gene)
TLR8
toll like receptor 8 (gene)
TLR9
toll like receptor 9 (gene)
TLR10
toll like receptor 10 (gene)
IFNG
interferon gamma (gene)
TNF
tumor necrosis factor alpha (gene)
IRAK1
interleukin-1 receptor-associated kinase 1 (gene)

IL1B
interleukin 1 beta (gene)
IL10
interleukin 10 (gene)
NRAMP1
Natural Resistance-Associated Macrophage Protein 1 (gene)
SLC11A1
solute carrier 11 member 1 (gene)
FOXP3
Forkhead box protein P3 (gene)
VDR
Vitamin D receptor (gene)
MR1
Major histocompatibility complex class I-related gene protein (gene)
HIV
human immunodeficiency viruses
BCG
bacillus Calmette–Guérin
EDTA
ethylenediaminetetraacetic acid
HWE
Hardy–Weinberg Equilibrium
SNP
single nucleotide polymorphism
MALDI-TOF
matrix-assisted laser desorption/ionization time-of-flight
PCR
polymerase chain reaction
RFLP
restriction fragment length polymorphism
SSPs
sequence-specific primers
MAF
minor allele frequency
OR
odds ratio
CI
confidence interval
LD
linkage disequilibrium
NF-κB
nuclear factor kappa-light-chain-enhancer of activated B cells
MyD88
myeloid differentiation primary response 88
CEU
Utah residents with Northern and Western European ancestry
FIN
Finnish in Finland
GBR
British from England and Scotland
IBS
Iberian populations in Spain
TSI
Toscani in Italia

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethic Committee of the Institute of Phthisiopneumology (Republic of Moldova) and was conducted in accordance with the Declaration of Helsinki. All participants gave their written informed consent.

Consent for publication

All authors consent to the publication of this manuscript.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The raw data (i.e., sample-linked data on the sex, age and genotypes) analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AV wrote the manuscript. AV and IVD designed the study and analyzed the data. ET designed the study and collected the data. AV and HG performed the experiments. TI had primary responsibility for the final content. All authors read and approved the final manuscript.

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Figures

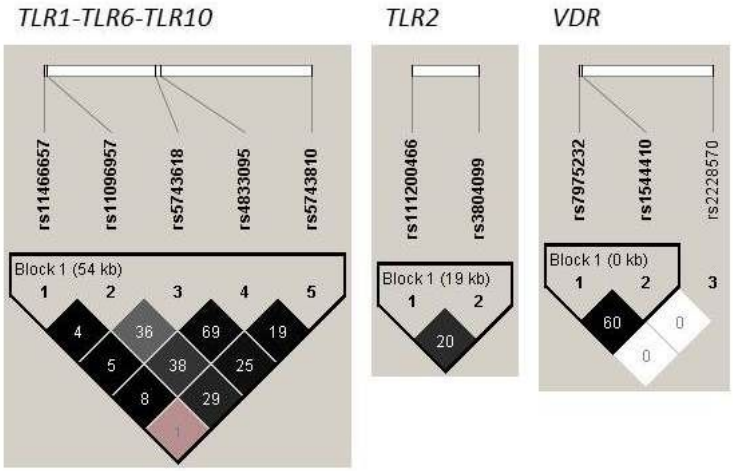

Figure 1

Linkage disequilibrium (LD) plots of TLR2, VDR and TLR1-TLR6-TLR10 polymorphisms in the combined group of cases and controls. The colours of the squares represent D' values, with black being D' = 1, and white D' = 0. The r2 values (%) are indicated on the squares (no value = 100%).

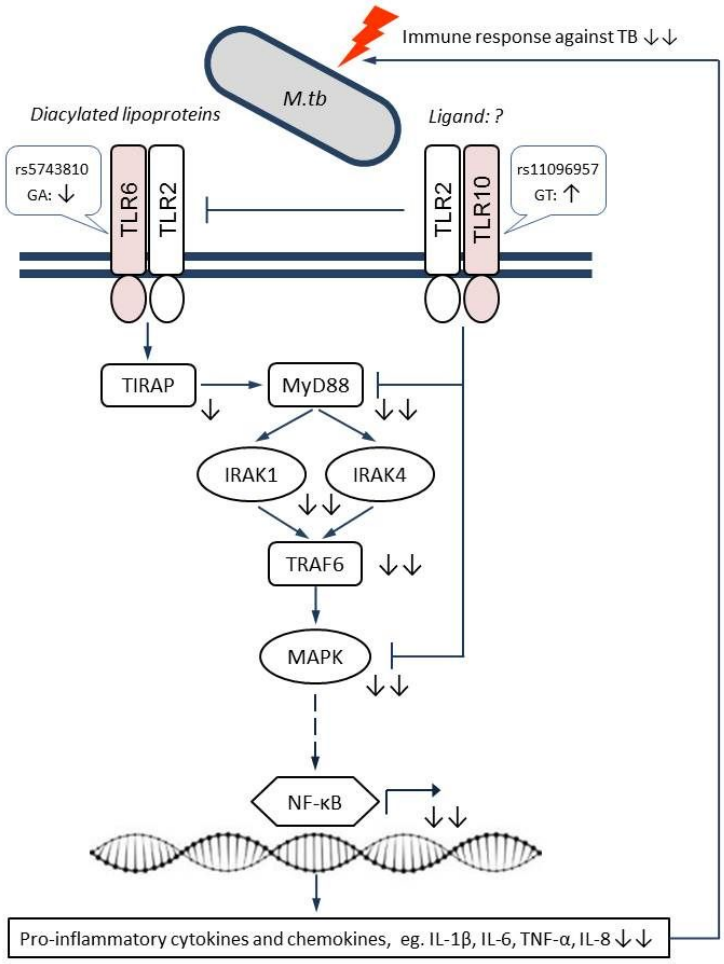

Figure 2

Schematic representation of the proposed epistatic/synergistic interaction between SNPs TLR6 rs5743810 and TLR10 rs11096957 in conferring susceptibility to TB based on the present results. Symbols indicate the following: sharp arrow - positive interaction; blunt-pointed arrow - negative interaction; solid line – direct interaction; dashed line - indirect interaction; question mark - unknown ligand partner; (↑) - up-regulation/activation; (↓) - down-regulation/suppression. TLR6 and TLR10 have largely opposite effects on the immune response, with TLR10 mainly having a suppressive function. Simultaneous suppression of TLR6 signaling and activation of TLR10 signaling caused by genetic variations may result in decreased pro-inflammatory responses against M.tb, and thus increase the risk of TB. Figure adapted from Oosting et al. 2014.

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