

## **TOLLIP rs5743854 and rs116938768 Gene Polymorphisms in Bacillus Calmette-Guerin Osteitis**

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### **Abstract**

**Background:** Toll interacting protein (TOLLIP) encoded by the *TOLLIP* gene is mainly an inhibitory protein connected to toll-like receptors (TLR). TOLLIP regulates TLR2-mediated signalling, and in our previous studies, *TLR2* gene variations increased the risk of osteitis after Bacillus Calmette-Guerin (BCG) vaccination.

**Methods:** We determined two single nucleotide polymorphisms (SNP) of the *TOLLIP* gene, the rs5743854 and rs116938768, in 132 study subjects who presented with BCG osteitis after newborn vaccination and compared the genotype and allele frequencies between them and population controls. In addition, we compared eleven serum cytokines between carriers of the wild versus variant genotypes.

**Results:** The genotype or allele frequencies of the *TOLLIP* rs5743854 or rs116938768 did not differ between cases and controls. Serum concentrations of eleven cytokines did not differ between carriers of the wild versus variant genotypes.

**Discussion:** The findings suggest that TOLLIP may not have an impact on the osteitis risk after newborn BCG vaccination.

**Keywords:** *Bacillus Calmette-Guerin; BCG Osteitis; Gene Polymorphism; Toll-Interacting Protein*

### **Abbreviations**

BCG: Bacillus Calmette-Guerin; DNA: Deoxyribonucleic Acid; HWE: Hardy-Weinberg Equilibrium; IL: Interleukin; IFN: Interferon; mRNA: Messenger Ribonucleic Acid; SNP: Single Nucleotide Polymorphisms; TOLLIP: Toll-Interacting Protein; TLR: Toll-Like Receptor; TNF: Tumor Necrosis Factor

### **Introduction**

Finnish newborns were vaccinated as part of the national vaccination program with the Bacillus Calmette-Guerin (BCG) vaccine from the 1940s until 2006, and 222 children presented with BCG osteitis from 1960 to 1988 [1,2].

Toll-like receptors (TLR), especially TLR1, TLR2, TLR4 and TLR6 seem to be involved in immune responses against mycobacteria [3,4]. Toll interacting protein (TOLLIP), encoded by the *TOLLIP* gene is an inhibitory protein connected to human TLRs. TOLLIP regulates TLR2 and TLR4 functions and further, production of cytokines such as interleukins (IL), interferons (IFN) and tumour necrosis factors (TNF) [5,6].

Our previous study included 132 of 222 former Finnish BCG osteitis patients, and they differed from population controls with respect to *TLR1*, *TLR2* and *TLR6* polymorphisms [7]. The *TLR2* variant genotypes were associated with a higher risk of BCG osteitis and lower IFN- $\gamma$  production in BCG-stimulated cell cultures [7,8]. However, no associations were found between *TLR1*, *TLR2* or *TLR6* polymorphisms and serum concentrations of 11 cytokines when measured in adulthood [9].

### **Aim of the Study**

The aim of the study was to compare the presence of the wild versus variant genotypes and the minor versus major allele frequencies of the *TOLLIP* rs5743854 and rs116938768 between BCG osteitis patients and population controls. In addition, we compared serum IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, IL17-F, IFN- $\gamma$  and TNF- $\alpha$ , concentrations between the carriers of the wild versus variant genotypes.

### **Materials and Methods**

#### **Subjects**

The diagnostics of invasive complications caused by the BCG vaccine in Finland was centralized in one national laboratory, the Public Health Institute, Helsinki, from 1960 to 1988. At that time, BCG vaccination was provided to all Finnish newborns, and altogether 222 BCG osteitis cases after newborn vaccination were diagnosed based on culture of the BCG strain and/or on typical histology of the bone sample [1,2]. In 2007 - 2008, 132 former BCG osteitis patients gave blood samples for further studies on susceptibility to BCG vaccination complications [7,8]. At that time, the study subjects were 21 to 49 years old. Fresh whole blood samples were taken in local laboratories across the country and were sent within 24 hours to the laboratory of the National Institute for Health and Welfare, Turku, Finland [7,10]. DNA was isolated from whole blood, and DNA and serum samples were frozen at -70°C for further analyses. In the present study, the frozen samples were transferred to the laboratory of Medical Microbiology and Immunology at the University of Turku, Turku, Finland

#### **Genetic testing**

The *TOLLIP* rs5743854 and rs116938768 polymorphisms were determined using PCR-based sequencing and following primers: (forward) 5'-TTCGGACGTGCGACCC-3' and (reverse) 5'-AACCGCGCCCATCTTTA-3'. The primers were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The sequencing was performed at the Institute for Molecular Medicine Finland laboratories, Helsinki, Finland.

#### **Control group**

Data on the *TOLLIP* rs5743854 and rs116938768 polymorphisms were available from 99 healthy Finnish subjects in the publicly available database of the 1000 Genomes Project [11].

#### **Measurement of serum cytokines**

Serum concentrations of IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, IFN- $\gamma$  and TNF- $\alpha$  were measured by the Bio-Plex Pro human immunoassay kit (Bio-Rad, Helsinki, Finland), as described previously in details for IL-17A [9,12]. Serum IL17-F concentration was measured by a commercial ELISA kit (DuoSet ELISA, Human IL-17F, R&D systems, Abingdon, the UK) [9]. The detection limit was 12.5 mg/mL for IL-17F and 2.5 mg/mL for the other 10 cytokines.

#### **Subjects of the present study**

The number of good-quality samples for genotyping of the *TOLLIP* SNPs was 132 and for serum cytokine measurements 130.

#### **Statistics**

The Statistical Package of SPSS for Windows, version 23 (IBM Corp, Armonk, NY, USA) was used for statistical analyses. The distributions of the genotypes and allele frequencies between cases and controls were compared using Chi square and Fisher's exact tests, as appropriate. The Mann-Whitney U test was used to compare the cytokine concentrations as continuous variables. Deviations from the Hardy-Weinberg equilibrium (HWE) were studied with the HWE Calculator at [www.changbiocentence.com](http://www.changbiocentence.com) and the alleles of the two *TOLLIP* SNPs included were in the HWE.

## Ethics

The study subjects gave their voluntary, informed, written consent for studies including permission to perform genetic analyses concerning susceptibility to BCG vaccination complications. The study was approved by the Ethics Committee of the Tampere University Hospital District, Tampere, Finland.

## Results

The frequency of the variant *TOLLIP* rs5743854 genotype was 35.6% in BCG osteitis patients and 30.3% in controls ( $p = 0.312$ ). The MAFs were 18.6% versus 14.6% ( $p = 0.266$ ). The variant homozygous GG genotype was present in two cases (Table 1).

The frequency of the variant *TOLLIP* rs116938768 genotype was 11.4% in BCG osteitis patients and 7.1% in controls ( $p = 0.271$ ). The MAFs were 5.7% versus 3.5% ( $p = 0.284$ ). The variant homozygous TT genotype was present in no case (Table 1).

Genotypes and allele frequencies	Cases N = 132	Controls N = 99	p value
<b><i>TOLLIP</i> rs5743854</b>			
CC, wild	85	70	--
CG/GG, variant	47(35.6%) <sup>#</sup>	29(30.3%) <sup>&amp;</sup>	0.312
MAF(G)	49/264(18.6%)	29/198 (14.6%)	0.266
<b><i>TOLLIP</i> rs116938768</b>			
CC, wild	117	92	--
CT, variant	15(11.4%) <sup>§</sup>	7(7.1%) <sup>§</sup>	0.271
MAF (T)	15/264(5.7%)	7/198(3.5%)	0.284

**Table 1:** The *TOLLIP* rs5743854 and rs116938768 genotypes in 132 study subjects with BCG osteitis after newborn vaccination compared with 99 population controls from the FIN data of the 1000 Genome Project.

<sup>#</sup>: Two homozygous GG genotypes; <sup>&</sup>: No homozygous GG genotypes; <sup>§</sup>: No homozygous TT genotypes; MAF: Minor Allele Frequency.

Serum concentrations of the 11 studied cytokines did not differ between the subjects with wild and variant genotypes of the *TOLLIP* rs5743854 (Table 2) or the *TOLLIP* rs116938768 (Table 3).

Cytokine	Wild, CC Median (25% - 75% range) N = 85	Variant CG or GG Median (25% - 75% range) N = 47	P value
IL-1-beta	0.92 (0.68 - 1.6)	0.8 (0 - 1.4)	0.200
IL-4	0.28 (0.12 - 0.68)	0.32 (0 - 0.68)	0.990
IL-6	4.5 (1.9 - 11.3)	5.8 (2.2 - 12.8)	0.572
IL-8	60.8 (26.5 - 136.8)	52.0 (25.8 - 168.3)	0.880
IL-10	12.4 (7.0 - 31.5)	9.3 (6.4 - 22.5)	0.321
IL-12	16.0 (8.0 - 44.3)	18.2 (17.4 - 49.4)	0.779
IL-17A	25.8 (10.3 - 62.9)	29.2 (16.1 - 60.9)	0.276
IL-17F†‡	60.1 (0 - 200.2)	22.3 (0 - 151.9)	0.400
IL-23	0.42 (0 - 10.2)	0 (0 - 8.7)	0.491
TNF-alpha	0 (0 - 2.5)	0 (0 - 1.9)	0.616
IFN-gamma	30.4 (3.5 - 94.8)	25.6 (0 - 99.7)	0.575

**Table 2:** Serum concentrations of 11 cytokines in 130 former BCG osteitis patients in relation to wild versus variant genotypes of the *TOLLIP* rs5743854 at 20 - 49 years of age

\*: The 75% limit 2.5 pg/mL (the detection limit); †: The detection limit was 12.5 pg/mL; ‡: 128 samples; and §: Exact concentrations were measured until 4000.0 pg/mL.

Cytokine	Wild, CC Median (25% - 75% range) N = 117	Variant, CT Median (25% - 75% range) N = 15	P value
IL-1-beta	0.8 (0.6 - 1.5)	1.1 (0.6 - 1.9)	0.574
IL-4	0.3 (0.08 - 0.68)	0.3 (0.1 - 16.6)	0.711
IL-6	4.8 (1.9 - 11.5)	5.8 (2.7 - 14.7)	0.473
IL-8	63.2 (29.1 - 149.4)	41.4 (22.1 - 73.7)	0.084
IL-10	9.7 (6.6 - 26.4)	15.9 (6.7 - 32.2)	0.519
IL-12	15.8 (7.9 - 44.8)	19.8 (11.5 - 51.0)	0.388
IL-17A	28.7 (13.7 - 63.2)	34.2 (14.0 - 53.2)	0.971
IL-17F†‡	37.8 (0 - 194.7)	46.5 (0 - 164.4)	0.604
IL-23	0 (0 - 8.9)	0 (0 - 16.6)	0.775
TNF-alpha	0 (0 - 2.2)	0.4 (0 - 2.6)	0.702
IFN-gamma	28.2 (3.5 - 94.9)	43.0 (0 - 103.1)	0.960

**Table 3:** Serum concentrations of 11 cytokines in 130 former BCG osteitis patients in relation to wild versus variant genotypes of the TOLLIP rs116938768 at 20–49 years of age

\*: The 75% limit 2.5 pg/mL (the detection limit); †: The detection limit was 12.5 pg/mL; ‡: 128 samples; and §: Exact concentrations were measured until 4000.0 pg/mL.

## Discussion

The main finding of the present study was that there were no significant associations between the TOLLIP rs5743854 or rs116938768 variations and BCG osteitis after newborn BCG vaccination. In the study of Shah, *et al.* [6], the TOLLIP-deficient genotype GG at the rs5743854, which was studied also in the present study, was associated with decreased BCG-specific memory cell proliferation. In that study, TOLLIP rs5743899 and rs4963035 SNPs were also determined, and no such associations were found [6].

In this our cohort, TLR2 and TLR6 variant genotypes were associated with a higher risk and TLR1 variant genotype with a lower risk of BCG osteitis [7]. TOLLIP regulates functions of TLRs including TLR2-mediated signalling, and further, production of mediators of innate immunity [3,4].

In a study from China, the researchers genotyped 11 polymorphisms of the TLR2, TLR4 and TOLLIP genes in 410 adults with latent or active pulmonary tuberculosis and in 204 healthy controls [13]. The TOLLIP rs5743889 and rs5743867 variations, which are located in the promoter region of the gene close to the TOLLIP rs5743854 applied in the present study, were risk factor for the progression of tuberculosis [13]. Instead, TLR2 or TLR4 variations were not associated with tuberculosis [13], but TLR2 and TLR4 polymorphisms were associated with tuberculosis in a meta-analysis [3]. In our BCG osteitis cohort, the TLR2 subfamily variations were associated with BCG osteitis risk [7], but the TOLLIP variations were not, as documented in this paper.

The TOLLIP rs5743854 applied in the present study is no doubt functional, but functionality data are lacking concerning the TOLLIP rs116938768, which we also determined. The TOLLIP single nucleotide polymorphisms (SNP) was associated with decreased TOLLIP mRNA expression (rs3750920) after stimulation with TLR2 and TLR4 in monocytes of healthy adult volunteers [5]. In the Vietnamese population, the TOLLIP rs3750920 and rs5743899 were associated with tuberculosis [6]. The TOLLIP rs5743854 was associated with decreased TOLLIP mRNA expression in monocytes of infants vaccinated with BCG, but this finding was significant only when the variant genotypes were homozygous [6]. This can be one explanation for our unexpected negative result, since only two of the variant genotypes were homozygous. Of these three SNPs with functionality data available, the TOLLIP rs5743854 was determined in the present study.

We were not able to confirm any significant associations between the TOLLIP rs5743854 or rs116938768 SNPs and serum concentrations of 11 cytokines, although the TOLLIP rs5743899 suppressed the production of anti-inflammatory IL-16 and induced the production

of pro-inflammatory IL-10 after stimulation with TLR2 and TLR4 in monocytes of healthy adult volunteers [5]. Cytokine production needs a stimulus, such as ongoing infection or inflammation. Our requirement for the sampling was that the study subjects had to be symptom-free without any acute infections or exacerbations of chronic diseases during sampling [7,10].

The main strength of the present study is the unique cohort of more than 130 patients who presented with firmly diagnosed BCG osteitis after receiving BCG vaccination as newborns. Other studies on BCG osteitis have comprised only individual cases or small case series. Our initially 222 patients formed two-thirds of all 341 thus far globally detected BCG osteitis patients [14]. Despite this, the number of our patients may be too small for genetic studies.

## Conclusion

The genotype or allele frequencies of the *TOLLIP* rs5743854 or rs116938768 genes did not differ between former BCG osteitis patients and population controls. Serum concentrations of eleven cytokines did not differ between carriers of the wild versus variant genotypes of the *TOLLIP* genes. The results offer preliminary evidence that *TOLLIP* seems not be involved in BCG vaccination complications.

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