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LAURA PÖYHÖNEN

Immunity in Bacillus Calmette-Guérin Osteitis

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To Jesus and my Family

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, referred to in the text by Roman numerals I–IV, and unpublished data:

I Pöyhönen, L., Kröger, L., Gröndahl-Yli-Hannuksela, K., Vuononvirta, J., Huhtala, H., He, Q., Korppi M. (2013). Variant MBL2 genotypes producing low mannose-binding lectin may increase risk of Bacillus Calmette-Guérin osteitis in vaccinated newborns. *Acta Paediatrica*, 102, 1095–1099.

II Pöyhönen, L., Nuolivirta, K., Vuononvirta, J., Kröger, L., Huhtala, H., Mertsola, J., He, Q., Korppi M. (2015). Toll-like receptor 2 subfamily gene polymorphisms are associated with Bacillus Calmette-Guérin osteitis following newborn vaccination. *Acta Paediatrica*, 104, 485–490.

III Pöyhönen, L., Kröger, L., Huhtala, H., Mäkinen, J., Mertsola, J., Martinez-Barricarte, R., Casanova, J. L., Bustamante, J., He, Q., Korppi M. Interferon-gamma-dependent immunity in Bacillus Calmette-Guérin vaccine osteitis survivors. *The Pediatric Infectious Disease Journal*, in press.

IV Pöyhönen, L., Teräsjärvi, J., Nuolivirta, K., Vuononvirta, J., Kröger, L., Gröndahl-Yli-Hannuksela, K., Huhtala, H., Ilonen, J., Peltola, V., Mertsola, J., Korppi, M., He, Q. (2015). Interleukin-10 gene promoter region polymorphisms are not associated with BCG osteitis in vaccinated infants. *International Journal of Tuberculosis and Lung Disease*, 19, 1158–1162.

ABBREVIATIONS

A	Adenine
AD	Autosomal dominant
aOR	Adjusted odds ratio
APC	Antigen-presenting cell
AR	Autosomal recessive
BCG	Bacillus Calmette-Guérin
Breg	Regulatory B cell
C	Cytosine
CD	Cluster of differentiation
CI	Confidence interval
CLR	C-type lectin receptor
CSF	Colony-stimulating factor
CYBB	Cytochrome b-245 beta subunit gene
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Environmental mycobacteria
G	Guanine
GAVI	Global Alliance for Vaccines and Immunization
GWAS	Genome-wide association study
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
HWE	Hardy-Weinberg Equilibrium
IFNGR1	Interferon-gamma receptor 1 gene
IFNGR2	Interferon-gamma receptor 2 gene
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IGRA	Interferon-gamma release assay

IL-10	Interleukin-10
IL10	Interleukin-10 gene
IL12B	Interleukin-12 B gene
IL12RB1	Interleukin-12 receptor beta 1 gene
IRAK-4	Interleukin-1 receptor-associated kinase 4
IRF8	Interferon regulatory factor 8 gene
ISG15	Interferon-stimulated protein 15 gene
LBW	Low-birth-weight
LRR	Leucine-rich repeat
MBL	Mannose-binding lectin
MBL2	Mannose-binding lectin gene
M. bovis	*Mycobacterium bovis*
MDR	Multi-drug-resistant
MHC	Major histocompatibility complex
MSMD	Mendelian susceptibility to mycobacterial disease
M. tb	*Mycobacterium tuberculosis*
NEMO	NF-kappa-B essential modulator gene
NK	Natural killer (cell)
NLR	Nod-like receptor
NTM	Nontuberculous mycobacteria
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
QFT	QuantiFERON TB (test)
rBCG	Recombinant BCG
RNA	Ribonucleic acid
SD	Standard deviation
SNP	Single nucleotide polymorphism
STAT1	Signal transducer and activator of transcription 1 gene
T	Thymine
TB	Tuberculosis

Th	T helper (cell)
THL	Terveyden ja hyvinvoinnin laitos
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TLR	Toll-like receptor gene
Treg	Regulatory T cell
TST	Tuberculin skin test
UNICEF	The United Nations Children's Fund
WES	Whole exome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant
XR	X-chromosomal recessive

ABSTRACT

BACKGROUND: Tuberculosis (TB) is the most common infectious disease in the world; 9.6 million new cases occur every year. Vast morbidity and mortality is associated with TB, and one third of the world's population are infected with *Mycobacterium tuberculosis* (*M. tb*). The active disease develops in 10% of infected people, and 1.5 million lives are lost to TB annually. The Bacillus Calmette-Guérin (BCG) vaccine was developed nearly a century ago, and it remains the only vaccine available against TB. Some complications have been associated with the vaccinations of newborns, including lymphadenitis, abscess, and osteitis. As a consequence of this – and the low rate of TB infections – many developed countries have eliminated the vaccine from their vaccination programs. The BCG vaccine is still used in third world countries, where TB is a major health problem affecting the whole of society, and risk groups in the developed countries continue to be vaccinated. The possibilities of creating new vaccines against TB are being studied continuously, but no breakthrough has occurred. Therefore, it is essential to study immune responses to the BCG vaccine in different populations, especially in patients that have suffered from severe complications such as BCG osteitis. BCG vaccination of newborns was in general use in Finland from 1941 to 2006. From 1951, the coverage of the vaccinations was nearly 100%. The microbe-specific diagnostics of invasive complications caused by BCG vaccinations was centralized in one national laboratory (National Public Health Institute, Helsinki, Finland) from 1960 to 1988, and during these 29 years, a total of 222 BCG osteitis cases were diagnosed.

AIMS: The aim of this study was to investigate whether there is an immunity-related genetic predisposition to BCG osteitis. The more specific issues addressed were: (1) the association of mannose-binding lectin (MBL) concentration and mannose-binding lectin encoding gene (*MBL2*) polymorphisms with BCG osteitis; (2) the association of Toll-like receptor 2

(TLR2) subfamily gene polymorphisms with BCG osteitis; (3) the role of the interleukin-12/interferon-gamma (IL-12/IFN-γ) pathway and Mendelian susceptibility to mycobacterial disease (MSMD) -related gene mutations in BCG osteitis; and (4) the association of interleukin-10 encoding gene (*IL10*) polymorphisms with BCG osteitis.

SUBJECTS AND METHODS: In 2007–2008, a questionnaire and invitation to join the study was sent to the 203 former BCG osteitis patients in Finland with a current address available, and 160 (78.8%) replied. The questionnaire comprised questions on chronic illnesses, long-term use of medicines, repeated or chronic infections, and mycobacterial infections. Whole blood samples were obtained from 132 of the 160 former BCG osteitis patients who agreed to participate in this study. In addition, 25 transfer control samples were taken from healthy persons. The blood samples were collected at laboratories around the country and immediately transported to the Tuberculosis Reference Laboratory, National Institute for Health and Welfare, Turku, Finland. The samples were then analyzed for innate immunity -related markers. Genetic studies were conducted in Turku and at the Rockefeller University, New York, USA.

RESULTS: Of the former BCG osteitis patients, 56 (42.4%) had the non-wild variant genotype A/O or O/O, compared with 133 (32.3%) in the controls (p=0.033). There was no significant difference in MBL concentrations between patients and controls.

The *TLR1* genotype was GG in 106 cases (80.3%), GT in 25 cases (18.9%), and TT in one case (0.81%) (p=0.012 vs. controls). The *TLR2* genotype was GG in 117 cases (88.7%), GA in 15 cases (11.4%), and there were no AA cases (p=0.033). The *TLR6* genotype was CC in 30 cases (22.8%), CT in 69 cases (52.2%), and TT in 33 cases (25.0%) (p=0.006 vs. controls). Only the combination of the *TLR1* wild genotype and the *TLR2* and *TLR6* variant genotypes differed significantly between the cases and controls. The *TLR1* variant genotype decreased the risk of BCG osteitis (aOR 0.48), and both the *TLR2* (aOR 2.16) and the *TLR6* (aOR 2.11) variant genotypes increased the risk of BCG osteitis significantly and independently of each other.

In stimulations of white blood cells, by the limit of the $<5^{th}$ percentile, *ex vivo* IL-12 concentration and increase in concentration was low in five cases, and *ex vivo* IFN-γ concentration and increase in concentration was low in

six cases. Two cases had low concentrations and low increases in both IL-12 and IFN-γ. By the limit of the $<10^{th}$ percentile, *ex vivo* IL-12 concentration and increase in concentration was low in an additional six cases, and *ex vivo* IFN-γ concentration and increase in concentration was low in an additional four cases. With two exceptions, low *ex vivo* concentrations and low increases in concentrations picked up the same cases. None of the patients presented with a low ($<10^{th}$ percentile) stimulated IFN-γ and a high ($>90^{th}$ percentile) stimulated IL-12 concentration, or with a low ($<10^{th}$ percentile) stimulated IL-12 and a high ($>90^{th}$ percentile) stimulated IFN-γ concentration in cell cultures. There were four subjects with a low production of both IL-12 and IFN-γ. Known gene mutations of MSMD or the IL-12/IFN-γ pathway were studied in 20 former BCG osteitis patients by whole exome sequencing, and no mutations were found in the coding regions of these genes.

In the IL-10 encoding gene, the frequencies of genotypes, and allele frequencies were similar in former BCG osteitis patients and controls. There were no significant differences in the frequencies of the eight possible combinations of the three *IL10* single nucleotide polymorphisms (SNPs) (*IL10* rs1800896, *IL10* rs1800871, and *IL10* rs1800872) located in the same proximal promoter region of the gene.

CONCLUSIONS: There are differences in the polymorphisms of the MBL encoding gene and TLR2 subfamily genes in BCG osteitis patients compared to the controls in the study. The patients do not have known genetic mutations related to MSMD illness, but some of them have a defect in the production of IFN-γ and IL-12. *IL10* polymorphisms do not differ between the patients and the controls. The results of this dissertation indicate that the gene polymorphisms involved in regulating innate immunity are associated with the risk of osteitis after BCG vaccination.

TIIVISTELMÄ

TAUSTAA: Tuberkuloosi (TB) on maailman yleisin infektiotauti; 9,6 miljoonaa uutta tapausta ilmaantuu joka vuosi. Tuberkuloosin aiheuttama sairastavuus ja kuolleisuus on valtavaa, ja kolmasosalla maailman väestöstä on *Mycobacterium tuberculosis* (*M. tb*) -infektio. Tartunnan saaneista 10 % sairastuu aktiiviin tuberkuloosiin, ja siihen kuolee 1,5 miljoonaa ihmistä vuosittain. Bacillus Calmette-Guérin (BCG) -rokote kehitettiin lähes vuosisata sitten, mutta on vieläkin ainoa rokote tuberkuloosia vastaan. Vastasyntyneiden rokottamiseen on liittynyt joitakin komplikaatioita, kuten lymfadeniittia, abskesseja ja osteiittia. Tämän seurauksena – sekä tuberkuloosi-infektioiden alhaisen esiintyvyyden vuoksi – monet kehittyneet maat ovat poistaneet rokotteen rokotusohjelmastaan. Kolmannen maailman maat, joissa tuberkuloosi on suuri koko yhteiskuntaan vaikuttava terveysongelma, käyttävät edelleen BCG-rokotetta, ja riskiryhmät kehittyneissä maissa rokotetaan. Uusia tuberkuloosirokotteita yritetään kehittää jatkuvasti, mutta läpimurtoa ei ole tapahtunut. Sen vuoksi on olennaista tutkia BCG-rokotteen immuunivasteita eri väestöissä, varsinkin potilailla, jotka ovat kärsineet vakavista komplikaatioista kuten BCG-osteiitista. Vastasyntyneiden BCG-rokotus oli yleisessä käytössä Suomessa vuosina 1941–2006. Vuodesta 1951 alkaen rokotuskattavuus oli lähes 100 %. BCG-rokotusten invasiivisten komplikaatioiden mikrobispesifinen diagnostiikka oli keskitetty yhteen kansalliseen laboratorioon (Kansanterveyslaitos, Helsinki, Suomi) vuosina 1960–1988, ja näiden 29 vuoden aikana diagnosoitiin yhteensä 222 BCG-osteiittitapausta.

TUTKIMUKSEN TAVOITE: Tutkimuksen tavoite oli selvittää, onko BCG-osteiitin taustalla immuniteettiin liittyvä geneettinen alttius. Tarkemmin selvitettävät aiheet olivat: (1) MBL-pitoisuuden ja mannoosia sitovaa lektiiniä koodaavan geenin (*MBL2*) polymorfismien yhteys BCG-osteiittiin; (2) TLR2-perheen geenien polymorfismien yhteys BCG-osteiittiin; (3) IL-12/IFN-γ-polun ja MSMD-geenimutaatioiden yhteys BCG-osteiit-

tiin; ja (4) interleukiini-10:ä koodaavan geenin (*IL10*) polymorfismien yhteys BCG-osteiittiin.

AINEISTO JA MENETELMÄT: Vuosina 2007–2008 lähetettiin kyselylomake ja kutsu tutkimukseen 203:lle entiselle BCG-osteiittipotilaalle, joiden osoitetiedot oli löydettävissä. Potilaista 160 (78,8 %) vastasi. Kyselylomakkeessa oli kysymyksiä kroonisista sairauksista, lääkkeiden pitkäaikaisesta käytöstä, toistuvista tai kroonisista infektioista ja mykobakteeri-infektioista. Kokoverinäytteet otettiin 132/160:lta entiseltä BCG-osteiittipotilaalta, jotka suostuivat osallistumaan tutkimukseen. Myös 25:ltä terveeltä ihmiseltä otettiin matkakontrollinäytteet. Verinäytteet otettiin laboratorioissa eri puolilla maata ja kuljetettiin välittömästi Terveyden ja hyvinvoinnin laitoksen Tuberkuloosilaboratorioon Suomen Turkuun. Näytteistä analysoitiin synnynnäiseen immuniteettiin liittyviä tekijöitä. Geneettisiä tutkimuksia tehtiin Turussa ja Rockefellerin yliopistossa New Yorkissa USA:ssa.

TULOKSET: Entisistä BCG-osteiittipotilaista 56:lla (42,4 %) oli "non-wild" variantti-genotyyppi A/O tai O/O, verrattuna 133:een (32,3 %) kontrolleista (p=0.033). MBL-pitoisuuksissa ei ollut merkitsevää eroa potilaiden ja kontrollien välillä.

TLR1-genotyyppi oli GG 106:lla (80,3 %) tapauksista, GT 25:lla (18,9 %) tapauksista ja TT yhdellä (0,81 %) tapauksella (p=0.012 vs. kontrollit). *TLR2*-genotyyppi oli GG 117:lla (88,7 %) tapauksista, GA 15:lla (11,4 %) tapauksista, ja yhtään AA-tapausta ei ollut (p=0.033). *TLR6*-genotyyppi oli CC 30:lla (22,8 %) tapauksista, CT 69:lla (52,2 %) tapauksista ja TT 33:lla (25,0 %) tapauksista (p=0.006 vs. kontrollit). Vain yhdistelmä "*TLR1* wild, *TLR2* variant, *TLR6* variant" erosi merkitsevästi potilailla ja kontrolleilla. *TLR1* varianttigenotyyppi pienensi osteiittiriskiä (aOR 0.48), ja sekä *TLR2* varianttigenotyyppi (aOR 2.16) että *TLR6* varianttigenotyyppi (aOR 2.11) lisäsivät osteiittiriskiä merkitsevästi ja toisistaan riippumattomasti.

Valkosolujen stimulaatioissa viidennen persentiilin rajan mukaan IL-12:n *ex vivo* -pitoisuus ja pitoisuuden nousu oli matala viidellä ja IFN-γ:n *ex vivo* -pitoisuus ja pitoisuuden nousu kuudella potilaalla. Kahdella potilaalla oli matala pitoisuus ja matala nousu sekä IL-12:ssa että IFN-γ:ssa. Kymmenennen persentiilin rajan mukaan IL-12:n *ex vivo* -pitoisuus ja pitoisuuden nousu oli matala lisäksi kuudella ja IFN-γ:n *ex vivo* -pitoisuus ja pitoisuuden nousu lisäksi neljällä potilaalla. Kahta poikkeusta lukuun ottamatta matalat *ex vivo* -pitoisuudet ja matalat nousut pitoisuuksissa va-

litsivat samat potilaat. Yhdelläkään ei ollut matalaa (<10. persentiili) stimuloitua IFN-γ- ja korkeaa (>90. persentiili) stimuloitua IL-12 -pitoisuutta, tai matalaa (<10. persentiili) stimuloitua IL-12- ja korkeaa (>90. persentiili) stimuloitua IFN-γ-pitoisuutta soluviljelmissä. Neljällä potilaalla oli sekä IL-12:n että IFN-γ:n matala tuotanto. MSMD:n tai IL-12/IFN-γ -polun tunnetut geenimutaatiot tutkittiin 20:ltä entiseltä BCG-osteiittipotilaalta koko eksomin sekvenoinnilla, ja yhtään mutaatiota ei löytynyt näiden geenien koodaavilta alueilta.

IL-10:ä koodaavassa geenissä genotyyppifrekvenssit ja alleelifrekvenssit olivat samanlaiset entisillä BCG-osteiittipotilailla ja kontrolleilla. Kahdeksan mahdollisen kombinaation frekvenssit *IL10*:n kolmella SNP:llä (*IL10* rs1800896, *IL10* rs1800871 ja *IL10* rs1800872), jotka sijaitsevat geenin samalla proksimaalisella promootterialueella, eivät eronneet merkitsevästi toisistaan.

JOHTOPÄÄTÖKSET: *MBL2*:n ja TLR2-perheen geenien polymorfismeissa on eroja BCG-osteiittipotilailla verrattuna tutkimuksen kontrolleihin. Potilailla ei ole MSMD-sairauksiin liittyviä tunnettuja geenimutaatioita, mutta joillakin heistä on vikaa IFN-γ:n ja IL-12:n tuotannossa. *IL10*:n polymorfismit eivät eroa potilaiden ja kontrollien kesken. Väitöskirjatyön tulokset osoittavat, että geenien polymorfismit, jotka säätelevät synnynnäistä immuniteettia, liittyvät BCG-rokotuksen jälkeiseen osteiittiriskiin.

1 INTRODUCTION

Tuberculosis (TB) is the most common infectious disease in the world. It kills 1.5 million people every year (Zhang et al. 2013), especially in developing countries with a high prevalence of the human immunodeficiency virus (HIV) and poor access to health care (Ahmad et al. 2011, Abel et al. 2014). TB can infect almost any part of the body, but most commonly the lungs are the site and also the route of infection. TB is a huge burden for society because of its infectious nature, chronic progression, and long treatment. It has been estimated that one third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tb*) – 9.6 million people every year – and 10% of this group gets active TB (Ahmad et al. 2011, Abel et al. 2014). Others are latently infected, serving as a reservoir from which active TB cases will continue to develop (Gideon et al. 2011). Interindividual genetic variation, as well as variation between different populations, contributes to the differing immune responses against *M. tb*, resulting in varying susceptibility to active TB (Abel et al. 2014). Twin studies, whole exome sequencing (WES) studies, and recent genome-wide association (GWAS) studies demonstrate that host genetics clearly influences susceptibility to TB (Azad et al. 2012).

The emergence of multi-drug-resistant (MDR) TB, extensively drug-resistant (XDR) TB, and the current TB-HIV epidemic have raised great concern in different parts of the world. MDR-TB and XDR-TB are closely associated with HIV/AIDS infection, which has contributed to the slowing down of the decline of TB incidence over the last two decades. This has been one of the most important barriers to the elimination of TB (Pontali et al. 2015). The majority of the drug-resistant TB cases have been reported in South Africa, Russia, and India. Worldwide, in 2012, 450,000 people had MDR-TB, and as many as 170,000 died of it. MDR-TB is resistant to at least two of the most important TB drugs, isoniazid and rifampicin. XDR-TB is also resistant to fluoroquinolones and aminoglycosides (Soini et al. 2014).

In poor countries, infants may get congenital TB from their infected mother. Most commonly though, they acquire the disease postnatally through contact with an infected adult (Skevaki et al. 2005). The infant immune system is at an early stage of development, and in encountering foreign antigens, it must learn to distinguish nonthreatening antigens from those that are expressed by dangerous pathogens (Vanden Driessche et al. 2013). The innate and adaptive immune cells of infants produce fewer proinflammatory cytokines than those of adults. As a consequence, several factors critical to controlling *M. tb* infection are not in effective use. Furthermore, infant T cells are less capable of differentiating into more mature cells, such as IFN-γ -producing T cells, and this leads to a 5–10 times greater risk of developing active TB compared to adults. Infants also have higher rates of severe disseminated disease, including miliary TB and meningitis (Vanden Driessche et al. 2013).

The Bacillus Calmette-Guérin (BCG) vaccine is the only effective vaccine against TB. It has been in use for nearly a hundred years, since it was developed at the beginning of the twentieth century in France (Luca et al. 2013). The first BCG vaccine was given to a healthy newborn baby in 1921 in Paris. Since then, the BCG vaccine has become the most widely used vaccine in the world, and has been given to more than four billion people. However, it only protects against meningitis and disseminated TB, and is mainly for infants (Colditz et al. 1995, Hanekom et al. 2005). Worldwide, at least 20 genetically distinct BCG vaccine strains are in use. This is because of genetic changes that have resulted from the repeated subculture in different countries. It is unknown which strain provides the best protection against TB, but it is known that immune responses against various strains of BCG differ depending on the strain (Davids et al. 2006, Ritz et al. 2009, Ritz et al. 2012). In addition, mild and severe complications have been associated with the vaccinations, although the BCG vaccine is considered to be one of the safest vaccines, and the majority of adverse effects require no medical treatment. Different BCG strains have caused varying complications in different populations (Wilson et al. 1995, Norouzi et al. 2012, Yuk et al. 2014), and the incidence rates of BCG vaccination complications vary greatly depending on the country and population.

In recent decades, universal BCG vaccinations have been discontinued in Western countries, but risk groups are still vaccinated. These include infants whose parents are from countries with a high prevalence of TB, or whose close relatives suffer from or have suffered from TB. General vaccinations in Western countries have been discontinued because of emerging numbers of complications and the fact that the incidence of TB has declined. In developing countries, the BCG vaccine is still in general use because of the heavy disease burden of TB. Research on new TB vaccines is actively ongoing, but no significant discoveries have been made (McShane et al. 2011, da Costa et al. 2014).

In Finland, the BCG vaccine has been in use since the late 1920s, and up to 2006, the national coverage was over 98% (Salo 2006). The incidence of complications has been higher than in other countries, despite the variation in BCG strains over the decades (Kröger et al. 1994, Kröger et al. 1995). The complications have included abscesses, lymphadenitis, and osteitis, and of these, osteitis is by far the most serious complication. In the 1970s, the rate of BCG osteitis cases increased dramatically in Finland (Wasz-Höckert et al. 1976, Wasz-Höckert et al. 1979), and in 2006, vaccinations of the general Finnish population were discontinued because of a continuously high number of complications (Salo 2006).

This dissertation concentrates on the study of the innate immunity of former BCG osteitis patients. In Finland, reported complications of the BCG vaccine, such as BCG osteitis, have been clearly more numerous than in other countries, and the cases have been recorded in national registers for many decades. Innate immunity is the first step of protection against mycobacteria in the host. The main purpose of this study was to analyze different parts of the innate immunity of former BCG osteitis patients to find a possible genetic predisposition to BCG osteitis. The Finnish population is homogenous, and we have excellent national registers on patients and diseases. These facts provide a good basis for the study of this unique group of patients.

2 REVIEW OF THE LITERATURE

2.1 History of the Bacillus Calmette-Guérin vaccine

Mycobacterium tuberculosis (*M. tb*) is an intracellular pathogen that causes TB. It was discovered in 1882 by Robert Koch. The BCG vaccine against TB was developed nearly a century ago in France by Albert Calmette and Camille Guérin (Luca et al. 2013). They began their research in 1900 at the Pasteur Institute in Lille, and in 1908, they started to culture a virulent bovine strain of tubercle bacillus (*Mycobacterium bovis, M. bovis*) on a bile, glycerin, and potato medium, and then proceeded to subculture it at roughly three weekly intervals. In 1913, they were initiating a vaccination trial with this cultured bacillus in cattle, but World War I interrupted it. Throughout the German occupation of Lille, subculturing was continued, and by 1919 Calmette and Guérin had developed a tubercle bacillus (Bacillus Calmette-Guérin) which did not produce progressive TB in injected animals. In 1921, the first BCG vaccine was given at the Charité Hospital in Paris to a healthy newborn baby of a mother who had died of TB just a few hours after giving birth. The given oral dose of the BCG vaccine seemed to be safe, as it lead to no complications. By 1924, 664 infants had received the oral BCG vaccine, and the Pasteur Institute in Lille began mass production of the vaccine. From 1924 to 1928, 114,000 infants were vaccinated without serious complications. TB mortality clearly reduced among those susceptible infants that had received the BCG vaccine, and vaccinations were also begun outside France. Arvid Wallgren in Gothenburg and Johannes Heimbeck in Oslo pioneered the cutaneous administration of the BCG vaccine (Luca et al. 2013).

Calmette and Guérin were strongly criticized in Great Britain and the United States despite the successful beginning of vaccinations, and in 1929 it was reported in the USA that a virulent tubercle bacillus had been isolated

in a specimen supplied by Calmette. The developers of the vaccine still remained confident that the BCG vaccine was safe to use, but then, in 1930, "the Lübeck disaster" happened. A scheme to vaccinate newborn babies was undertaken in Lübeck General Hospital. The BCG vaccine was supplied from the Pasteur Institute in Paris, but prepared in the TB laboratory in Lübeck. The oral route was used in the administration, and after four to six weeks, a large number of the 250 vaccinated infants developed TB. In the first year after being vaccinated, 73 of them died. Another 135 were infected, but fortunately recovered. It was concluded that the BCG vaccine was the cause of the disaster, due to negligent contamination of the vaccine by virulent tubercle bacilli in the Lübeck laboratory. Two of the doctors concerned were given sentences of imprisonment. After these events, confidence in the BCG vaccine was shattered, although the disaster had nothing to do with the vaccine Calmette and Guérin had developed.

Studies on BCG continued despite the disaster in Lübeck, and over 20 years they had provided evidence that the vaccine is indeed useful in protection against TB. After World War II, TB was emerging, and the use of the BCG vaccine was encouraged by the United Nations Children's Fund (UNICEF), the World Health Organization (WHO), and the Scandinavian Red Cross societies. The campaigns also spread to the developing countries. The majority of the world followed the lead of Europe and the WHO, and introduced routine BCG vaccinations according to various schedules, for example at birth, or upon school entry. This led to mass vaccinations and the fact that to date, the BCG vaccine has been given to four billion people worldwide.

2.2 The BCG vaccine today and development of new vaccines

The BCG vaccine is given to over 120 million children every year (Zufferey et al. 2013). The majority are vaccinated with BCG strains supplied by UNICEF, distributed on behalf of the Global Alliance for Vaccines and Immunization (GAVI). UNICEF uses only four BCG vaccine suppliers. Three different vaccine strains are in use:

1) BCG-Denmark, produced by the Statens Serum Institute, Denmark

2) BCG-Russia, (genetically identical to BCG-Bulgaria) produced by Bulbio (BB-NCIPD), Bulgaria and the Serum Institute, India

3) BCG-Tokyo, produced by the Japan BCG Laboratory, Japan

Although the BCG vaccine is in widespread use around the world, it is difficult to access information on recent changes in BCG vaccine use and immunization policies (Dierig et al. 2015). The WHO publishes information on BCG immunization coverage regularly. A recent study has also collected up-to-date data on the use of the BCG vaccine in Europe (Dierig et al. 2015). It concluded that immunization indications varied greatly, depending, for example, on the national TB incidence rate and immigration. Clearly a regular collection of up-to-date information from different countries is needed in order to develop more efficient and better targeted vaccination regimes and to guide the choice of the BCG strains used.

A recent meta-analysis concluded that the BCG vaccine protects against *M. tb* infection as well as progression from infection to disease (Roy et al. 2014). However, the efficacy of the BCG vaccine varies in different parts of the world. In vaccination studies, study sites at a greater distance from the equator are associated with a higher efficacy, and geographic latitude alone accounts for as much as 41% of between-study variance. This geographical variation could be explained by the fact that the BCG vaccine does not seem to protect against TB when it is given to subjects that have already been infected or sensitized to environmental mycobacteria. In several studies, it has been shown that the efficacy of the BCG vaccine can be affected by a variety of reasons, including the susceptibility of human hosts, the pathogenicity of the organism, and host-agent interactions. Socioeconomic conditions, the genetic composition of the population, climate and exposure to sunlight, diet and nutrition, the presence of nontuberculous mycobacteria (NTM) in the environment, the quality of follow-up in vaccination studies, the virulence of locally prevalent strains of *M. tb*, and the strain, storage, and viability of the BCG vaccine make up a complex combination that affects the efficacy of the vaccine (Wilson et al. 1995). This complexity makes the studies of vaccine efficacy challenging. In addition,

the limitations of TB tests in the past have raised the question of whether the BCG vaccine is effective against *M. tb* infection. The oldest of the TB tests is the tuberculin skin test (TST). Its limitations include difficulty in administration and interpretation, the need for a return visit by the patient, and false-positive results caused by significant cross-reaction with the BCG vaccine and many NTM (Starke et al. 2014). Interferon-γ (IFN-γ) release assays (IGRAs) are blood tests that measure *ex vivo* T lymphocyte release of IFN-γ after stimulation by antigens specific for *M. tb*. Because these antigens are not found on BCG or most NTM, IGRAs are more specific tests than the TST, yielding fewer false-positive results. However, IGRAs have little advantage over the TST in sensitivity, and both methods have reduced sensitivity in immunocompromised children, including children with severe TB. In addition, neither method distinguishes between TB infection and TB disease (Starke et al. 2014). The ability of IGRA tests to distinguish *M. tb* infection from previous BCG vaccination yields more specific information for studies on vaccine efficacy (Roy et al. 2014).

The BCG vaccine that is in use today is a live attenuated vaccine, and it mediates immune protection through the production of IFN-γ by cluster of differentiation (CD) 4(+) T cells. IFN-γ activates macrophages to kill *M. tb* (Abebe 2012). On the other hand, some recent studies have reported a lack of correlation between IFN-γ production by CD4(+) T cells and BCG-induced immune protection. Other T cell subsets, such as gammadelta (γδ) T cells, CD8(+) T cells, and NK cells may also play a vital role in immune protection against *M. tb* infection and BCG-induced immune responses (Zufferey et al. 2013, Kleinnijenhuis et al. 2014). It has been shown that γδ T cells and NK cells develop immunological memory upon re-encountering the same pathogen (Abebe 2012).

Increasing epidemiological evidence has shown recently that BCG, in addition to its obvious effects on mycobacterial diseases, has beneficial non-specific, heterologous effects (Shann 2010, Ritz et al. 2012). The BCG vaccine reduces infant and child mortality to various diseases, offering protection, for example, against non-mycobacterial infections. Early BCG vaccination of low-birth-weight (LBW) newborns reduces neonatal mortality by over 40% due to the prevention of septicemia and pneumonia. A recent study concluded that four weeks after immunization, BCG-vaccinated infants

had a significantly increased production of cytokines, particularly T helper cell type 1 (Th1), typically monocyte-derived pro-inflammatory cytokines (Jensen et al. 2015). Thus, the BCG vaccine may accelerate the development of the neonatal immune system, mediating comprehensive protection against infections and mortality. Furthermore, a decrease in the incidence of allergic diseases has been seen in association with BCG vaccinations (Marks et al. 2003, Kiraly et al. 2015). Interestingly, the BCG vaccine can also be used in the treatment of certain malignancies, for example, bladder cancer (Redelman-Sidi et al. 2014). These effects are mediated partly by non-specific effects on adaptive immunity, but also on the potentiation of innate immune responses, such as the reprogramming of monocytes. A recent finding of interest is that the innate immune system clearly has adaptive characteristics that involve a heterologous memory of past insults (Blok et al. 2015). Innate immunity can remember a previous exposure to a micro-organism and respond differently during a second exposure (Lerm et al. 2015). Although the specificity and memory of innate immunity cannot compete with the highly sophisticated adaptive immune response, its contribution to host defense against infection and to vaccine-induced immunity is important. The line between innate and adaptive immunity is blurred, and the whole process of defense against foreign organisms can be seen more as an entity of interactions.

Both experimental models and clinical trials have shown that innate immune cells, such as monocytes, macrophages, and natural killer (NK) cells, can provide protection against certain infections in vaccination models independently of lymphocytes (Blok et al. 2015). This process is regulated through epigenetic reprogramming of innate immune cells and has been termed "trained immunity." It has been hypothesized that the induction of trained immunity is responsible for the protective, non-specific effects induced by vaccines, such as the BCG vaccine, the measles vaccine, and other whole-micro-organism vaccines. Trained immunity leads to increased cytokine production in response to non-related pathogens. In addition to monocytes, NK cells may contribute to the heterologous beneficial effects of BCG vaccination (Kleinnijenhuis et al. 2014). In addition, the process of trained immunity may in fact play a role in the beneficial effects of BCG against *M. tb* infection and the development of active TB. This finding

in turn could guide the development of new TB vaccines and improved vaccination strategies (Netea et al. 2014, Kleinnijenhuis et al. 2015, Lerm et al. 2015).

Currently, as the only vaccine available against TB – but only partly protective – the BCG vaccine is under modification to make it more efficient (Delogu et al. 2014, Delogu et al. 2015), especially against TB in adults and pulmonary TB (Graves et al. 2015). A number of new candidate TB vaccines are in different phases of clinical trial, and many are under advanced clinical assessment (Kaufmann 2013). They are either recombinant forms of BCG (rBCG) or prime boosters of BCG, and their immunogenicity is tested using BCG as a benchmark. In these trials, IFN-γ produced by CD4(+) T cells is measured and acts as a protective immune marker. The aim of these new vaccines is to elicit robust and long-lived T cell responses against *M. tb* antigens (Delogu et al. 2014, Delogu et al. 2015).

Several different strategies are used to develop a more efficient vaccine against TB. These include (da Costa et al. 2014):

1) overexpression of *M. tb* immunodominant antigens already present in BCG

2) gene insertion of immunodominant antigens from *M. tb* absent in the BCG vaccine

3) A combination of the introduction and overexpression of genes that are lost during the attenuation process of BCG

4) BCG modifications for the induction of CD8(+) T cell immune responses and cytokines expressing rBCG

A recent study hypothesized that rBCG overexpressing immunodominant antigens expressed at different growth stages of *M. tb* could provide more comprehensive protection against primary and latent *M. tb* infection (Liang et al. 2015). In the study, a new combination of rBCG strains was produced, and the following immune protection was attributed to stronger antigen-specific CD4(+) Th1 responses (Liang et al. 2015).

2.3 BCG vaccine strains and the incidence of vaccination complications in Finland

The BCG vaccine was first used in Finland in the late 1920s, but only on a small scale (Salmenkallio 1972). In 1951, a mass campaign against TB was organized, including vaccination of all newborn babies, and the first vaccine strain used was the Gothenburg strain produced by a Swedish BCG laboratory (Kröger et al. 1994).

From 1960 to 1970, the incidence of BCG osteitis was 2.7–13.0/100,000 BCG-vaccinated infants (mean 7.3, median 6.9). From 1971, the strain was the same, but the vaccine was produced in Copenhagen, Denmark. Between 1971 and 1978, the annual incidence of BCG osteitis increased markedly to 15.3–72.9/100,000 BCG-vaccinated infants (mean 36.9, median 30.4). Because of this, the vaccine was replaced by the Glaxo vaccine in 1978. The incidence of BCG osteitis decreased after the change to 1.7–10.1/100,000 BCG-vaccinated infants (mean 6.4, median 7.2) (Kröger et al. 1994), which was still higher than that reported from other countries.

In 2002, the BCG vaccine strain was changed again in Finland, because the manufacturing of the Glaxo BCG vaccine ended. From 2002 to 2006, the Copenhagen 1331 strain manufactured by Statens Serum Institute (SSI), Denmark, was in use. After the change, the incidence of complications multiplied ten-fold. The complications were mainly abscesses in the lymph nodes (1.5/1,000 BCG-vaccinated infants). Between 2002 and 2004, the incidence of BCG osteitis/arthritis was 10/100,000 BCG-vaccinated infants. In 2005, the National Advisory Committee on Vaccines recommended that only risk groups should be vaccinated. This change in the BCG vaccination policy took place in 2006.

2.4 BCG osteitis

2.4.1 Incidence

BCG osteitis is a very rare disease. The incidence of this complication of the BCG vaccine varies greatly in different populations, and the highest incidence rates have been reported in Finland and Sweden (25–33/100,000 vaccinations) (Dahlström et al. 1977, Wasz-Höckert et al. 1979, Böttiger et al. 1982, Lotte et al. 1988). Japan is on the other end of the scope, with a prevalence of BCG osteitis of only 0.01/million vaccinations (Milstien et al. 1990). In Japan, the BCG-Tokyo strain has been in use.

2.4.2 Pathogenesis and symptoms

Osteitis is the most common disseminated complication of BCG vaccination (Lotte et al. 1984, Lotte et al. 1988). The pathogen of the vaccine, live attenuated *Mycobacterium bovis* (*M. bovis*), reaches the bones by lymphohematogenous dissemination (Yamada et al. 2009). In complications of the BCG vaccine, the lesion site is not necessarily associated with the injection site (Aftimos et al. 1986), but can occur almost anywhere in the body. BCG osteitis usually occurs in the tibia, femur, vertebrae, sternum, or ribs. The lower limbs are most commonly affected (Aftimos et al. 1986, Kröger et al. 1995). The bone lesion of BCG osteitis presents with slow progression and mild symptoms, and diagnosis can often be difficult. The symptoms can lead to suspicion of trauma instead of infection. Initial symptoms usually include sensitivity, pain, and limited mobility in the affected region. Fever may be present, but is low. The general status of the patient remains good (Lin et al. 1999). Clinical manifestations of BCG osteitis can appear over a long time, from a few months to five years after vaccination. Usually, the complication occurs approximately 18 months after vaccination (Kröger et al. 1995). In a Japanese study, 73% of BCG osteitis cases were seen within 9–18 months of vaccination, and the defect and cavity formation of the affected bone was shown by radiography (Koyama et al. 2009).

Medical records of Finnish children, based on nationwide registration from 1960 to 1988, were retrospectively analyzed in a study by Kröger et al. (1995). The age at onset of BCG osteitis that fulfilled the diagnostic criteria in the studied group of 222 children was 0.3–5.7 years (mean 1.5 years). The most common sites of osteitis were the metaphyses of long bones. The lower extremity (58%) was affected more often than the upper (14%). Osteitis was situated in the sternum of 36 patients (15%) and in the ribs of 27 patients (11%) (Kröger et al. 1995). The general condition of the Finnish osteitis patients usually remained unaltered (Kröger et al. 1995).

2.4.3 Diagnosis

The diagnosis of BCG osteitis can be challenging due to the rarity of the disease. X-ray findings include lytic lesions with a sclerotic halo and a periosteal reaction with periarticular osteoporosis (Moreno et al. 1990, Kröger et al. 1994, Lin et al. 1999). In the majority of the Finnish patients, X-rays showed these typical features of BCG osteitis, and the diagnosis was confirmed by culture of the BCG strain, by typical histology and age, or by both (Kröger et al. 1994). Histopathological findings of BCG osteitis show granulomatous inflammation with epithelioid cells, with or without caseous necrosis. Acid-fast bacilli are detected in approximately half of all BCG osteitis cases (Peltola et al. 1984, Kröger et al. 1994).

In 1971, Foucard and Hjelmstedt proposed criteria for the diagnosis of BCG osteitis (Foucard et al. 1971):

1) BCG vaccination in the neonatal period

2) a period of less than four years between vaccination and symptom onset

3) no contact between the child and any adults with TB

4) a consistent clinical profile

5) histopathology suggestive of TB

In Japan, definitive diagnosis of BCG osteitis has been made by the detection of BCG from pus or biopsied materials (Koyama et al. 2009). In Taiwan, pathologic diagnosis of a *Mycobacterium* infection from bony specimens was recorded for 92% of patients in a BCG osteitis study (Nan-Chang et al. 2015). Recently, a multiplex polymerase chain reaction (PCR) method has been taken into use to identify the etiological agent of osteitis, and it has been proven to be rapid and reliable in diagnostics. The difference in a single nucleotide can distinguish *M. tb* from *M. bovis* (Lin et al. 1999). Different strains of BCG can be distinguished with PCR, as in a South-Korean study, where BCG-Tokyo strains were identified in osteomyelitis patients (So Hee et al. 2008). As for other TB tests, TST and the QuantiFERON TB (QFT) test have given varying results in BCG osteitis patients. In a Taiwanese study, 53% of BCG osteitis patients had positive TST results (Nan-Chang et al. 2015). In study subjects of a Japanese study, TST was positive, but QFT was negative in all tested cases (Koyama et al. 2009).

2.4.4 Treatment and prognosis

Early diagnosis and treatment of BCG osteitis is necessary. The treatment regime usually includes long-term antituberculous therapy and surgical drainage. Most of the patients are cured after 6-12 months of chemotherapy without any complications. Medical treatment of the Finnish BCG osteitis patients initially included one month of streptomycin combined with either ethionamide or isoniazid. Later, the combination was changed to streptomycin, isoniazid, and rifampicin. After the first month of medications, four months of isoniazid combined with ethionamide or rifampicin was used, and isoniazid monotherapy was used to complete the total course of 6–24 months of antituberculous therapy (Kröger et al. 1994).

If the diagnosis and treatment of BCG osteitis are delayed, it is possible that defects will occur in the bone, and restriction occurs in articular movements (Koyama et al. 2009). In a Taiwanese study, 84% of BCG osteitis patients underwent surgery, which included excision, debridement, and open biopsy (Nan-Chang et al. 2015). Some of the children in the study needed arthrotomy. As medical treatment usually has a good outcome, extensive debridement should be avoided. In Finland, it has also been concluded

that the short-term prognosis of most BCG osteitis patients is good with adequate treatment. Bone sequelae or growth deficit are described in only 3% of cases (Kröger et al. 1995).

2.5 Single nucleotide polymorphisms of genes

A single nucleotide polymorphism (SNP) is the simplest form of deoxyribonucleic acid (DNA) variation among individuals (Shastry 2009); it is a mutation in one nucleotide. There are approximately 10 million SNPs in the human genome, occurring in every 300 nucleotides. SNPs are the most common type of genetic variation among people; they are found at a frequency of over 1%. Human populations vary in the frequency of different SNPs depending on geography and ethnicity. SNPs can be assigned a minor allele frequency. It is the lowest allele frequency at a defined locus that is observed in a particular population. SNPs occur within coding sequences of genes, non-coding regions of genes, or in intergenic regions. They may change the structure of the encoded protein, affect the promoter characteristics of the gene, or be completely silent (Schröder et al. 2005). Synonymous SNPs are those with different alleles encoding for the same amino acid (silent mutation). Non-synonymous SNPs have different alleles that encode different amino acids (Thada et al. 2013).

Most SNPs have no effect on health or development. However, some SNPs underlie differences in susceptibility to disease and sensitivity to medications (Calabrese et al. 2009, Shastry 2009). Identification of numerous variations in genes and analysis of their effects contribute to a better understanding of their impact on gene function and the health of an individual. New SNP markers can be used in medical testing and safer individualized medication regimes (Shastry 2009). Furthermore, SNPs can be used to track the inheritance of disease genes within families. Genome-sequencing studies have rendered novel information on genes that are involved in immune reactions. It has been shown that these genes exhibit a high number of polymorphisms (Schröder et al. 2005) that influence the effectiveness of immune responses against various pathogens, both in individuals and ethnic groups. For example, several non-synonymous

SNPs, which alter the amino acid sequence of the encoded protein, have been described in genes involved in immune recognition (Schröder et al. 2005).

2.6 Immunity in BCG osteitis

2.6.1 Innate immunity

Innate immunity acts as a first line of defense against foreign pathogens. It is crucial in defense against mycobacteria like *M. tb* (Kleinnijenhuis et al. 2011, Hossain et al. 2013, Mortaz et al. 2014). Innate immunity is a complex system that has a wide array of soluble and cellular pattern-recognition receptors (PRRs) for microbial antigens (Rämet et al. 2011). These receptors recognize pathogen-associated molecular patterns (PAMPs), facilitate the phagocytosis and lysis of intruding micro-organisms, and activate signaling pathways that lead to the production of cytokines and other molecules responsible for anti-inflammatory processes (Hallman et al. 2001, Worthley et al. 2005, Rämet et al. 2011, Thada et al. 2013). Several classes of PRRs are involved in the recognition of *M. tb*, including TLRs (mainly TLR1, TLR2, and TLR6), C-type lectin receptors (CLRs), and Nod-like receptors (NLRs) (Kleinnijenhuis et al. 2011).

Mycobacteria are intracellular pathogens through phagocytosis, and thus, they are recognized by, and infect, macrophages and dendritic cells, where they avoid elimination by interfering with host defense mechanisms (Bruns et al. 2014). Phagocytosis is obviously the critical first step in *Mycobacterium*-phagocyte interaction, and the process is aided by mannose-binding lectin (MBL), a protein that enhances the uptake of microbes by phagocytic cells and activates the complement system (Bonar et al. 2005). It has been thought that acidification of phagosomes is the means by which macrophages destroy mycobacteria. Recently, it has been discovered that human macrophages have additional antimicrobial effector functions, such as autophagy and the secretion of antimicrobial peptides. These mechanisms restrict mycobacterial proliferation (Bruns et al. 2014).

2.6.1.1 T cells and natural killer cells

Neonatal vaccination with BCG stimulates T cells, which have a complex pattern of cytokine expression and phenotypes (Soares et al. 2008). Significant increases in the proportions of mycobacteria-specific T cells have been observed after BCG immunization (Ritz et al. 2012). In addition, NK cells play a key role in the innate immune reactions following vaccination (Zufferey et al. 2013). They are large granular lymphocytes that express many inhibitory and activating receptors for major histocompatibility complex (MHC) class I and class I-like molecules, classical co-stimulatory ligands, and cytokines (Horowitz et al. 2012). NK cells may in fact be among the very early responders in the host during infection, due to their ability to be rapidly activated by inflammatory cytokines, to secrete effector cytokines, and to kill infected host cells. NK cells also contribute to adaptive immune responses and have regulatory and immunomodulatory functions (Horowitz et al. 2012).

2.6.1.2 Cytokines

Cytokines are centrally involved in immune reactions. They are polypeptide regulators and comprise interleukins, lymphokines, monokines, interferons, colony-stimulating factors (CSFs), chemokines, and a variety of other proteins. They have multiple activities in innate and adaptive immunity (Haddad 2002, Akdis et al. 2011). Cytokines target cells by specific receptors and are divided into Th1 cytokines (for example, IL-2, and IFN-γ), Th2 cytokines (for example, IL-4, IL-5, IL-9, and IL-13), and Treg (regulatory T) cytokines (for example, IL-10) (Ozdemir et al. 2009). The host response to mycobacterial infection is mediated by the type I cytokine pathway (cell-mediated immunity). Deficiencies in this response result in susceptibility to infections caused by poorly pathogenic mycobacterial species, such as BCG and environmental mycobacteria (Cottle 2011). Many of the polymorphisms in cytokine-encoding genes are located in promoter regions, with the potential to affect transcription and activity of cytokines and chemokines (Azad et al. 2012).

2.6.1.3 Mannose-binding lectin and *MBL2* polymorphisms

MBL is an acute phase pattern recognition protein secreted by the liver. Its basic structural unit is a homotrimer of MBL peptides that form a collagen-like triple helix (Worthley et al. 2005). Functional MBL is a higher-order multimer of the structural unit. MBL is involved in the control of different micro-organisms, such as bacteria, fungi, parasites, and viruses. It recognizes the surface structures of pathogens, including mycobacteria, and initiates the lectin pathway of complement activation (Worthley et al. 2005, Ip et al. 2009, Matsushita et al. 2013).

MBL is encoded by *MBL2* located on chromosome 10. *MBL2* has four exons, and deficiency states of MBL arise from polymorphisms in the structural and promoter sequences of the gene. There are three polymorphisms in the MBL encoding gene, and they are located at codons 52, 54, and 57. The structural gene variants are referred to as D, B, and C, respectively, and they are collectively referred to as O. The wild-type genotype is A/A (Minchinton et al. 2002, Denholm et al. 2010), which is generally associated with high MBL levels. Subjects with variant *MBL2* genotypes (A/O or O/O) have clearly presented with lower serum MBL concentrations than those with the wild genotype. The homozygotic variants for both alleles (O/O) have consistently presented with lower MBL production than heterozygotes (A/O) in several studies (Minchinton et al. 2002, Eisen et al. 2008, Denholm et al. 2010).

MBL2 variants and low MBL levels have been associated with increased susceptibility to and severity of many different infections (Eisen et al. 2008, Eisen et al. 2010, Gröndahl-Yli-Hannuksela et al. 2013). Particularly infants and young children with immature adaptive immunity are susceptible to these infections (Israels et al. 2010, Ozkan et al. 2012). Variant *MBL2* genotypes have also been linked to more severe clinical pictures and an increased risk for asthma in young infants hospitalized for bronchiolitis at under six months of age (Koponen et al. 2012, Nuolivirta et al. 2012). Studies on MBL and TB have been inconsistent regarding the results. In a meta-analysis, TB was not associated with variant *MBL2* genotypes, and interestingly, patients with active TB presented with even higher MBL

concentrations than healthy controls (Denholm et al. 2010), even though MBL has been low in patients with other infections.

2.6.1.4 Toll-like receptors and *TLR1*, *TLR2*, and *TLR6* polymorphisms

TLRs are pattern recognition receptors that play an important role in innate immunity (Takeda et al. 2003, Schröder et al. 2005). They are type I transmembrane proteins (Medzhitov et al. 1997) that are located on the surface of white blood cells, such as antigen-presenting cells (APCs). Their extracellular domain is characterized by leucine-rich repeats (LRRs), which are responsible for TLR-ligand interactions (Schröder et al. 2005). The intracellular domain of TLRs contains a Toll/interleukin-1 receptor (TIR) domain, which in turn activates the signaling cascade that leads to the induction of pro-inflammatory cytokines (Schröder et al. 2005). TLRs recognize and convey information about different pathogens, initiating an inflammatory and immunological response in the host (Takeda et al. 2003, Akira et al. 2004, Schröder et al. 2005, Akira et al. 2006). The TLR2 subfamily is the main mediator of macrophage activation in recognizing pathogens such as mycobacteria, including *M. tb*, and initiating the innate immunity response (Underhill et al. 1999, Thada et al. 2013). TLRs activate macrophages and dendritic cells through specific adaptor proteins (Zhang et al. 2013). The TLR2 subfamily comprises TLR1, TLR2, TLR6, and TLR10 (Tapping et al. 2007). The genes that encode these receptors are located in a gene cluster on chromosome four (Veltkamp et al. 2012). As co-receptors for TLR2, TLR1 and TLR6 enhance the pathogen recognition signal through TLR2 (Bulut et al. 2001), which can form heterodimers with either TLR1 or TLR6 to sense the PAMPs of *M. tb* (Zhang et al. 2013).

TLR1, TLR2, and TLR6 are encoded by the genes *TLR1*, *TLR2*, and *TLR6*, respectively. The ability to respond properly to TLR ligands may be impaired by SNPs within these TLR-encoding genes, which leads to increased susceptibility to infectious diseases (Schröder et al. 2005). Altered signaling in association with *TLR2* polymorphisms, for example rs5743708, has been detected in functional studies (Schröder et al. 2005). *TLR1* rs5743618 regulates signaling in response to lipopeptides, including those in mycobacteria, and essentially defines TLR1 deficiency in humans

(Randhawa et al. 2011). SNPs in *TLR1* and *TLR6* have been associated with altered BCG-induced immune responses after vaccination (Randhawa et al. 2011). TLR2 subfamily gene SNPs have also been associated with TB, because the activation of innate immunity responses against mycobacteria occurs only through functional TLRs (Thada et al. 2013). In a recent meta-analysis of 16 studies, *TLR1*, *TLR2*, and *TLR6* polymorphisms were associated with the risk of TB (Zhang et al. 2013).

2.6.1.5 Interleukin-12/interferon-γ pathway and MSMD

IL-12 and IFN-γ are key cytokines in the defense against mycobacteria and the immune response to the BCG vaccine (Rosenzweig et al. 2005). Monocytes and macrophages are the main producers of IL-12 (Feinberg et al. 2004). NF-kappa-B essential modulator (NEMO) and interleukin-1 receptor-associated kinase 4 (IRAK-4) are intracellular molecules involved in signaling innate immune responses from TLRs. They are important for IL-12 production and subsequent IFN-γ production. IFN-γ is produced by T cells and NK cells, and activates cytotoxic T cells (Boehm et al. 1997). It regulates mycobacterial growth, granuloma formation, and the initiation of the adaptive immune response to *M. tb* (Hossain et al. 2013). Stimulation of the whole blood by live BCG triggers the IL-12/IFN-γ pathway by an IRAK-4- and NEMO-dependent interaction between monocytes, NK cells, and T lymphocytes (Figure 1) (Feinberg et al. 2004).

The understanding of the pathogenesis of mycobacterial diseases has been greatly improved by studies of Mendelian susceptibility to mycobacterial disease (MSMD). It is a rare genetic condition that makes an individual susceptible to weakly virulent mycobacteria, such as BCG (Casanova et al. 2002, Filipe-Santos et al. 2006, Al-Muhsen et al. 2008, Cottle 2011, Bustamante et al. 2014, Ramirez-Alejo et al. 2014, Haverkamp et al. 2014) and atypical mycobacteria. MSMD patients are also vulnerable to the more virulent *M. tb* (Boisson-Dupuis et al. 2011, Bustamante et al. 2014, Boisson-Dupuis et al. 2015), and half of them have suffered from typhoidal or non-typhoidal salmonellosis (Cottle 2011, Bustamante et al. 2014, Ramirez-Alejo et al. 2014). These patients are also prone to candidiasis, and more rarely to infections with other intramacrophagic bacteria, fungi, or parasites,

and possibly even a few viruses. MSMD patients have a limited defect in their innate immunity, and they are usually otherwise healthy. They have no overt abnormalities in routine hematological and immunological tests. Mycobacterial disease usually begins in childhood or, rarely, during adolescence or adulthood (Bustamante et al. 2014).

The first genetic etiology of MSMD to be identified was autosomal recessive (AR) complete IFN-γR1 deficiency, and other forms of IFN-γR1 deficiency were subsequently discovered. Investigation of related candidate genes led to the identification of mutations in five other genes, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, and *NEMO* (Figure 1), all of which are involved in IL-12-dependent IFN-γ-mediated immunity. Nine genes causing MSMD have been discovered since the first observations in 1996 (Jouanguy et al. 1996, Cottle 2011, Bustamante et al. 2014). Seven of these genes are autosomal (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, and *IRF8*), and two are X-linked (*NEMO* and *CYBB*). The seven autosomal gene defects are either autosomal dominant (AD) or autosomal recessive (AR), and the resulting defect of the protein can be complete functional or partial functional. Two of the MSMD genes are X-chromosomal recessive (XR). Up to 2015, 18 different disorders of the IL-12/IFN-γ pathway have been defined. This relatively high number is due to the high level of allelic heterogeneity. These disorders are linked to the function of the pathway, impairing either the production of IFN-γ or the response to IFN-γ (Bustamante et al. 2014), which in part alters the immune response to mycobacteria. However, these defects account for only about half of the known MSMD cases. Patients with genetic defects causing MSMD may display other infectious diseases, or even remain asymptomatic. Because of the case-definition phenotype of MSMD, most of these inborn errors do not show complete clinical penetrance (Bustamante et al. 2014). Age at onset and clinical outcome vary considerably as a function of the gene involved, the type of mutation, and the time and manner in which the affected individual became exposed – i.e. whether the individual received the BCG vaccination at birth or acquired environmental mycobacterial (EM) infection via natural routes. Even subtle clinical features may depend on the genetic defect involved. For example, dominant partial IFN-γR1 deficiency is almost always associated with

osteomyelitis. Careful descriptions of the clinical features of each genetic defect will become possible as more patients with MSMD are identified.

Many patients with clinical and laboratory evidence of primary immunodeficiency do not have a gene-specific diagnosis. Next generation sequencing, particularly WES, is a powerful tool in identifying the disease-causing genes in some of these patients. At least 34 new gene defects have been identified in the last few years. Thus, it can be concluded that there is a great heterogeneity in the phenotype of patients with mutations in the same gene. Some patients have loss-of-function mutations and others have gain-of-function mutations (Conley et al. 2014). For example, IFN-γ receptor 1 (IFNGR1) deficiency is one of the genetic disorders of MSMD. It is autosomal recessive, a complete functional defect, with a poor prognosis due to early-onset, recurrent, and disseminated mycobacterial infections (Moilanen et al. 2009, Kong et al. 2010). Hematopoietic stem cell transplantation (HSCT) is the only curative treatment, and it has been successful in some cases (Moilanen et al. 2009).

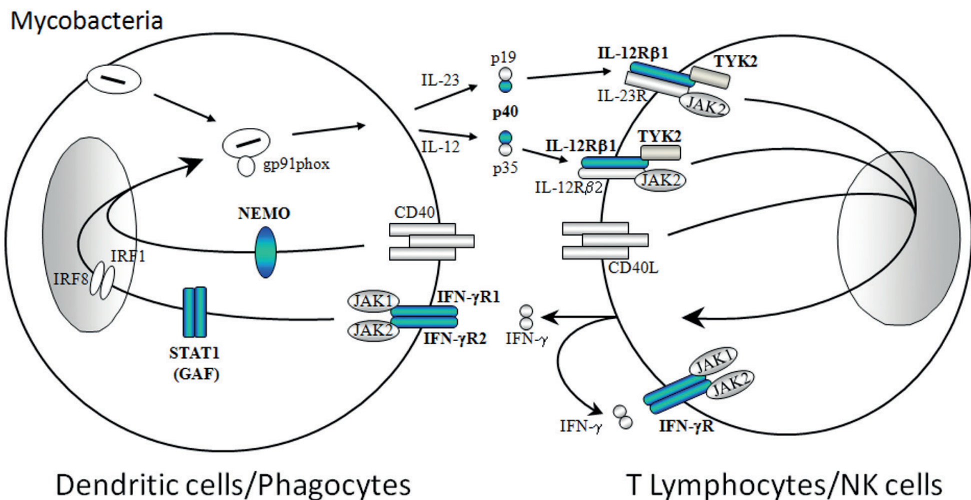

Figure 1. Inborn errors of the IL-12/IFN-γ pathway in patients with MSMD.

Schematic diagram of IL-12 and IFN-γ production and cooperation between myeloid (phagocytes/dendritic cells) and lymphoid (NK/T) cells. The IL-12/IFN-γ pathway is crucial for protective immunity against mycobacteria. MSMD-causing mutations in *IL12B* and *IL12RB1* impair the IL-12-dependent production of IFN-γ. MSMD-causing mutations in *IFNGR1*, *IFNGR2*, and *STAT1* impair the cellular responses to IFN-γ. MSMD-causing mutations in *NEMO* impair the T-cell- and CD40 -dependent production of IL-12.

2.6.1.6 Interleukin-10 and *IL10* polymorphisms

IL-10 is an essential cytokine in immune reactions. It is produced by Th1, Th2, and a subset of Th17 and Th22 cells, including macrophages, monocytes, T cells, B cells, dendritic cells, mast cells, and eosinophils. Interestingly, the structures of IL-10 and IFN-γ are similar (Sabat et al. 2010). IL-10 acts as an important immunoregulatory and anti-inflammatory cytokine that downregulates the expression of Th1 cytokines through macrophage deactivation (Zhang et al. 2011) and the blocking of IFN-γ release by Th1 lymphocytes (Hsu et al. 1995). Thus, the principal function of IL-10 is to limit, and ultimately terminate, inflammatory responses (Moore et al. 2001). IL-10 inhibits the synthesis of IL-12 and the development of Th1 immunity (Sabat et al. 2010). It is also capable of inhibiting MHC class I and class II expression and the expression of co-stimulating and adhesion molecules (Sabat et al. 2010). On the other hand, IL-10 enhances humoral immunity, for example, by promoting the proliferation and differentiation of B cells (Rousset et al. 1995, Moore et al. 2001). In addition, a new family of regulatory B cells (Bregs) has been discovered. These cells produce IL-10 and control the immune responses at the innate and adaptive levels. Innate CD5(+) Bregs negatively control innate inflammation and dendritic cell functions in neonatal mice by producing large amounts of IL-10 following TLR triggering (Lo-Man 2011).

The IL-10 encoding gene, *IL10*, is located on the long arm of chromosome 1, and it is composed of five exons and four introns (Eskalde et al. 1999, Reuss et al. 2002). It is highly polymorphic, and point mutations in the proximal promoter region, including rs1800896, rs1800871, and rs1800872, form distinct haplotypes associated with IL-10 production (Turner et al. 1997, Crawley et al. 1999). Three haplotypes are common in the Caucasian population: GCC, ACC, and ATA (Turner et al. 1997).

The ability to produce IL-10 varies between individuals due to genetically regulated differences (Eskdale et al. 1999). IL-10 has been associated in previous studies with many diseases, including TB (Ramaseri Sunder et al. 2012). The roles of *IL10* SNPs, as well as IL-10 production, have varied depending on the infection and studied population.

3 AIMS OF THE STUDY

The aim of this dissertation was to study the innate immunity of former BCG osteitis patients. Further, the aim was to find possible hereditary reasons that increase susceptibility to BCG osteitis, and to achieve this goal, different innate immunity gene polymorphisms were studied in the patients and the controls.

The specific aims were to evaluate:

1. The occurrence of *MBL2* polymorphisms and concentration of MBL in former BCG osteitis patients;

2. The occurrence of TLR2 subfamily gene polymorphisms in former BCG osteitis patients;

3. The function of the IL-12/IFN-γ pathway and occurrence of MSMD-related gene defects in former BCG osteitis patients;

4. The occurrence of *IL10* polymorphisms in former BCG osteitis patients.

4 MATERIALS AND METHODS

4.1 Study design

Registers of the National Public Health Institute were investigated to find former BCG osteitis patients from the years 1960-1988 in Finland. The registers revealed 222 patients (Kröger et al. 1995), and in 2007, addresses were found for 203 of them. Thus, 203 invitations to participate in the study were sent, and 160 of the patients replied. Blood samples were taken from 132 former BCG osteitis patients in different parts of Finland. The samples were then transported to Turku, Finland, and analyzed for factors involving the innate immunity of the patients. Genetic analyses were performed in Turku and at the Rockefeller University, New York, USA. Data from the 28 patients that replied to the invitation but did not give their blood samples was collected with a questionnaire. Also, data was requested from Statistics Finland on the causes of death of the missing patients.

4.1.1 Finnish BCG osteitis patients

The study group comprised 132 Finnish patients aged 21–49 years. They had developed BCG osteitis as children after receiving the BCG vaccine. The mean age when the first signs of osteitis appeared was 1.5 years (range 0.3–5.7 years). The median age of the 132 former BCG osteitis patients was 33.0 years (range 21–49 years), and 72 (54.4%) of the group were females. Their blood samples were taken in 2007–2008, and analyzed for different immunological and genetic markers.

4.1.2 Questionnaire data

The former BCG osteitis patients were given a questionnaire (Appendix) on their illnesses. In the questionnaire, the study subjects were asked if they had suffered from TB or any other mycobacteriosis or other severe or uncommon infections after infant BCG osteitis. Those 160 subjects who returned the questionnaire, including those 132 who provided blood samples, did not report any such infections. Among the 160 patients, 8% reported allergy, 7% asthma, 3% hypothyreosis, 3% psoriasis, 2% rheumatoid arthritis, 2% epilepsy, 2% multiple sclerosis, and less than 1% diabetes, spondylarthritis, stroke, breast cancer, or migraine. Seven patients (5%) reported an earlier *Salmonella* infection.

4.1.3 Register for causes of death

No residence information was available for 13 subjects with BCG osteitis in early childhood, but their dates of birth were recorded in the study documents. The register for causes of death (Statistics Finland) charted at a group level the causes of death of all deceased persons who had been born on the same day as the 13 former BCG osteitis patients without any residence information available, and nobody had died of TB or any other infection.

4.2 *Ex vivo* IL-12 and IFN-γ measurements and MBL

Fresh whole blood samples were obtained from 132 of the 160 former BCG osteitis patients who agreed to attend the study. Blood samples were collected into heparinized tubes at laboratories around the country, and they were immediately (within 12 hours) transported to the Tuberculosis Reference Laboratory, National Institute for Health and Welfare, Turku, Finland. Blood samples were also taken from 25 travel controls and transported to Turku. The cell cultures and stimulations were performed, and the blood samples were then centrifuged and the sera and peripheral blood mononuclear cells (PBMCs) were frozen at -70°C.

4.2.1 Stimulation of white blood cells

At the laboratory, the blood samples were diluted to 1:2 with RPM 1640 medium (GibcoBRL) supplemented with penistreptomycin at 10000 U/mL (GibcoBRL). The detailed protocol of whole blood culture and stimulation has been described previously (Feinberg et al. 2004). In brief, 6 mL of the diluted blood sample was dispensed into four wells (1.5 mL/well) of a 24-well plate (Nunc). They were cultured and stimulated with BCG (4x10E7 cells/0.1mL), BCG and recombinant IFN-γ (50 µl, 165000U/mL), or BCG and recombinant IL-12 (50 µL, 700 ng/mL), respectively. For each sample, whole blood without stimulation was used as a negative control. After 18 hours of incubation, 450 µL supernatant was collected from each well for the determination of IL-12, and after 48 hours of incubation, all supernatants left in each well were collected for the determination of IFN-γ. The cytokine concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D systems) according to the manufacturer's protocol. The detection limit of the test was 0.7 pg/mL for IL-12 and 17.6 pg/mL for IFN-γ. The differentials of white blood cells were counted in 119/132 (90%) study subjects before the samples were transported, and the IL-12 and IFN-γ concentrations in the cell cultures are expressed per PBMCs, including lymphocytes and monocytes. The IL-12 and IFN-γ concentrations (in pg/mL) per PBMCs (1.0 x 10E9/L) were used in the analyses. Data on PBMC counts were available in 119 cases, data on stimulated IL-12 concentrations in 125 cases, and data on stimulated IFN-γ concentrations in 131 cases. Calculated concentrations per PBMCs were present in 115 (IL-12) and 118 (IFN-γ) cases, respectively.

4.2.2 MBL

Serum concentrations of MBL were measured using an ELISA-based method, as described earlier (Gröndahl-Yli-Hannuksela et al. 2013). Briefly, mouse monoclonal antihuman MBL immunoglobulin G1 (IgG1) antibodies (8 µg/mL) (HYB 131-01, Statens Serum Institut (SSI), Copenhagen, Denmark) were used for coating. Samples were diluted

to 1:100 in PBS-0.05% Tween 20. The absorbance was read at 450 nm with a Multiskan EX reader (Thermo Fisher Scientific, Vantaa, Finland). Standard serum containing 3200 ng/mL (SSI, Copenhagen, Denmark) of oligomerized MBL was used to create the standard curve. The detection limit of this assay was 50 ng/mL. The concentration below the detection limit was given the value 25 ng/mL. Sera with an MBL concentration higher than the upper detection limit of the standard curve (6400 ng/mL) were further diluted to 1:200 and re-tested. MBL-deficient serum (SSI) was included in each run.

4.3 Genetic methods

4.3.1 Genotyping of *MBL2*

MBL2 genotypes in exon 1, codons 52 (allele D, rs5030737), 54 (allele B, rs1800450), and 57 (allele C, rs1800451) were determined by a PCR followed by pyrosequencing (PSQ TM 96MA Pyrosequencer, Biotage, Uppsala, Sweden), using a PSQ TM 96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol and as described earlier (Roos et al. 2006, Vuononvirta et al. 2011). The genomic DNA was extracted from peripheral blood using the Qiagen QiAmp DNA blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers used for *MBL2* PCR were: forward 5'-biotin-CCTTCCCTGAGTTTTCTCAC-3', reverse 5'-AACAGCCCAACACGTACCTG-3', sequencing 5'-CGTACCTGGTTCCCCCTTTTCT-3'. All the primers were purchased from Sigma-Aldrich, Helsinki, Finland. The size of the *MBL2* PCR product was 240 bp. The genotyping process for *MBL2* was identical in the former BCG osteitis patients and the controls.

4.3.2 Genotyping of *TLR1*, *TLR2*, and *TLR6*

The genotyping of the SNPs *TLR1* rs5743618 (-1805T/G), *TLR2* rs5743708 (-2258G/A), and *TLR6* rs5743810 (-745C/T) was performed by pyrosequencing at the laboratory of National Institute for Health

and Welfare, Turku, Finland, as described earlier (Nuolivirta et al. 2012, Nuolivirta et al. 2013). The genomic DNA was extracted from peripheral blood using the Qiagen QiAmp DNA blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All the primers were purchased from Sigma-Aldrich, Helsinki, Finland. PCRs were studied with selected primer pairs for the *TLR*s, and the PCR amplifications were carried out as described earlier (Vuononvirta et al. 2011, Nuolivirta et al. 2012, Nuolivirta et al. 2013). The genotyping process for *TLR1*, *TLR2*, and *TLR6* was identical in the former BCG osteitis patients and the controls.

4.3.3 Analysis of MSMD genes

Given the implication of the IFN-γ pathway in mycobacterial infections, the nine known genes of MSMD (18 known gene defects) were studied in 20 study subjects as a pilot study. The samples were picked up to include cases with low and high IL-12 concentrations after BCG+IFN-γ stimulation, low and high increases in stimulated concentration, and those with low and high IFN-γ concentrations after BCG+IL-12 stimulation, and low and high increases in stimulated concentration, respectively. WES was performed by the New York Genome Center using an Illumina HiSeq 2500 machine, with an Agilent 71 Mb SureSelect exome kit. The reads were aligned to the human reference genome with a BWA aligner, and then re-calibrated and annotated with GATK (McKenna et al. 2010), PICARD (http://picard.sourceforge.net/), and ANNOVAR (Wang et al. 2010). The variations were further filtered and investigated with an in-house online server. The known gene defects of *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO*, and *CYBB* were examined as described earlier (Bustamante et al. 2014).

4.3.4 Genotyping of *IL10*

Genotyping of the *IL10* rs1800896 (-1082G/A) SNP was carried out using the ABI PRISM® 7000 Sequence Detection System for both PCR and allelic discrimination, as described earlier.

IL10 rs1800890 (-3575T/A) genotypes were determined by PCR followed by pyrosequencing, as described previously. Primers were designed to recognize the polymorphic site in the *IL10* promoter region (A changes to T).

The detection of *IL10* polymorphism sites rs1800871 (-819A/G) and rs1800872 (-592T/G) was performed using PCR and the sequencing of the amplified region. *IL10* rs1800871 and rs1800872 are in full linkage in Caucasian populations. The SNPs at position -592 and -819 were detected simultaneously in one PCR reaction.

The sequences of PCR primers were: forward 5'-TAGGTCTCTGGGCCTTAGTT-3' and reverse 5'-AAGGCCAATTTAATCCAAGGTT-3'. The correct PCR product size (440 bp) was verified with agarose gel electrophoresis. The forward primer was also used for the sequencing reaction. The SNP at location -1082 was amplified with primers forward 5'-TCTCCAGCACATAGAATGAAACC-3' and reverse 5'-CTTCCCCAGGTAGAGCAACA-3'. The PCR product size was 330 bp. The forward primer was also used for the sequencing reaction. Sequencing reactions were performed at the Institute for Molecular Medicine Finland (FIMM, Helsinki). All PCR reactions were performed in the following conditions: initial denaturation at 95°C, denaturation at 95°C for 2 minutes, annealing at 60°C for 30 seconds, and extension at 72°C for 40 seconds. After 40 cycles, there was a final denaturation at 72°C for 7 minutes.

Our test gave the reverse transcription sequence, instead of the forward transcription sequence used in most previous publications. Therefore, to aid comparison with other studies, we express the alleles G/A (*IL10* rs1800871) and G/T (*IL10* rs1800872) as C/T (*IL10* rs1800871) and C/A (*IL10* rs1800872), respectively.

Genotyping of 401 controls (rs1800896, rs1800871, and rs1800872) from extracted DNA was performed using the Sequenom mass ARRAY iPlex Gold® system (Sequenom Inc, CA, USA) at the University of Eastern Finland, Kuopio, Finland. The genotyping process for *IL10* was identical in the former BCG osteitis patients and the controls.

4.4 Statistical analyses

The SPSS 19.0 package (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) was used for statistical analyses.

4.4.1 MBL and *MBL2*

The MBL concentrations are expressed as medians, interquartile ranges (25th–75th percentile), and minimum and maximum values, presented as box-and-whiskers diagrams when appropriate. The Mann-Whitney U -test was used for comparing the medians of MBL concentrations between cases and controls, and between *MBL2* genotypes. Chi square and Fisher's exact tests were used for comparing *MBL2* genotypes between cases and controls.

4.4.2 *TLR1*, *TLR2*, and *TLR6*

Chi square and Fisher's exact tests, when appropriate, were used for comparing genotypes and allele frequencies between cases and controls. Bonferroni correction was applied in paired comparisons. Logistic regression was used in adjusted analyses, and the results were expressed as adjusted odds ratios (aOR) and their 95% confidence intervals (95%CI). Deviations from Hardy-Weinberg Equilibrium (HWE) were evaluated using the FINETTI program.

4.4.3 IL-12/IFN-γ pathway

Exploratory data analyses revealed that the IL-12 and IFN-γ concentrations and increases in concentrations were non-normally distributed, and therefore, the results are expressed as medians, 5th, 10th, 25th, 75th, 90th, and 95th percentiles, and ranges. The PBMC counts (cells x 10E9/L) are expressed as means, standard deviations (SD), and ranges. IL-12 and IFN-γ concentrations are expressed per PBMCs.

4.4.4 *IL10*

Chi square and Fisher's exact tests were used, when appropriate, for comparing genotypes and allele frequencies of the cases and controls. Allele combinations are presented as percentages and their 95% confidence intervals (95%CI).

4.5 Ethics

The study was accepted by the Ethics Committee of the Tampere University Hospital District, Finland. Written consent was obtained from the study subjects, including permission to perform genetic studies concerning susceptibility to mycobacterial infections. The study protocol concerning the genetic control group of children was approved by the Ethics Committee of the Hospital District of South-Western Finland, Turku, Finland. Written consent was obtained from the children's parents to use their children's blood samples.

5 RESULTS

5.1 *MBL2* polymorphisms and MBL (I)

The study comprised 132 former BCG osteitis patients, and among them, 76 (57.6%) were A/A homozygotes, 53 (40.2%) A/O heterozygotes, and only 3 (2.3%) O/O homozygotes (Table 1). Thus, 56 (42.4%) study subjects had the non-wild variant genotype A/O or O/O, compared with 133 (32.3%) in the controls (p=0.033).

Data of serum MBL concentration was available for 130 of the former BCG osteitis patients, and there was no significant difference in MBL concentrations between the patients and the controls. The lower limit of the interquartile range was higher than 500 ng/mL in both groups. Twenty-one patients (16.2%) and 21 controls (25.3%; p=0.114) had MBL <500 ng/mL. Six patients (4.6%) and six controls (7.2%; p=0.543) had MBL <50 ng/mL (the detection limit of the test).

In the 74 A/A homozygotes, the median of the serum MBL concentrations was 2673 ng/mL (range 391–8543 ng/mL), compared to 736 ng/mL (25–2458; p<0.001) in the 53 A/O heterozygotes. The median of the serum MBL concentrations was below the detection limit (<50 ng/mL) in the three O/O homozygotes. Thus, the variant genotypes were associated with lower serum MBL concentrations than the wild type genotype. However, the number of cases homozygous for the variant genotype was too low for statistical analyses. The six low-producers of MBL (<50 ng/mL) had genotype AB in four cases (66.7%) vs. 26.6% in the 124 non-low-producers. The respective proportions were 16.7% vs. 0% for the BB and 16.7% vs. 8.1% for the BD genotypes.

Table 1. Genotypes of *MBL2* in 132 former BCG osteitis patients and 412 population controls.

Genotypes	Former osteitis patients n=132		Population controls n=412[&]	
	n	%	n	%
A/A	76	57.6	279	67.7
A/O	53[¤]	40.2[¤]	124	30.1
A/B	37	28.0	86	20.9
A/C	3	2.3	1	0.2
A/D	13	9.8	37	9.0
O/O	3"	2.3"	9	2.2
B/B	1	0.8	5	1.2
C/C	0	0	0	0
D/D	0	0	1	0.2
B/D	2	1.5	1	0.2
A/O or O/O	56[#]	42.4[#]	133	32.3

[&]Population-based cohort of three-month old infants, as published previously (Gröndahl-Yli-Hannuksela et al. 2013). The figures are nearly identical to those published from 473 Finnish military constricts (Rantala et al. 2008): A/A 64.8%, A/O 30.3%, and O/O 4.9%. [#]p=0.033 vs. controls; [¤]p=0.128 vs. controls; ["]p=0.405 vs. controls

5.2 *TLR1*, *TLR2*, and *TLR6* polymorphisms (II)

The *TLR1* genotype was GG in 106 cases (80.3%), GT in 25 cases (18.9%), and TT in one case (0.81%) (p=0.012 vs. controls). The frequency of the GT or TT variant genotype was 19.7% in the cases and 33.6% in the controls (p=0.003), and the frequency of the minor allele T was 10.2% and 17.9%, respectively (p=0.004) (Table 2).

The *TLR2* genotype was GG in 117 cases (88.7%), GA in 15 cases (11.4%), and there were no AA cases (p=0.033). The frequency of the variant GA genotype was 5.7% in the controls (p=0.033 vs. cases), and the frequencies of the minor allele A were 5.7% and 2.8% (p=0.036) (Table 2).

The *TLR6* genotype was CC in 30 cases (22.8%), CT in 69 cases (52.2%), and TT in 33 cases (25.0%) (p=0.006 vs. controls). The frequency of the CT or TT variant genotype was 77.3% in the cases and 61.6% in the controls

(p=0.001), and the frequency of the minor allele T was 51.1% and 40.6%, respectively (p=0.004) (Table 2).

The genotypes of *TLR1* rs5743618 (-1805T/G), *TLR2* rs5743708 (-2258G/A), and *TLR6* rs5743810 (-745C/T) of the control population were in HWE.

Different combinations of *TLR1*, *TLR2*, and *TLR6* wild and variant genotypes were compared with the combination of a wild genotype in all three *TLR*s. This showed that only the combination of the *TLR1* wild and the TL*R*2 variant and the *TLR6* variant genotypes differed significantly between the cases and the controls (Table 3).

The presence of the *TLR2* or *TLR6* variant genotypes increased the risk of belonging to the BCG osteitis group and the presence of the *TLR1* variant genotype decreased the risk (Table 3). Therefore, all three variant genotypes were included simultaneously in a logistic regression model, and this showed that the *TLR1* variant genotype decreased the risk (aOR 0.48), while both the *TLR2* variant genotype (aOR 2.16) and the TLR6 variant genotype (aOR 2.11) increased the risk significantly and independently of each other (Table 4).

Table 2. The distribution of *TLR1* (rs5743618), *TLR2* (rs5743708), and *TLR6* (rs5743810) genotypes in 132 cases with BCG osteitis in infancy and 318 population-based controls.

Genotypes	Cases n=132		Controls n=318		p-value
	n	%	n	%	
TLR1					0.012
GG	106	80.3	211	66.3	
GT	25	18.9	100	31.5	
TT	1	0.8	7	2.2	
Variant type (GT or TT)	26	19.7	107	33.6	0.003
Major allele (G)	237/264	89.8	522/636	82.1	0.004
Minor allele (T)	27/264	10.2	114/636	17.9	
TLR2					0.033
GG	117	88.6	300	94.3	
GA	15	11.4	18	5.7	
AA	0	0	0	0	
Variant type (GA or AA)	15	11.4	18	5.7	0.033
Major allele (G)	247/262	94.3	618/636	97.2	0.036
Minor allele (A)	15/262	5.7	18/636	2.8	
TLR6					0.006
CC	30	22.8	122	38.3	
CT	69	52.2	134	42.1	
TT	33	25.0	62	29.6	
Variant type (CT or TT)	102	77.3	196	61.6	0.001
Major allele (C)	129/264	48.9	378/636	59.4	0.004
Minor allele (T)	135/264	51.1	258/636	40.6	

Table 3. The association between different combinations of *TLR1*, *TLR2*, and *TLR6* wild and variant genotypes and BCG osteitis in infancy.

Combinations					**Compared to wild-wild-wild**
TLR1 (rs5743618) wild GG variant GT or TT	***TLR2*** (rs5743708) wild GG variant GA	***TLR6*** (rs5743810) wild CC variant CT or TT	***Case*** N=132 (%)	***Control*** N=318 (%)	***p-value***
Wild	Wild	Wild	18	68	-
Wild	Wild	Variant	73 (55.3)	131 (41.1)	0.013[1]
Wild	Variant	Wild	2	4	0.608
Wild	Variant	Variant	12 (9.0)	8 (2.5)	0.002[2]
Variant	Wild	Wild	9	45	0.661
Variant	Wild	Variant	16	56	0.849
Variant	Variant	Wild	1	5	0.999
Variant	Variant	Variant	0	1	0.999
Overall p-value 0.001					

[1]p=0.091 after Bonferroni correction by multiplying the p-value by 7 (the number of paired comparisons),
[2]p=0.014 after Bonferroni correction by multiplying the p-value by 7 (the number of paired comparisons)

Table 4. Logistic regression: *TLR1*, *TLR2*, and *TLR6* variant genotypes as predictive factors of BCG osteitis.

Genotypes	**Cases** (N=132)	**Controls** (N=318)	**aOR**(95%CI)[1]
***TLR1* variant** (N=133)	26 (19.7%)	107 (33.6%)	0.554 (0.336–0.911)
***TLR2* variant** (N=33)	15 (11.4%)	18 (5.7%)	2.154 (1.026–4.521)
***TLR6* variant** (N=298)	102 (77.3%)	196 (61.6%)	1.907 (1.183–3.075)

[1]Adjusted for sex. *TLR1*, *TLR2*, and *TLR6* variant genotypes were included in the same model.

5.3 IL-12/IFN-γ pathway and MSMD genes (III)

5.3.1 Peripheral blood mononuclear cells

In the blood samples of the former BCG osteitis patients, the mean peripheral blood mononuclear cell (PBMC) count (SD, range) was 2.80 x10E9/L (0.89, 0.60–5.49). The figures for lymphocytes were 2.35 (0.81, 0.54–4.80) and for monocytes 0.45 (0.18, 0.06–1.10).

5.3.2 IL-12 and IFN-γ concentrations

Before BCG stimulation of white blood cells, IL-12 was measurable (>0.7 pg/mL) in 17 (13%) samples and IFN-γ was measurable (>17.6 pg/mL) in 15 (11%) samples. The respective figures were 84 (64%) and 131 (99%) after stimulation with BCG. Thus, even after BCG stimulation, IL-12 concentration was below the detection limit in 48 (36%) cases.

The median IL-12 concentration per PBMCs was 38.1 pg/mL after BCG+IFN-γ stimulation. The median IFN-γ concentration per PBMCs was 16708.7 pg/mL after BCG+IL-12 stimulation (Table 5).

The median increase of the IL-12 concentration per PBMCs was 36.5 pg/mL between BCG alone and BCG+IFN-γ stimulations. The median increase of the IFN-γ concentration per PBMCs was 12516.0 pg/mL between BCG alone and BCG+IL-12 stimulations (Table 6).

By the limit of the $<5^{th}$ percentile, *ex vivo* IL-12 concentration and the increase in concentration was low in five cases and *ex vivo* IFN-γ concentration and the increase in concentration was low in six cases; two cases with low concentrations and low increases in both IL-12 and IFN-γ are included (Table 7).

By the limit of the $<10^{th}$ percentile, *ex vivo* IL-12 concentration and increase in concentration was low in an additional six cases, and *ex vivo* IFN-γ concentration and increase in concentration was low in additional four cases; two cases with low concentrations and low increases in both IL-12 and IFN-γ are included (Table 6). With two exceptions, low *ex vivo*

concentrations and low increases in concentrations picked up the same cases.

No cases presented with a low (<10th percentile) stimulated IFN-γ and a high (>90th percentile) stimulated IL-12 concentration, or with a low (<10th percentile) stimulated IL-12 and a high (>90th percentile) stimulated IFN-γ concentration in the cell cultures (data not shown).

The reported current or earlier diseases in low (<5th or 5–10th percentiles) IL-12 or IFN-γ producers are presented in Table 7. There were four subjects with low production of both IL-12 and IFN-γ, and one of them reported asthma, one diabetes, one stroke, and one reported no disease (Table 7).

5.3.3 Genes of the IL-12/IFN-γ pathway

Known gene mutations of MSMD, or the IL-12/IFN-γ pathway – including *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO* and *CYBB* – were studied in 20 former BCG osteitis patients by WES. The samples were picked up to include cases with low and high IL-12 concentrations after BCG+IFN-γ stimulation, and low and high increases in stimulated concentration, and cases with low and high IFN-γ concentrations after BCG+IL-12 stimulation, and low and high increases in stimulated concentration, respectively.

No mutations were found in the coding regions of the genes studied. Seven of the patients belonged to the groups of low (<10th percentile) stimulated *ex vivo* IL-12 concentration and low increase in concentration (N=4), or low (<10th percentile) stimulated *ex vivo* IFN-γ concentration and low increase in concentration (N=3).

Table 5. IL-12 and IFN-γ concentrations in cell cultures per PBMCs in 115 and 118 adults with BCG osteitis in infancy.

	IL-12 concentration (pg/mL) **after BCG+IFN-γ stimulation** (N=115)	**IFN-γ concentration** (pg/mL) **after BCG+IL-12 stimulation** (N=118)
5th percentile	3.5	2229.4
10th percentile	5.2	4474.2
25th percentile	12.4	8597.8
Median	38.1	16708.7
75th percentile	66.0	31330.9
90th percentile	109.6	45801.5
95th percentile	152.4	59741.3
Range	2.9–274.7	543.0–81402.3

Table 6. Increases in IL-12 and IFN-γ concentrations per PBMCs after BCG+IFN-γ or BCG+IL-12 stimulations, respectively, compared to concentrations after BCG stimulation alone. The detection limit was used for calculations if the concentration after BCG stimulation was non-measurable.

	Increase in IL-12 concentration (pg/mL) (N=114)	**Increase in IFN-γ concentration** (pg/mL) (N=110)
5th percentile	3.5	2236.9
10th percentile	5.1	4390.3
25th percentile	11.7	7035.0
Median	36.5	12516.0
75th percentile	61.2	22393.9
90th percentile	108.9	32620.3
95th percentile	147.7	43708.4
Range	-3.0–260.5	990.3–66182.5

Table 7. Twenty low IL-12 or IFN-γ producers identified with four criteria based on results after BCG+IFN-γ or BCG+IL-12 stimulations of cell cultures, respectively (the *ex vivo* concentration or increase in concentration of IL-12 or IFN-γ <5th percentile or <10th percentile, respectively).

No	Sex (m/f), age (years)	IL-12 concentration low	IL-12 increase low	IFN-γ concentration low	IFN-γ increase low
	<5th percentile				
25	f, 45			x	x
64	f, 32	x	x	x	x
65	m, 36*			x	x
66	f, 34			x	x
67	m, 34*	x	x		
72[1]	f, 22	x	x	x	x
74	f, 47			x	x
82	m, 28			x	
128[2]	f, 41*	x	x		
132[3]	f, 34*	x	x		
	5th–10th percentile				
26	m, 33			x	x
59	f, 38*	x	x		
62	m, 35				x
110	f, 28	x	x		
115[4]	f, 37	x	x		
130[5]	f, 35*	x	x	x	x
137[6]	f, 48*	x	x	x	x
141[1,3]	f, 46		x		
142	f, 49			x	x
155	f, 21	x	x		

**IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO*, and *CYBB* genes were studied in seven cases, and all tests gave a negative result.
Reported diseases: [1]Asthma; [2]Allergy; [3]*Salmonella* infection; [4]Multiple sclerosis; [5]Diabetes; [6]Stroke

5.3.4 Association of *MBL2*, *TLR1*, *TLR2*, and *TLR6* polymorphisms with *ex vivo* IL-12 and IFN-γ production (unpublished data)

In this cohort, three patients had the homozygous variant *MBL2* genotype (O/O), and in one of them, IFN-γ concentration was at the 5th percentile level after stimulation with BCG+IL-12. The heterozygous variant *MBL2* genotype (A/O) was present in 53 patients, and no association was found with low IFN-γ or IL-12 production by either the 5th or 10th percentile limits (data not shown).

In 14 patients with the *TLR2* variant genotype (GA), 28.6% had low IFN-γ production by the 10th percentile limit, compared to 7.8% in the 103 patients with the *TLR2* wild genotype (GG) (p=0.037). The respective figures were 14.3% and 2.9% by the 5th percentile limit (p=0.108). There were no significant associations between *TLR1* or *TLR6* polymorphisms (or variant or wild genotypes) and stimulated *ex vivo* IL-12 or IFN-γ concentrations (data not shown).

5.4 *IL10* polymorphisms (IV)

In *IL10* rs1800896 (-1082G/A), the genotype was AA in 43 (33.9%) cases, AG in 56 (44.1%) cases, and GG in 28 (22.0%) cases (vs. 34.4%, 47.1%, and 18.5% in controls, p=0.655). The frequency of the variant genotype (AG or GG) was 66.1% (vs. 65.6% in controls, p=0.908), and the frequency of minor allele G was 44.1% (vs. 42.0% in controls, p=0.560).

In *IL10* rs1800871 (-819C/T), the genotype was CC in 79 (61.2%) cases, CT in 42 (32.6%) cases, and TT in 8 (6.2%) cases (vs. 59.1%, 34.4%, and 6.5% in controls, p=0.911). The frequency of the variant genotype (CT or TT) was 38.8% (vs. 40.9% in controls, p=0.667), and the frequency of minor allele T was 22.5% (vs. 23.7% in controls, p=0.690).

In *IL10* rs1800872 (-592C/A), the genotype was CC in 79 (61.2%) cases, CA in 42 (32.6%) cases, and AA in 8 (6.2%) cases (vs. 59.1%, 34.4%, and 6.5% in controls, p=0.911). The frequency of a variant genotype (CA or AA) was 38.8% (vs. 40.9% in controls, p=0.667), and the frequency of minor allele A was 22.5% (vs. 23.7% in controls, p=0.690).

In *IL10* rs1800890 (-3575 T/A), the genotype was AA in 55 (41.7%) cases, AT in 56 (42.4%) cases, and TT in 21 (15.9%) cases (vs. 44.7%, 44.4%, and 10.8% in controls, p=0.307). The frequency of variant genotype (AT or TT) was 58.3% (vs. 55.3% in controls, p=0.544) and the frequency of minor allele T was 37.1% (vs. 33.1% in controls, p=0.232).

Since the frequencies of genotypes and allele frequencies were surprisingly similar in the former BCG osteitis cases and the controls, we calculated the frequencies of the eight possible combinations of the three *IL10* SNPs (*IL10* rs1800896, *IL10* rs1800871, and *IL10* rs1800872) located in the same proximal promoter region of the gene. Again, there were no significant differences in the frequencies of these allele combinations between the cases and the controls (Table 8). They were in HWE.

Table 8. Allele combinations of *IL10* rs1800896 (-1082G/A), *IL10* rs1800871 (-819C/T), and *IL10* rs1800872 (-592C/A) in 127 former BCG osteitis patients and 400 population-based controls.

Combination	Cases No	Cases % (95%CI)	Controls No	Controls % (95%CI)
GCC	84	22.6 (18.6–27.1)	262	21.6 (19.4–24.0)
GCA	22	5.9 (3.9–8.8)	71	5.9 (4.7–7.3)
GTC	22	5.9 (3.9–8.8)	71	5.9 (4.7–7.3)
GTA	22	5.9 (3.9–8.8)	71	5.9 (4.7–7.3)
ACC	91	24.5 (20.4–29.1)	300	24.8 (22.4–27.3)
ACA	41	11.0 (8.2–14.6)	137	11.3 (9.6–13.2)
ATC	41	11.0 (8.2–14.6)	137	11.3 (9.6–13.2)
ATA	49	13.2 (10.1–17.0)	163	13.4 (11.6–15.5)

6 DISCUSSION

6.1 *MBL2* polymorphisms and MBL in BCG osteitis

Serum MBL concentrations were measured in the former BCG osteitis patients of this study in adulthood. The results showed that they were no lower in the patients than in the controls of the same age group, and the findings were similar in the analyses with MBL included as a continuous and categorized variable. However, a difference was found in the genetic studies, as a significantly higher percentage of the former BCG osteitis patients had variant *MBL2* genotypes than the population controls. In addition, the variant *MBL2* genotypes were associated with low serum MBL concentrations. This has also been a common finding in various previous studies (Minchinton et al. 2002, Eisen et al. 2008, Denholm et al. 2010).

MBL plays a key role in the complement pathway of innate immunity against different pathogens (Eisen et al. 2008, Eisen et al. 2010), including *M. tb* (Azad et al. 2012). Despite this, the impact of low MBL production on susceptibility to or the severity of infections is still unresolved, as the results of different studies have been controversial. For example, no association was found between *MBL2* genotypes or MBL production and invasive pneumococcal infections such as pneumonia in a recent review (Garcia-Laorden et al. 2013). Furthermore, a large European study found that variants in *MBL2* do not associate with sepsis susceptibility or survival (Mills et al. 2015). On the other hand, one meta-analysis showed that low MBL concentrations were associated with increased susceptibility to septic infections in newborns (Israels et al. 2010).

In a recent meta-analysis of 17 publications on MBL and TB, no association was found between *MBL2* genotypes and TB infection (Denholm et al. 2010). This indicates that the variant, usually low MBL producing genotypes are not associated with susceptibility to TB. Instead, the reviewed studies

reported a consistent increase in serum MBL concentration in patients with active TB (Denholm et al. 2010). This suggests that MBL is elevated in active TB as part of an acute-phase reaction. One recent study detected higher serum MBL levels in patients with active TB than in household contacts and a strong association between *MBL2* exon 1 and promoter genotypes and MBL levels (Garcia-Gasalla et al. 2014). A fairly recent study found – as opposed to negative association studies – that *MBL2* exon 1 allele O was associated with susceptibility to TB; allele C at codon 57, as well as the AC genotype, were more frequent in TB patients than in the controls (da Cruz et al. 2013). In a Turkish study, the frequency of the *MBL2* polymorphism that produces low levels of MBL was lower in children with TB than in the controls (Cosar et al. 2008).

No previous studies have been made on the association of BCG infections and MBL production or genetics. Because of the fact that in some infectious diseases clearly low MBL levels have been detected (Rantala et al. 2008, Luo et al. 2014), we hypothesized that the former BCG osteitis patients of our study may have lower MBL levels than the controls. However, we did not find differences in MBL levels in the patients compared to the controls; instead, we found a difference in the frequencies of *MBL2* genotypes. As the cohort of former BCG osteitis patients had more variant genotypes than the control group, it can be concluded that variant *MBL2* genotypes may be associated with an increased risk of BCG osteitis in vaccinated newborns.

MBL2 and promoter genes together regulate the production of MBL (Minchinton et al. 2002, Eisen et al. 2008), but we did not study the promoter areas. Previous studies have evaluated gene polymorphisms in three regulatory areas of the MBL encoding gene (Eisen et al. 2008, Rantala et al. 2008). H, Y, and Q alleles have been associated with high MBL concentrations, and their counterparts, L, X, and P alleles, with low MBL concentrations. In Vietnam, the YA/YA diplotype, which exhibited high plasma MBL concentrations, was associated with protection against active TB in patients under 45 years old. The results indicate that high MBL concentration may protect against early development of pulmonary TB (Hijikata et al. 2014). In light of these studies and our present results, the promoter genes of *MBL2* would be interesting to study in BCG osteitis patients.

6.2 *TLR1*, *TLR2*, and *TLR6* polymorphisms in BCG osteitis

SNPs of the genes encoding three Toll-like receptors of the TLR2 subfamily, TLR1, TLR2, and TLR6, were analyzed in this study. Interestingly, polymorphisms in all these genes were clearly associated with BCG osteitis. The *TLR1* wild genotype and the *TLR2* and *TLR6* variant genotypes were significantly more common in the former BCG osteitis patients than in the controls. When different combinations of *TLR1*, *TLR2*, and *TLR6* wild and variant genotypes were studied, only the combination of the *TLR1* wild type and the *TLR2* and *TLR6* variant types was significantly associated with BCG osteitis.

TLR2 subfamily gene polymorphisms have been associated with an increased susceptibility to many diseases in previous studies. Associations have also been found with various infectious diseases, such as TB, borreliosis, and meningococcal meningitis (Ma et al. 2007, Misch et al. 2008, Dalgic et al. 2011, Van Well et al. 2013, Zhang et al. 2013). However, TLR2 subfamily gene polymorphisms do not seem to play a role in some infections, such as viral bronchiolitis (Nuolivirta et al. 2013). There are no previous studies on genes or gene polymorphisms regulating TLR expression in patients with complications due to the BCG vaccination, such as BCG osteitis. Some studies have documented cytokine responses to BCG antigens in cell cultures (Finan et al. 2008, van den Biggelaar et al. 2009) and TLR expressions in cell cultures after BCG exposure (van den Biggelaar et al. 2009). These studies provide *ex vivo* evidence that functionally defined innate immune gene variants are associated with the development of immune responses after *in vivo* vaccination against mycobacteria in humans. The BCG vaccination is known to activate the same pathways as TB, including TLR2 (Pompei et al. 2007). In addition to *TLR1* rs5743618 and *TLR6* rs5743810, some other *TLR1* polymorphisms have been associated with altered immune responses to BCG vaccination (Randhawa et al. 2011), and we found that the variant *TLR2* and *TLR6* genotypes were associated with susceptibility to BCG osteitis. In a meta-analysis of 16 studies, only *TLR1* rs5743618, *TLR2* rs5743708, and *TLR6* rs5743810 polymorphisms differed constantly between the TB patients and the controls (Zhang et al. 2013), and only these polymorphisms were analyzed in the present study.

The same *TLR1* and *TLR6* polymorphisms, which were analyzed in the present study, and *TLR1* rs3923547 and *TLR6* rs3821985 polymorphisms, which were not analyzed in the present study, were associated with *ex vivo* IFN-γ and/or IL-2 production in BCG-stimulated cell cultures (Randhawa et al. 2011).

In animal models, TLR2-deficient mice could not effectively control BCG growth *in vivo* due to defective innate and adaptive immune responses (Heldwein et al. 2003). These experimental findings are in line with our observation that people who suffered from BCG osteitis in early childhood were more likely to present with variant *TLR2* polymorphism. However, a later study in mice with TLR2, TLR4, and TLR6 deficiency was not able to confirm their poor control of BCG growth and deficient immunological responses (Nicolle et al. 2004).

TLR2 rs5743708 polymorphism was associated with both pulmonary and extra-pulmonary TB in children in a Turkish study. In particular, the *TLR2* polymorphism was associated with the speed of progression from infection to TB disease, and the minor allele A frequency was higher in children with TB diseases than in children with latent TB infections (Dalgic et al. 2011). *TLR6* polymorphism rs5743810, which is the same as the one used in the present study on BCG osteitis patients, was associated with altered secretion of IL-6 in South-African adults (Shey et al. 2010). In addition, the *TLR6* polymorphism rs3821985 was associated with altered IL-6 secretion and responses to both *M. tb* and BCG (Shey et al. 2010). The results of a meta-analysis (Zhang et al. 2013) showed that the *TLR2* polymorphism rs5743708 was associated with an increased risk of TB, especially in Asians and Europeans, and the *TLR1* polymorphism rs5743618 was associated with an increased risk of TB in Africans and American Hispanics (Zhang et al. 2013). However, the *TLR6* polymorphism rs5743810 was associated with a decreased risk of TB (Zhang et al. 2013). In this present Finnish study, the variant *TLR2* polymorphism rs5743708 was associated with an increased risk of BCG osteitis, which is in line with the association of TB and the *TLR2* polymorphism in Europeans.

In terms of *TLR1* polymorphism, the results were different compared to those in the meta-analysis, but that meta-analysis comprised subjects who were non-European, either of African or American Hispanic origin

(Zhang et al. 2013). In addition, *TLR1* rs5743618 has a very high population differentiation (Barreiro et al. 2009). The minor allele frequency of *TLR1* is much lower in the Finnish population than in other European populations (Crisan et al. 2012, Nuolivirta et al. 2013). In the present study, the minor allele frequency of *TLR1* was lower in the cases than in the population-based controls. Thus, some genetic properties – like the high prevalence of the *TLR1* wild type (GG) in the Finnish population – may explain the slow decrease of TB prevalence and the high incidence of BCG osteitis (Kröger et al. 1994, Kröger et al. 1995) in Finland.

6.3 Function of the IL-12/IFN-γ pathway, analysis of MSMD genes and association of *MBL2/TLR2* polymorphisms with IFN-γ in BCG osteitis

The IL-12/IFN-γ pathway is the main part of the immunity acting against mycobacteria, including BCG. Eighteen disorders of this pathway have been previously identified (Bustamante et al. 2014). We hypothesized that the cohort of 132 former BCG osteitis patients may include cases with defects in the IL-12/IFN-γ pathway, thus predisposing to complications with the BCG vaccine. Thus, we analyzed the *ex vivo* concentrations of IL-12 and IFN-γ in white blood cell cultures stimulated with BCG alone and BCG+IFN-γ or BCG+IL-12, respectively, in the former BCG osteitis patients. Since reference values were not available, we classified the concentrations and increases in concentrations as low by the limit of the 5th percentile or 10th percentile. Low stimulated *ex vivo* IL-12 concentrations and low increases in concentrations revealed the same cases – with only one exception – and low stimulated *ex vivo* IFN-γ concentrations and low increases in concentrations also revealed the same cases – with only two exceptions. There were four cases (20% of all those with low concentrations or low increases in either IL-12 or IFN-γ) with low stimulated concentrations and low increases in concentrations of both IL-12 and IFN-γ.

In a previous study (Feinberg et al. 2004), which was done using the same methodology as our study, all 50 healthy BCG-vaccinated controls responded to the stimulation with BCG with increased IFN-γ production

and all but two responded with increased IL-12 production in cell cultures. In line, IFN-γ was measurable in 11% of cases before BCG stimulation but in 99% after BCG stimulation in the present study. The respective figures for IL-12 were 13% and 64%. IL-12 was measurable in all cases after stimulation with BCG+IFN-γ. On average, the stimulated IFN-γ and IL-12 concentrations were lower in the patients of the present study than in the historical controls (Feinberg et al. 2004), but the comparisons with historical controls studied in different laboratories are generally not reliable. Regardless, our results suggest some weakness in the IL-12/IFN-γ pathway, although we could not reveal any increased infection morbidity or mortality after the patients had recovered from BCG osteitis.

An important result of the present study was that mutations in known genes affecting the IL-12/IFN-γ pathway were not found in those 20 cases that were examined as a pilot study, although seven of them presented with low stimulated *ex vivo* IL-12 or IFN-γ concentrations or low increases in concentrations, and two presented with low stimulated *ex vivo* concentrations and low increases of both IL-12 and IFN-γ.

In an Iranian study, 11 children presented with invasive complications after BCG vaccination, such as cervical lymphadenitis or osteitis. Ten (91%) of these children had an impaired *ex vivo* IFN-γ response to BCG+IL-12 stimulation (Parvaneh et al. 2014). Evidently, these patients had MSMD, a genetic defect either in IL-12 receptor function or in IFN-γ production, but gene tests were not done. In another study, no significant differences were found in stimulated IL-12 and IFN-γ concentrations in cell cultures between 11 patients with non-tuberculous mycobacterial cervical lymphadenitis and healthy controls from Israel (Serour et al. 2007). Despite the negative result, the authors considered MSMD possible. In the present study, only 64% of the former BCG osteitis patients had a measurable IL-12 concentration after BCG stimulation in cell cultures, but after BCG+IFN-γ stimulation, IL-12 was measurable in all cases. Instead, IFN-γ was measurable in all cell cultures after BCG stimulation. However, the study did not allow an evaluation of the magnitude of these responses due to the lack of appropriate healthy controls or reference values. Despite this, our results suggest that stimulation tests of white blood cells may help to select patients for genetic studies when thus far uncovered gene defects are being searched for.

Genes that are known to have defects in the IL-12/IFN-γ pathway are *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB1*, *ISG15*, *IRF8*, *NEMO*, and *CYBB* (Figure 1). The 18 known defects account for only half of the known MSMD cases (Bustamante et al. 2014). The most common MSMD is complete recessive IL-12Rβ1 deficiency, for which BCG disease is the most common infection (de Beaucoudrey et al. 2010, Bustamante et al. 2014). The clinical phenotype ranges from fatal infection in infancy to asymptomatic course throughout adulthood (de Beaucoudrey et al. 2010). Interestingly, BCG vaccination or BCG disease protects against subsequent environmental mycobacteriosis (de Beaucoudrey et al. 2010). Multifocal BCG osteitis occurs frequently in partial dominant IFNGR1 and signal transducer and activator of transcription 1 (STAT1) diseases (Dorman et al. 2004, Tsumura et al. 2012, Bustamante et al. 2014). However, BCG osteitis and multifocal osteitis are separate clinical entities, and all of our BCG osteitis patients presented with only one focus. The cellular phenotype of partial IFNGR1 disease is characterized by a reduced *ex vivo* response to IFN-γ (de Beaucoudrey et al. 2010). Likewise, the defect of the cellular IFN-γ response is partial in STAT1 disease, and the outcome of infections, including BCG disease, has mainly been good (Bustamante et al. 2014).

In MSMD patients, BCG infections and other mycobacterioses have diverse manifestations, ranging from localized to disseminated cases (Bustamante et al. 2014). Thus, the beneficial prognosis of early-life BCG osteitis in the patients of the present cohort does not rule out MSMD. Evidently, the high incidence of BCG osteitis in Finland reflects a selective genetic susceptibility to the disease, and the lack of severe or exceptional infections in later life rules out primary immunodeficiencies.

Known gene defects of the IL-12/IFN-γ pathway were not found in a sample of 20 adults from Finland with a history of BCG osteitis in infancy. On the other hand, there were marked differences in *ex vivo* IL-12 and IFN-γ concentrations after white blood cell stimulations when the whole cohort of 118 subjects were examined. This suggests that there may be defects in the production of IL-12 and/or IFN-γ, causing decreased immunity and increased susceptibility to BCG vaccination complications. Some of the former BCG osteitis patients had clearly low stimulated *ex vivo* IL-12 and/or IFN-γ production; this finding is commonly linked with susceptibility

to mycobacterial disease. MSMD genetics was available for two of the four BCG osteitis patients who had low *ex vivo* production of both IL-12 and IFN-γ. No mutations were identified. Based on these results, further genetic studies to find novel genetic defects are indicated, and these studies could first be focused on those patients with low *ex vivo* IL-12 or IFN-γ concentrations or low increases in concentrations (Itan et al. 2014).

Three patients in the cohort of 132 former BCG osteitis patients had the homozygous variant *MBL2* genotype (O/O), and in one of them, IFN-γ concentration was at the 5th percentile level after stimulation with BCG+IL-12. Since IFN-γ plays an important role in immunity against mycobacteria (Rosenzweig et al. 2005), this finding of a very low production of IFN-γ is in line with the result that the *MBL2* variant genotype was associated with an increased risk of BCG osteitis.

We hypothesized that variant *TLR* genotypes in former BCG osteitis patients might be associated with low cytokine production because of the connection of TLR signaling with the subsequent production of cytokines in innate immunity reactions against mycobacteria. One significant association was found. In the patients with the *TLR2* wild genotype, 7.8% had low IFN-γ production by the 10th percentile limit, but in the patients with the *TLR2* variant genotype, as many as 28.6% had such low IFN-γ production. The *TLR2* variant genotype increased the risk of BCG osteitis in the logistic regression model, and again, due to the crucial role of IFN-γ in immunity against mycobacteria (Rosenzweig et al. 2005), the low production of IFN-γ in the patients with this genotype agrees with our hypothesis.

6.4 *IL10* polymorphisms in BCG osteitis

The former BCG osteitis patients of the present study did not differ for the frequencies of polymorphisms rs1800896 (-1082G/A), rs1800871 (-819C/T), rs1800872 (-592C/A), and rs1800890 (-3575T/A) of *IL10* from the controls representing the Finnish population. The frequencies of the genotypes, allele frequencies, and the frequencies of the eight allele combinations of the *IL10* rs1800896 (-1082G/A), rs1800871 (-819C/T), and rs1800872 (-592C/A)

located in the same proximal promoter area of the IL-10 encoding gene were similar between the cases and the controls. However, it has been shown in previous studies that IL-10 plays a central role in various infections, and the SNPs of *IL10* predispose to, for example, allergic diseases such as atopic dermatitis and wheezing (Raedler et al. 2013). One study found that three of the four *IL10* linkage disequilibrium blocks, including rs1800890 and two distal promoter SNPs, presented as determinants for reduced Treg cell numbers and a potentially changed balance in immune regulation in SNP carriers (Raedler et al. 2013).

Distinct *IL10* promoter haplotypes have been associated with the transcriptional activity of IL-10. High, intermediate, and low IL-10 production has been associated with the *IL10* rs1800896/rs1800871/rs1800872 combinations GCC, ACC, and ATA, respectively (Turner et al. 1997, Crawley et al. 1999, Suarez et al. 2003). In addition, it has been shown that the innate cytokine response of neonates to BCG varies between populations (van den Biggelaar et al. 2009). For example, children with previous miliary or meningeal TB that had received the BCG vaccine did not have a major defect in their cytokine pathways (Sterling et al. 2007).

IL10 polymorphisms have been associated with increased susceptibility to TB in several studies (Zhang et al. 2011). One meta-analysis concluded that the rs1800896 polymorphism of *IL10* could be a risk factor for TB in Europeans (Zhang et al. 2011). In another recent meta-analysis, *IL10* rs1800896 polymorphism was associated with TB risk in Europeans and Americans, and it was also found that *IL10* rs1800871 and rs1800872 polymorphisms could be risk factors for TB in Asians (Liang et al. 2014). In Turkish TB patients, the *IL10* rs1800896 G allele frequency was significantly more common than in healthy controls (Oral et al. 2006). In a Tunisian study, the rs1800896 AG genotype was significantly associated with an increased risk of developing extrapulmonary TB (Ben-Selma et al. 2011), whereas the rs1800896 AA genotype seemed to be associated with resistance to pulmonary TB. The high-producer *IL10* GCC haplotype was associated with increased susceptibility to both pulmonary and extrapulmonary TB. In a recent study, genotypes of *IL10* rs1800896 polymorphism were significantly associated with TB patients versus healthy controls (Meenakshi et al. 2013). Household contacts of TB patients were at increased risk of developing TB

if they had the GA genotype of *IL10*. There were also significant differences in allele distribution of *IL10* rs1800872 in patients with TB compared with healthy controls in the Sudanese population (Mhmoud et al. 2013).

Some previous studies have found no association between *IL10* polymorphisms and TB. For example, *IL10* promoter rs1800896 region was not associated with the susceptibility or resistance to TB (Ulger et al. 2013). In Egyptian children, no differences were observed between TB patients and controls for the frequency of *IL10* rs1800896 alleles or genotypes, but a difference in the frequency of *IL10* rs1800896 GG genotype was found between patients with pulmonary and extrapulmonary TB (Mosaad et al. 2010). *IL10* polymorphisms also play a role in non-tuberculous mycobacterial diseases. In one study, the *IL10* rs1800896 AA genotype was significantly associated with pulmonary non-tuberculous mycobacterial disease (Affandi et al. 2013).

Several studies have revealed that ethnicity influences significantly the distribution of cytokine gene polymorphisms (Koss et al. 2000, Reynard et al. 2000, Hoffmann et al. 2002). Allele frequencies of 49 SNPs, including *IL10* SNPs, were studied in a community-based cohort of 9960 individuals, of whom 98% were Caucasian (Huang et al. 2007). The distribution of alleles was similar to the distribution in previous databases (SNP500 Cancer database, dbSNP database) except in African Americans.

The results of this study indicate that the SNPs rs1800896, rs1800871, rs1800872, and rs1800890 of *IL10* are not more common in former BCG osteitis patients than in population-based controls in Finland. This is an interesting finding, as the polymorphisms of the IL-10 encoding gene have been associated with susceptibility to TB in previous studies from other parts of the world.

6.5 Methodological aspects

The main strength of this study is the large number of BCG osteitis patients, spanning a period of 29 years and covering the whole country. The study group is unique on an international level. From 1960 to 1988, the microbiological diagnostics of BCG complications was centralized and

systematically recorded in one national register. The diagnosis of BCG osteitis was confirmed by culture of the BCG strain, by typical histology and age, or by both (Kröger et al. 1994). All the former BCG osteitis patients are of Finnish origin. The Finnish people is very homogenous, and this is obviously a great advantage in genetic studies.

Three of the four original studies of this dissertation had population-based control groups. The distribution of all the gene polymorphisms, such as the TLR2 subfamily gene polymorphisms, have not been studied between populations from different parts of Finland, but the frequencies of some other polymorphisms, such as those in the gene regulating MBL production, have been very similar (Rantala et al. 2008). We were able to collect fresh blood samples from 132/222 (60%) of the former BCG osteitis patients. This is mainly because of the long distances within the country, and therefore, a selection bias is unlikely. None of the study subjects who suffered from BCG osteitis in early childhood reported any mycobacterial infections or other severe or exceptional infections in later life.

Since references in healthy individuals were not available, the significance of the stimulated IL-12 and IFN-γ responses could not be assessed. On the other hand, to be representative of the population, the control group should be large, covering different age groups and different parts of the country. Since the distances in Finland are long and the transfer of some samples within 24 hours to the reference laboratory was challenging, we had 25 transfer control samples from healthy persons, but this material is too small and also non-representative to serve as a study control. Historical controls from other laboratories, even though the method was exactly the same, cannot be directly compared with the patients of this study. The measurements from the cases and the controls should be done during the same runs in the same laboratory.

6.6 Future considerations

The immunity and genome of the BCG osteitis patients will be studied in more depth in professor Jean-Laurent Casanova's laboratory at The Rockefeller University in New York. I am privileged to have been invited there to conduct my post-doctoral studies.

Future studies will include searching for monogenic inborn errors of immunity in the BCG osteitis patients. A hypothesis-based, candidate gene approach focused on genes involved in the IL-12/IFN-γ pathway and related to the known MSMD-causing genes will be used. The principal objective is to identify new genetic mutations causing susceptibility to BCG osteitis by following a complementary genome-wide screening approach. Genes will be characterized by genome-wide linkage and genome-wide deep sequencing (by whole exome sequencing). A novel method of genome-wide linkage has been developed in the laboratory of professor Casanova, and whole exome deep sequencing has been pioneered by his laboratory for other infectious diseases.

Our project will hopefully provide considerable and novel insights into the mechanisms of immunity to mycobacteria. The genetic dissection of BCG osteitis will for its part pave the way for developing a new, safer vaccine against tuberculosis.

7 CONCLUSIONS

The incidence of BCG osteitis has been significantly higher in Finland than in any other country. During the years 1960–1988 the incidence was at its highest at 72.9/100,000 BCG-vaccinated infants. Despite variation in the vaccine strains, the incidence remained high throughout the years, and general BCG vaccinations of the Finnish population were ended in 2006. The hypothesis of this dissertation was that there is a genetic predisposition to BCG osteitis in the Finnish population. This led to the study of innate immunity -related gene polymorphisms. Blood samples were taken from 132 former BCG osteitis patients and analyzed for MBL concentration and *MBL2* polymorphisms, TLR2 subfamily gene polymorphisms, MSMD-related gene defects, the production of IL-12 and IFN-γ in white blood cell stimulations, and *IL10* polymorphisms.

The occurrence of *MBL2* polymorphisms and concentration of MBL were studied. It was found that these may play a role in BCG osteitis, as the former BCG osteitis patients had more variant *MBL2* genotypes than the controls. In addition, the variant genotypes were associated with lower concentrations of MBL.

The occurrence of TLR2 subfamily gene polymorphisms was studied. It was found that TLR2 subfamily gene polymorphisms, namely *TLR1* wild type and the *TLR2* and *TLR6* variant type genotypes, are associated with an increased risk of BCG osteitis. This confirms the previous findings of other studies that the mycobacteria-binding receptors on white blood cells are crucial in the defense against mycobacterial infections. Furthermore, specific combinations of the SNPs of the genes encoding these receptors influence the incidence of BCG vaccination complications such as osteitis.

The function of the IL-12/IFN-γ pathway and occurrence of MSMD-related gene defects were studied. There were no known MSMD-related gene defects in the former BCG osteitis patients. However, it may well be that novel genetic mutations are found in the patients in future studies.

Some of the former BCG osteitis patients had clearly lowered production of IL-12 and/or IFN-γ, indicating that there may be a flaw in their innate immunity response to mycobacteria, and new mutations may be found in this selected group of patients.

The occurrence of *IL10* polymorphisms was studied, and it was found that they do not play a role in BCG osteitis. This is an interesting finding, as the polymorphisms of the IL-10 encoding gene have been associated with susceptibility to TB in previous studies of different populations.

In conclusion, the current research revealed that pattern recognition proteins MBL and TLR1, TLR2, and TLR6 are associated with BCG vaccination complications, but IL-10 is not. Preliminary evidence was found that there may be thus far unknown MSMD-related gene defects that increase susceptibility to BCG vaccination complications.

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APPENDIX

STUDY ON SUSCEPTIBILITY TO COMPLICATIONS OF THE BCG VACCINE IN THE FINNISH POPULATION

QUESTIONNAIRE

The purpose of the following questions is to investigate whether there is a predisposition to long-term illnesses after infant BCG osteitis, especially infections caused by *Salmonella* and other bacteria that belong to the same group as *Mycobacterium tuberculosis*.

We also request your permission to investigate the above-mentioned illnesses in your patient records when needed.

Please choose the correct option.

1. Has a doctor diagnosed you with any long-term illnesses?

   No ❐

   Yes ❐, I have been diagnosed with the following long-term illnesses:

   Illness Year of diagnosis Place of treatment (hospital/healthcare center)

   ____

   ____

2. Do you use or have you used regularly (over six months) medications prescribed by a doctor?

   No ❐

   Yes ❐, I use the following medications regularly/continuously:

   Medication Time of use

   ____

   ____

3. *Salmonella* bacteria can cause infections in the gastrointestinal tract (fever, chronic diarrhea, etc.) and/or joint infections. To your knowledge, have you suffered from an infection caused by *Salmonella*?

   No ❐

   Yes ❐, when? ___________________

4. Have you suffered from tuberculosis or other infections caused by other mycobacteria (bacteria similar to Mycobacterium tuberculosis)?

   No, or I do not know ❐

   Yes, I have ❐

   Year of diagnosis and place of treatment:

   ____

   ____

5. Have you suffered from other serious infections?

   No, or I do not know ❐

   Yes, I have ❐

   Year of diagnosis and place of treatment:

   ____

   ____

6. Have you suffered from other repeated pneumonias or bronchitis infections that have required treatment in hospital?

   No ❐

   Yes ❐

   Years of treatment and place of treatment:

   ____

   ____

7. Have you suffered from other repeated sinusitis infections that have required operative treatment?

   No ❐

   Yes ❐

   Years of treatment and place of treatment:

   ____

   ____

8. Have you received gammaglobulin treatment because of repeated infections?

   No ☐

   Yes ☐

   Years of treatment and place of treatment:

   ____

   ____

9. Do you have any long-term or permanent disablements from osteitis in childhood?

   No ☐

   Yes ☐

   What, and where has it been treated and followed:

   ____

   ____

I give permission to investigate my medical records when needed ☐
I do not give permission to investigate my medical records ☐

Signature and date: ________________________________

Time of birth: ________________________________

Address: ________________________________

____

Thank you for your response!

ORIGINAL PUBLICATIONS

I Pöyhönen, L., Kröger, L., Gröndahl-Yli-Hannuksela, K., Vuononvirta, J., Huhtala, H., He, Q., Korppi M. (2013). Variant MBL2 genotypes producing low mannose-binding lectin may increase risk of Bacillus Calmette-Guérin osteitis in vaccinated newborns. *Acta Paediatrica*, 102, 1095-1099.

II Pöyhönen, L., Nuolivirta, K., Vuononvirta, J., Kröger, L., Huhtala, H., Mertsola, J., He, Q., Korppi M. (2015). Toll-like receptor 2 subfamily gene polymorphisms are associated with Bacillus Calmette-Guérin osteitis following newborn vaccination. *Acta Paediatrica*, 104, 485-490.

III Pöyhönen, L., Kröger, L., Huhtala, H., Mäkinen, J., Mertsola, J., Martinez-Barricarte, R., Casanova, J. L., Bustamante, J., He, Q., Korppi M. Interferon-gamma-dependent immunity in Bacillus Calmette-Guérin vaccine osteitis survivors. T*he Pediatric Infectious Disease Journal*, in press.

IV Pöyhönen, L., Teräsjärvi, J., Nuolivirta, K., Vuononvirta, J., Kröger, L., Gröndahl-Yli-Hannuksela, K., Huhtala, H., Ilonen, J., Peltola, V., Mertsola, J., Korppi, M., He, Q. (2015). Interleukin-10 gene promoter region polymorphisms are not associated with BCG osteitis in vaccinated infants. *International Journal of Tuberculosis and Lung Disease*, 19, 1158-1162.

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REGULAR ARTICLE

Variant *MBL2* genotypes producing low mannose-binding lectin may increase risk of Bacillus Calmette–Guerin osteitis in vaccinated newborns

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Keywords
Bacillus Calmette–Guerin, Innate immunity, Mannose-binding lectin, *MBL2*, Osteitis, Vaccination

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ABSTRACT

Aim: The aim of this study was to evaluate whether mannose-binding lectin (MBL) plays a role in the development of osteitis after Bacillus Calmette–Guerin (BCG) vaccination as a newborn.

Methods: Blood samples were obtained from 132 former BCG osteitis patients, now aged 21–49 years, and analysed for MBL concentration and *MBL2* genotype in a controlled setting.

Results: Variant genotypes in the *MBL2* gene were more common in the former BCG osteitis patients (42.4%) than in the population controls (32.3%, p = 0.033). However, MBL concentrations at the age of 21–49 years were not lower in these patients than in the controls in the same age group. The variant *MBL2* genotypes were associated with low serum MBL concentrations, and moreover, MBL concentration was not measurable in two of those three patients who were homozygous for the variant *MBL2* genotype. Low serum MBL concentrations were not associated with any illnesses in the medical history of the BCG patients, their siblings or children.

Conclusion: Preliminary evidence was found that variant, low-MBL-producing genotypes may be associated with the increased risk of BCG osteitis in vaccinated newborns.

INTRODUCTION

Finnish newborns were universally vaccinated against tuberculosis with Bacillus Calmette–Guerin (BCG) vaccine from the 1940s until 2006 (1). Complications of adverse reactions to BCG vaccinations were registered by the National Institute of Public Health until 1988 (2,3). National registers revealed that 222 children developed osteitis between 1960 and 1988, confirmed by the positive culture of BCG strain, by typical histology or by both (2,4). The question why some children develop an invasive complication after BCG vaccination is still unresolved.

Mannose-binding lectin (MBL) is a multimeric protein that recognizes surface structures of different pathogens, including mycobacteria, and initiates the lectin pathway of complement activation (5,6). MBL is encoded by *MBL2* gene and forms multimers. *MBL2* variants and low MBL levels have been associated with increased susceptibility to, and severity of, many different infections (7–9), particularly in infants and young children with immature adaptive immunity (10,11). Recently, variant *MBL2* genotypes were linked with more severe clinical scenarios and increased risk of asthma in young infants hospitalized for bronchiolitis at under 6 months of age (12,13). In a meta-analysis, tuberculosis was not associated with variant *MBL2* genotypes, and patients with active tuberculosis presented with even higher MBL concentrations than healthy controls (14).

Variant *MBL2* genotypes are associated with lowered production of MBL protein. Subjects with variant genotypes have presented with lower serum MBL concentrations than those with the wild genotype. Among subjects with variant genotypes, homozygotes variant for both alleles have presented with lower MBL production than heterozygotes (7,15).

We obtained whole blood samples from 132 of the 222 former BCG osteitis patients for cell cultures, *MBL2* gene polymorphism determinations and MBL concentration measurements. Our hypothesis was that the low production

Key notes

- A higher percentage of the former BCG osteitis patients had variant genotypes of the *MBL2* gene than the population controls.
- Serum MBL concentrations in adulthood did not differ between former BCG osteitis patients and age-matched controls.
- Variant *MBL2* genotypes were associated with lower serum MBL concentrations than wild genotypes.

of MBL may expose BCG-vaccinated infants to complications such as BCG osteitis. The aim of the study was to evaluate *MBL2* genotypes and MBL concentrations in adulthood in the former BCG osteitis patients in a controlled setting. In addition, the association between the *MBL2* genotype and serum MBL concentration was studied.

SUBJECTS AND METHODS

Design

Bacillus Calmette–Guerin vaccination was in general use in Finland from 1941 to 2006. Starting in 1951, the vaccine was given to the newborns in the maternity hospital, and the coverage was very high, over 95%. The microbe-specific diagnostics of invasive complications caused by BCG vaccinations was centralized from 1960 to 1988 and, during this 29-year period, 222 BCG osteitis cases were diagnosed (2,4). In 2007–2008, a questionnaire and study invitation was sent to 203 former BCG osteitis patients with current address available. Of these, 160 responded, representing 72.1% of the 222 patients diagnosed with BCG osteitis.

The questionnaire comprised of questions on chronic illnesses, long-term use of medicines, repeated or chronic infections and mycobacterial infections in the study subjects and their siblings and children.

Fresh blood samples were collected from 132 of 160 (83.5%) study subjects and sent to the Mycobacterium Reference Laboratory at the National Institute for Health and Welfare, Turku, Finland. All blood samples were sent to the laboratory within 12 h of collection. DNA was isolated from 200 μL of whole blood, and both DNA and serum samples were frozen at −70°C for further analyses.

Laboratory analyses

Serum concentrations of MBL were measured using an ELISA-based method, as described earlier (9). Briefly, the mouse monoclonal antihuman MBL immunoglobulin G1 (IgG1) antibodies (8 μg/mL) (HYB 131-01; Statens Serum Institut (SSI), Copenhagen, Denmark) were used for coating. Samples were diluted at 1:100 in PBS–0.05% Tween 20. The absorbance was read at 450 nm with Multiskan EX reader (Thermo Fisher Scientific, Vantaa, Finland). Standard serum containing 3200 ng/mL (SSI) of oligomerized MBL was used for creating the standard curve. The detection limit of this assay was 50 ng/mL. The concentration below the detection limit was given the value of 25 ng/mL. Sera with MBL concentration higher than the upper detection limit of the standard curve (6400 ng/mL) were further diluted to 1:200 and were retested. MBL deficient serum (SSI) was included in each run.

MBL2 genotypes in exon 1, codons 52 (allele D, rs5030737), 54 (allele B, rs1800450) and 57 (allele C, rs1800451) were determined by PCR followed by pyrosequencing (PSQ TM 96MA Pyrosequencer; Biotage, Uppsala, Sweden), using a PSQ TM 96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol and as described earlier (16,17). Primers used for *MBL2* PCR were: forward 5′-biotin-CCTTCCCTGAGTTTTCTCAC-3′, reverse 5′-AACAGCCCAACACGTACCTG-3′ and for sequencing 5′-CGTACCTGGTTCCCCCTTTTCT-3′. All the primers were purchased from Sigma-Aldrich, Helsinki, Finland. The size of the *MBL2* PCR product was 240 bp.

Controls

Serum MBL concentrations were measured in 83 age-matched controls (21–49 years). The control samples were randomly selected from the sera submitted in 2004–2009 for the diagnosis of coeliac disease to the Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland. Only samples tested as negative for coeliac disease were used in this study. The results have been published previously (9).

MBL2 genotypes were determined in 412 controls recruited from a study called Steps to Children's Healthy Development and Wellbeing study, which is a prospective cohort study of approximately 1800 children. The control group comprised of Finnish infants aged three months old, who had not yet been vaccinated, were healthy and visited the study clinic from 2008 to 2010. The results have been published previously (16).

All data on the control subjects used in this study, except for age and gender, were anonymized.

Ethics

The study was accepted by the Ethics Committee of the Tampere University Hospital District. Written consent was obtained from the study subjects, including permission to perform genetic studies concerning susceptibility to mycobacterial infections. The study protocol concerning the genetic control group of children was approved by the Ethics Committee of the Hospital District of the southwestern Finland, Turku, Finland. Written consent was obtained from the parents to use their children's blood samples. No permission to use the anonymized blood samples of the control group for concentrations of MBL was needed, as no clinical data were handled.

Statistical analyses

The SPSS 19.0 package (IBM SPSS Statistics for Windows, version 19.0.; IBM Corp, Armonk, NY, USA) was used for statistical analysis. The results are expressed as medians, interquartile ranges (25–75th percentile), and minimum and maximum values, presented as box-and-whiskers pictures when appropriate. Mann–Whitney *U* test was used for comparing the medians of MBL concentrations between cases and controls, and between *MBL2* genotypes. Chi-square and Fisher's exact tests were used for comparing *MBL2* genotypes between cases and controls.

RESULTS

MBL2 genotypes

Blood samples were analysed for 132 former BCG osteitis patients. Of these, 76 (57.6%) were A/A homozygotes, 53 (40.2%) A/O heterozygotes and only three (2.3%) O/O

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homozygotes (Table 1). Thus, 56 (42.4%) study subjects had the nonwild variant genotype A/O or O/O, compared with 133 (32.3%) in the controls (p = 0.033).

MBL concentrations

The median age of the 132 former BCG osteitis patients was 33.0 years (range 21–49 years), and 72 (54.5%) were females. Among the 130 subjects with MBL data available, the median of serum MBL concentrations was 1633 ng/mL (range <50–8543 ng/mL) compared with 1372 ng/mL (<50–6400 ng/mL, p = 0.157 vs. cases) in controls (Fig. 1). Twenty-one patients (16.2%) and 21 controls (25.3%; p = 0.114) had MBL <500 ng/mL, suggested for the cut-off limit between deficient and nondeficient MBL production (8). Six patients (4.6%) and six controls (7.2%; p = 0.543) had MBL <50 ng/mL (detection limit of the test).

Association of MBL concentration with *MBL2* genotypes

The median of serum MBL concentrations was 2673 ng/mL (range 391–8543 ng/mL) in the 74 A/A homozygotes, compared with 736 ng/mL (<50–2458; p < 0.001) in the 53 A/O heterozygotes (Fig. 2), and below the detection limit (<50 ng/mL) in two of the three O/O homozygotes. In one, MBL concentration was 50 ng/mL. Thus, the variant genotypes were associated with lower serum MBL concentrations than the wild-type genotype, but the number of cases homozygous for the variant genotype was too low for statistical analyses.

MBL2 genotypes of the low producers of MBL

The six low producers of MBL (<50 ng/mL) had genotype AB in four cases and BB in one case and BD in one case.

Table 1 Genotypes and polymorphisms of the *MBL2* gene in 132 former Bacillus Calmette–Guerin (BCG) osteitis patients and 412 population controls

Genotypes and polymorphisms	Former BCG patients n = 132 n	%	Population controls n = 412† n	%
A/A	76	57.6	279	67.7
A/O	53**	40.2**	124	30.1
A/B	37	28.0	86	20.9
A/C	3	2.3	1	0.2
A/D	13	9.8	37	9.0
O/O	3***	2.3***	9	2.2
B/B	1	0.8	5	1.2
C/C	0	0	0	0
D/D	0	0	1	0.2
B/D	2	1.5	1	0.2
A/O or O/O	56*	42.4*	133	32.3

*p = 0.033 vs. controls; **p = 0.128 vs. controls; ***p = 0.405 vs. controls.

†Population-based cohort of 3-month-old infants, as published previously (9). The figures are nearly identical to those published from 473 Finnish military constricts (22): A/A 64.8%, A/O 30.3% and O/O 4.9%.

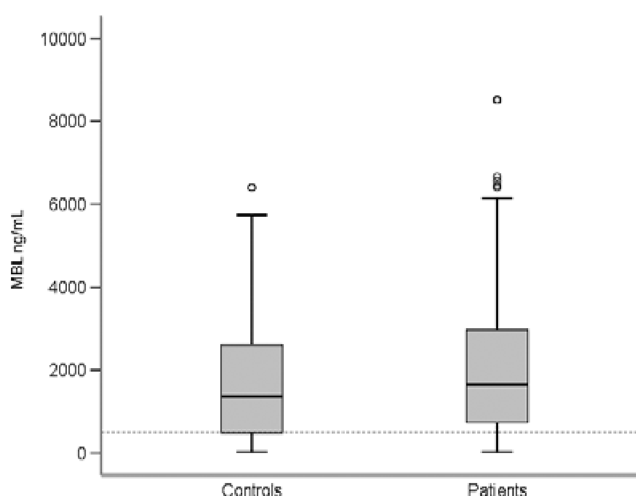

Figure 1 Serum mannose-binding lectin (MBL) concentrations in 130 former Bacillus Calmette–Guerin (BCG) osteitis patients and 83 controls expressed as medians, interquartile ranges, and minimum and maximum values. The results of the controls have been partly published previously (16). The broken line indicates MBL concentration 500 ng/mL.

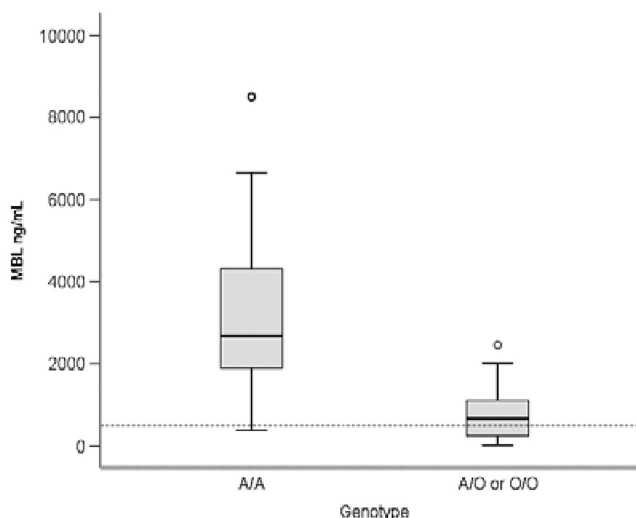

Figure 2 Serum mannose-binding lectin (MBL) concentrations in the 74 subjects with the wild, nonvariant genotype (A/A) and in the 56 subjects with variant genotype (A/O or O/O) expressed as medians, interquartile ranges, and minimum and maximum values. The results of the controls have been published previously (16). The broken line indicates MBL concentration 500 ng/mL.

None of the low producers reported any chronic illnesses, repeated infections or mycobacterioses in themselves, their siblings or children.

DISCUSSION

There are three main results in this study. First, serum MBL concentrations measured in adulthood were not lower in the former BCG osteitis patients than in the controls in the same age group. The result was similar in the analyses with

MBL included as a continuous and categorized variable. Second, a higher percentage of the former BCG osteitis patients had variant *MBL2* genotypes than population controls. Third, in line with many previous studies, the variant *MBL2* genotypes were associated with low serum MBL concentrations.

Mannose-binding lectin is an acute-phase protein secreted by the liver. It recognizes microbial surface carbohydrates and initiates the lectin pathway of complement activation (5,6). MBL plays an important role in the innate immunity against different pathogens (7,8), including *Mycobacterium tuberculosis* (18). On the other hand, the impact of low MBL production on susceptibility to, or severity of, infections is still unresolved. Although complement deficiencies are well-known risk factors for severe pneumococcal infections (19), no association was found between *MBL2* genotypes or MBL production and invasive pneumococcal infections such as pneumonia in a recent study and review (20). Seventeen publications on MBL and tuberculosis were included in a recent meta-analysis, and no significant association was found between *MBL2* genotypes and tuberculosis infection (14). This indicates that the variant, usually low-MBL-producing genotypes, is not associated with the susceptibility to tuberculosis. Instead, the reviewed studies reported a consistent increase in serum MBL concentration in patients with active tuberculosis (14), suggesting that MBL is elevated in active tuberculosis as part of an acute-phase reaction.

However, no studies have been published on the association between BCG infections and MBL production or genetics. In 1960–1988, when the subjects of the present study were infants and presented with BCG osteitis, BCG vaccinations were given to newborns. In a recent meta-analysis, low MBL concentrations were associated with increased susceptibility to septic infections in newborns (10). Therefore, we hypothesized that, due to the presence of high MBL production in active tuberculosis (14), and due to the importance of innate immunity in newborns (21), low-*MBL2*-producing genotypes may be associated with an increased susceptibility to BCG osteitis. We found preliminary evidence for the association. The difference in the prevalence of variant *MBL2* genotypes between the former BCG osteitis patients (42.4%) and controls (32.3%) was statistically significant.

In addition, our results confirmed that the subjects with variant *MBL2* genotypes had lower serum MBL concentrations than those with the wild, nonvariant genotype (7,15,22).

There are three noteworthy shortcomings in the present study. First, blood samples were only available from 60% of all of the identified former BCG osteitis patients. However, a better participation was difficult to achieve, because the study subjects lived around the country. Second, the controls were not recruited for the present study, and the number of the control subjects was limited. And third, most significantly, MBL concentrations were studied 20–45 years after the BCG infections. To minimize this error, MBL concentrations were studied at the same age, 21–49 years, in both cases and controls. The controls for the *MBL2* study were obtained from the nonselected population, although being infants and from one district in south-western Finland. However, the distribution of *MBL2* genotypes in them was nearly identical with the distribution in military constricts in north-east Finland (22). The Finnish population is rather homogenous, which increases the reliability of genetic findings.

In addition, polymorphisms of the promoter genes were not studied, although *MBL2* and promoter genes together regulate MBL production (7,15). Previous studies have evaluated polymorphisms in three regulatory areas (7,22), and H, Y and Q alleles have been associated with high MBL and their counterparts, L, X and P alleles, with low MBL. Instead, we studied serum MBL concentrations, although not during the BCG infection, which would be the optimal timing.

The main strength of the study is the unique material: former BCG osteitis patients collected for a period of 29 years and including all diagnosed cases from the whole country.

In conclusion, preliminary evidence was found that variant *MBL2* genotypes may be associated with an increased risk of BCG osteitis in vaccinated newborns. In Finland, universal BCG vaccinations were discontinued in 2006, but newborns and young children at increased risk of tuberculosis are still vaccinated. Therefore, studies on the risk factors for complications such as BCG osteitis are still needed. Contrary to earlier decades, genetic methods are now widely available, and genetic risk factors can be found and may be clinically useful in the future.

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REGULAR ARTICLE

Toll-like receptor 2 subfamily gene polymorphisms are associated with *Bacillus Calmette-Guérin* osteitis following newborn vaccination

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ABSTRACT

Aim: Toll-like receptor (TLR) 1, 2, 6 and 10, the TLR2 subfamily, are known to be associated with immunity against tuberculosis. We evaluated whether polymorphisms in genes encoding TLR1, TLR2 and TLR6 were associated with osteitis in infants who received the *Bacillus Calmette-Guérin* (*BCG*) vaccination soon after birth.

Methods: Blood samples from 132 adults aged 21–49 who had *BCG* osteitis in early childhood were analysed in a controlled study for *TLR1* T1805G (rs5743618), *TLR2* G2258A (rs5743708) and *TLR6* C745T (rs5743810) gene single nucleotide polymorphisms.

Results: The frequencies of the variant genotypes differed between the cases and controls: 11.4% versus 5.7% for *TLR2* G2258A (p = 0.033) and 77.3% versus 61.6% for *TLR6* C745T (p = 0.001). The *TLR2* and *TLR6* variant genotypes were associated with a higher risk of *BCG* osteitis, with adjusted odds ratios (aOR) of 2.154 (95%CI 1.026–4.521) and 1.907 (95%CI 1.183–3.075), respectively. The frequency of the *TLR1* T1805G variant genotype was 19.7% in the cases and 33.6% in the controls (p = 0.003). The *TLR1* variant genotype was associated with a lower risk of *BCG* osteitis (aOR 0.554, 95%CI 0.336–0.911).

Conclusion: Gene polymorphisms that regulate the function of the TLR2 subfamily play a role in the development of *BCG* osteitis in vaccinated infants.

The *Bacillus Calmette-Guérin* (*BCG*) vaccine is used globally but only provides partial protection against tuberculosis (TB), mainly severe infant diseases like TB meningitis (1). Although the *BCG* vaccine has a good safety profile, mild complications are common, such as localised abscesses and regional lymphadenitis. In most populations, severe complications like *BCG* osteitis are rare (1), for example in Japan they only account for 0.2 cases per 100 000 vaccinations (2).

Toll-like receptors (TLRs) are pattern recognition proteins that play a key role in innate immunity. They sit on the surface of the white blood cells and recognise, and convey information about, different pathogens, initiating an inflammatory and immunological response in the host. They include the TLR2 subfamily, which recognises a diverse set of pathogens, including *Mycobacterium tuberculosis*, and is the principal mediator of macrophage activation in response to mycobacteria (3). The TLR2 subfamily comprises TLR1, TLR2, and TLR6 (4) and the genes that encode these receptors are located in a gene cluster on chromosome number 4 (5). TLR1 and TLR6 are co-receptors for TLR2 and enhance the pathogen recognition signal through TLR2 (6). In the *TLR2* gene, the polymorphism G2258A has been associated with altered signalling in functional studies (7). *TLR1* T1805G regulates signalling in response to lipopeptides, including mycobacteria, and essentially defines TLR1 deficiency in humans (8).

Key notes

- The Toll-like receptor (TLR) 2 subfamily plays an important role in immunity against tuberculosis.
- In a controlled study of 132 former *Bacillus Calmette-Guérin* (*BCG*) osteitis patients, *TLR2* and *TLR6* variant genotypes were associated with a higher risk of *BCG* osteitis and *TLR1* variant genotypes were associated with a lower risk.
- The gene polymorphisms that regulate the TLR2 subfamily function are associated with *BCG* osteitis in infants vaccinated soon after birth.

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Single nucleotide polymorphisms (SNPs) in genes encoding TLR1 and TLR6 have been associated with altered *BCG*-induced immune responses after vaccination (8). In a recent meta-analysis, *TLR1, TLR2* and *TLR6* gene polymorphisms were associated with the risk of TB (9).

The *BCG* vaccine against TB was given to Finnish newborns from 1941 to 2006 and invasive complications like *BCG* osteitis were systematically registered until 1988 (10). According to the national registers, 222 children developed *BCG* osteitis between 1960 and 1988 (11,12). Between 1971 and 1978, the annual incidence of *BCG* osteitis increased to a mean of 36.9 per 100 000 vaccinated newborns and it then decreased after the vaccine was changed to a mean of 6.4 per 100 000 vaccinated newborns (11,12). Even this lower incidence of *BCG* osteitis in Finland was higher than the rates reported by other countries (1,2).

The aim of this study was to investigate *TLR2* subfamily gene polymorphisms in former *BCG* osteitis patients in a controlled setting. Our hypothesis was that the SNPs of the *TLR1, TLR2* and *TLR6* genes may be associated with the risk of severe complications like osteitis after *BCG* vaccinations in newborn infants. All three of the SNPs analysed in this study result in amino acid changes in the respective TLRs.

SUBJECTS AND METHODS

The National Public Health Institute in Helsinki, Finland, studied all samples and kept centralised Finnish records for microbe-specific diagnoses of invasive complications caused by *BCG* vaccinations from 1960 to 1988. During those 29 years, 222 *BCG* osteitis cases were diagnosed by a *BCG* strain culture, by typical histology or by both methods (11,12). In 2007–2008, we managed to obtain the current address of 213 of those 222 former *BCG* osteitis patients and when we sent them a questionnaire and study invitation, 160 (78.8%) of them replied. The questionnaire comprised questions about chronic illnesses, long-term use of medicines, repeated or chronic infections and mycobacterial infections in the study subjects.

We obtained whole blood samples from 132 of the 160 former *BCG* osteitis patients who agreed to participate in the study, including genotyping of the TLR2 subfamily encoding genes. The blood samples were collected at laboratories around the country and immediately transported to the Tuberculosis Reference Laboratory at the National Institute for Health and Welfare, Turku, Finland, where they were frozen at −70°C.

The samples from the control population came from the Steps to Healthy Development and Well-being of children (STEPS) study, a prospective cohort study of 1827 children, who visited the study clinic from 2008 to 2010 (13). The *TLR2* subfamily genotypes were identified in 318 healthy 2-month-old infants from this study population, who were all of Finnish origin and had not yet received any vaccinations. The design and results of this study on TLR2 and TLR4 genetics have been published previously (14).

Laboratory analyses

The SNPs of *TLR1* T1805G (rs5743618), *TLR2* G2258A (rs5743708) and *TLR6* C745T (rs5743810) were selected based on their obvious functional impact on TLR signalling (8,15) and their known association with TB and *BCG* infections (9). In a meta-analysis, a significant difference between patients with pulmonary TB and controls was only found in *TLR1* G1805T (rs5743618), *TLR2* G2258A (rs5743708) and *TLR6* C745T (rs5743810) polymorphisms (9). In an experimental study, SNPs of *TLR1* T1805G (rs5743618) and *TLR6* C745T (rs5743810) were associated with *ex-vivo* interferon-gamma and interleukin-2 production in *BCG*-stimulated cell cultures (8).

The genotyping was performed by pyrosequencing at the laboratory of National Institute for Health and Welfare, Turku, Finland, as described earlier (16,17). The genomic DNA was extracted from peripheral blood using the Qiagen QiAmp DNA blood Mini Kit 250 (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All the primers were purchased from Sigma-Aldrich, Helsinki, Finland. Polymerase chain reactions (PCRs) were studied with selected primer pairs for the *TLRs,* and the PCR amplifications were carried out as described earlier (14,16,17). The genotyping process for *TLR1, TLR2* and *TLR6* was identical in the former *BCG* osteitis patients and controls.

Ethics

The study was approved by the Ethics Committee of the Tampere University Hospital District, and written consent was obtained from the study subjects, who were now adults, including permission to perform genetic studies concerning susceptibility to mycobacterial infections. The study protocol for the genetic control group was approved by the Ethics Committee of the Hospital District of south-west Finland, Turku, Finland and parental written consent was obtained to use the children's blood samples.

Statistical analyses

The SPSS 19.0 package (IBM SPSS Statistics for Windows, Version 19.0, IBM Corp, Armonk, NY, USA) was used for statistical analyses. Chi-square and Fisher‘s exact tests were used, when appropriate, to compare genotypes and allele frequencies between cases and controls. Bonferroni correction was applied in paired comparisons. Logistic regression was used in adjusted analyses, and the results were expressed as adjusted odds ratios (aOR) and their 95% confidence intervals (95%CI). The *TLR1, TLR2* and *TLR6* wild and variant genotypes, and the subject's sex, were simultaneously included in the final logistic regression model.

Deviations from the Hardy–Weinberg equilibrium were evaluated using the FINETTI programme.

RESULTS

The median age of the 132 former *BCG* osteitis patients, who all received the *BCG* vaccination shortly after birth,

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was 33 years (range 21–49), and 72 (54.5%) were females. None of the 132 study subjects reported any issues with TB or mycobacteriosis, other than *BCG* osteitis in infancy.

The *TLR1* genotype was GG in 106 cases (80.3%), GT in 25 cases (18.9%) and TT in one case (0.81%) (p = 0.012 versus controls). The frequency of the GT or TT variant genotype was 19.7% in the cases and 33.6% in the controls (p = 0.003) and the frequency of the minor allele T 10.2% and 17.9%, respectively (p = 0.004) (Table 1). The *TLR2* genotype was GG in 117 cases (88.7%), GA in 15 cases (11.4%) and there were no AA cases (p = 0.033). The frequency of the variant GA genotype was 5.7% in the controls (p = 0.033 versus cases), and the frequencies of the minor allele A were 5.7% and 2.8% (p = 0.036) (Table 1). The *TLR6* genotype was CC in 30 cases (22.8%), CT in 69 cases (52.2%) and TT in 33 cases (25.0%) (p = 0.006 versus controls). The frequency of the CT or TT variant genotype was 77.3% in the cases and 61.6% in the controls (p = 0.001), and the frequency of the minor allele T was 51.1% and 40.6%, respectively (p = 0.004) (Table 1). The genotypes of *TLR1* T1805G, *TLR2* G2258A and *TLR6* C745T of the control population were in Hardy–Weinberg equilibrium.

We also compared different combinations, or haplotypes, of *TLR1, TLR2* and *TLR6* wild and variant genotypes with the combination of a wild genotype in all three *TLR*s. This showed that only the combination of *TLR1* wild and *TLR2* variant and *TLR6* variant genotype differed significantly between the cases and controls (Table 2).

As seen in Table 1, the presence of the *TLR2* or *TLR6* variant genotypes increased the risk of belonging to the *BCG* osteitis case group and the presence of the *TLR1* variant genotype decreased the risk. Therefore, we included all three variant genotypes simultaneously in a logistic regression model and this showed that the *TLR1* variant genotype decreased the risk (aOR 0.48) and both the *TLR2* variant genotype (aOR 2.16) and the *TLR6* variant genotype (aOR 2.11) increased the risk significantly and independently of each other (Table 3).

DISCUSSION

This study examined the polymorphisms of the genes regulating TLR1, TLR2 and TLR6 and the key finding was that polymorphisms in all three TLRs encoding genes were associated with *BCG* osteitis in infancy after newborn *BCG* vaccination. The *TLR1* wild genotype and the *TLR2* and *TLR6* variant genotypes were significantly more common in the former *BCG* osteitis patients than in the controls. TLR1 and TLR6 are considered to be TLR2 co-receptors (6) and are therefore called the TLR2 subfamily. When different combinations of *TLR1*, *TLR2* and *TLR6* wild and variant genotypes were studied, only the combination of the *TLR1* wild type and *TLR2* and *TLR6* variant types were significantly associated with *BCG* osteitis.

Toll-like receptors are pattern recognition proteins of innate immunity, conveying information about pathogens and initiating an inflammatory and immunological response in the host. *TLR2* subfamily gene polymorphisms have been

Table 1 The distribution of *TLR1* (rs5743618), *TLR2* (rs5743708) and *TLR6* (rs5743810) genotypes in 132 cases with *BCG* osteitis in infancy and 318 population-based controls

	Cases n = 132		Controls n = 318		
Genotypes	n	%	n	%	p-value
TLR1					
GG	106	80.3	211	66.3	0.012
GT	25	18.9	100	31.5	
TT	1	0.8	7	2.2	
Variant type (GT or TT)	26	19.7	107	33.6	0.003
Major allele (G)	237/264	89.8	522/636	82.1	0.004
Minor allele (T)	27/264	10.2	114/636	17.9	
TLR2					
GG	117	88.6	300	94.3	0.033
GA	15	11.4	18	5.7	
AA	0	0	0	0	
Variant type (GA or AA)	15	11.4	18	5.7	0.033
Major allele (G)	247/262	94.3	618/636	97.2	0.036
Minor allele (A)	15/262	5.7	18/636	2.8	
TLR6					
CC	30	22.8	122	38.3	0.006
CT	69	52.2	134	42.1	
TT	33	25.0	62	29.6	
Variant type (CT or TT)	102	77.3	196	61.6	0.001
Major allele (C)	129/264	48.9	378/636	59.4	0.004
Minor allele (T)	135/264	51.1	258/636	40.6	

Table 2 The association between different combinations of *TLR1, TLR2* and *TLR6* wild and variant genotypes and *BCG* osteitis in infancy

Combinations					Compared to wild–wild–wild
TLR1 (rs5743618) wild GG variant GT or TT	*TLR2* (rs5743708) wild GG variant GA	*TLR6* (rs5743810) wild CC variant CT or TT	Case N = 132 (%)	Control N = 318 (%)	p-value
Wild	Wild	Wild	18	68	–
Wild	Wild	Variant	73 (55.3)	131 (41.1)	0.013*
Wild	Variant	Wild	2	4	0.608
Wild	Variant	Variant	12 (9.0)	8 (2.5)	0.002†
Variant	Wild	Wild	9	45	0.661
Variant	Wild	Variant	16	56	0.849
Variant	Variant	Wild	1	5	0.999
Variant	Variant	Variant	0	1	0.999

Overall p-value 0.001.
*p = 0.091 after Bonferroni correction by multiplying the p-value by 7 (the number of paired comparisons).
†p = 0.014 after Bonferroni correction by multiplying the p-value by 7 (the number of paired comparisons).

Table 3 Logistic regression: *TLR1, TLR2* and *TLR6* variant genotypes as predictive factors of *BCG* osteitis

Genotypes	Cases (N = 132)	Controls (N = 318)	aOR (95%CI)*
TLR1 variant (N = 133)	26 (19.7%)	107 (33.6%)	0.554 (0.336–0.911)
TLR2 variant (N = 33)	15 (11.4%)	18 (5.7%)	2.154 (1.026–4.521)
TLR6 variant (N = 298)	102 (77.3%)	196 (61.6%)	1.907 (1.183–3.075)

*Adjusted for sex. *TLR1*, *TLR2* and *TLR6* variant genotypes were included in the same model.

associated with an increased susceptibility to various infectious diseases, such as TB, meningococcal meningitis and borreliosis (9,18,19), but they do not seem to play a major role in some infections, such as viral bronchiolitis (16). There are no previous studies on genes or gene polymorphisms regulating *TLR* gene expression in patients with complications arising from the *BCG* vaccination, like *BCG* osteitis.

Earlier studies suggested associations between *TLR* gene polymorphisms and increased TB susceptibility (9,20,21). In addition, there have been experimental studies that have documented the association of TLR encoding gene polymorphisms with cytokine responses to *BCG* antigens in cell cultures (8,22,23) and with *TLR* gene expressions in cell cultures after *BCG* exposure (23). In a meta-analysis of 16 studies, only *TLR1* G1805T, *TLR2* G2258A and *TLR6* C745T polymorphisms differed constantly between TB patients and controls (9) and only these polymorphisms were used in the present study. The same *TLR1* and *TLR6* polymorphisms, which were used in the present study, and *TLR1* A1188T and *TLR6* G1083C polymorphisms, which were not used in the present study, were associated with *ex-vivo* interferon-gamma and, or, interleukin-2 production in *BCG*-stimulated cell cultures (8).

In animal models, TLR2 deficient mice could not effectively control *BCG* growth *in vivo* due to defective innate and adaptive immune responses (24). These experimental findings are in line with our observation that people who suffered from *BCG* osteitis in early childhood were more likely to present with variant *TLR2* gene polymorphism. However, a later study in mice with TLR2, TLR4 and TLR6 deficiency was not able to confirm their poor control of *BCG* growth and deficient immunological responses (25). In South African infants, *TLR6* polymorphism C745T, which is the same as the one we used in the present study, was associated with altered IL-6 secretion (26), and *TLR6* polymorphism G1083C was associated with altered IL-6 secretion and responses to both *Mycobacterium tuberculosis* and *BCG* strain (26).

In a systematic review of *TLR2* subfamily gene polymorphisms and susceptibility to pulmonary TB (9), *TLR2* polymorphism G2258A was associated with an increased TB risk, especially in Asians and Europeans, and *TLR1* gene polymorphism G1805T was associated with an increased TB risk in Africans and American Hispanics. However, *TLR6* gene polymorphism C745T was associated with a decreased TB risk. In this present Finnish study, the variant *TLR2* gene polymorphism G2258A was associated with an increased risk of *BCG* osteitis, which is in line with the association between of TB and *TLR2* gene polymorphism in Europeans. In terms of *TLR1* gene polymorphism, the results were different to those in the meta-analysis, but that study comprised subjects who were non-European, either of African or American Hispanic origin. In addition, *TLR1* T1805G has a very high population differentiation (15). The minor allele frequency of *TLR1* is much lower in the Finnish population than in other European populations (16,27). In the present study, the minor allele frequency of *TLR1* was lower in the cases than in the population-based controls. Thus, some genetic properties, like the high

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prevalence of the *TLR1* wild type in the Finnish population, may, at least partly, explain the slow decrease of TB prevalence (28) and the high incidence of *BCG* osteitis (11,12) in Finland.

In a Turkish study, *TLR2* G2258A polymorphism was associated with both pulmonary and extrapulmonary TB in children. In particular, *TLR2* gene polymorphism was associated with the speed of progression from infection to TB disease and the minor allele A frequency was higher in children with TB diseases than in children with latent TB infections (20).

The *BCG* vaccination is known to activate the same pathways as TB, including TLR2 (29). In addition to *TLR1* T1805G and *TLR6* C745T, some other *TLR1* gene polymorphisms have been associated with altered immune responses to *BCG* vaccination (8) and we found that the variant *TLR2* and *TLR6* genotypes were associated with susceptibility to *BCG* osteitis. For example, the *TLR1* GG genotype, which is a wild genotype in the Finnish population, was associated with *BCG* osteitis, which is in line with the high rate of *BCG* complications reported in Finland (11,12).

The strength of this study is the large number of *BCG* osteitis patients, spanning a period of 29 years and covering the whole country (11,12). During the study period, 1960–1988, the microbiological diagnostics of *BCG* complications was centralised and systematically recorded in one national register. In addition, the patients were all of Finnish origin and homogeneity is an advantage in genetic studies. The cases were also compared with a strictly population-based control group from south-west Finland (13). The distribution of *TLR2* subfamily gene polymorphisms has not been studied between populations from different parts of Finland, but the frequencies of some other polymorphisms, such as those in the genes regulating mannose-binding lectin production, were very similar in populations in the south and west and in the east and north (30). None of the study subjects who suffered from *BCG* osteitis in early childhood reported any mycobacterial infections or other severe or exceptional infections in later life. On the other hand, these 132 subjects only represented 60% of the original group of 222 *BCG* osteitis patients (11,12). However, we also collected data from 28 additional cases, using a questionnaire, and none of those reported any significant infection morbidity. Although the number of cases was rather small for genetic studies, significant differences in all three studied *TLR* genotypes were found between the cases and controls. The result was confirmed with adjusted analyses that simultaneously included all three *TLR* genotypes in the model.

Our hypothesis was that TLR2 subfamily genetics played a role in the development of osteitis after *BCG* vaccination in newborns. We found that when we compared the study group of 132 former *BCG* osteitis patients with the controls, the study group displayed differences in polymorphisms in the genes encoding TLR1, TLR2 and TLR6. Our findings suggest that the Finnish population possesses an innate immunity-related genetic predisposition that leads to complications with the *BCG* vaccine.

COMPETING OF INTEREST
None.

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INTERFERON-GAMMA-DEPENDENT IMMUNITY IN BACILLUS CALMETTE-GUÉRIN VACCINE OSTEITIS SURVIVORS

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ABSTRACT

Background: Inborn errors of interferon-gamma (IFN-γ) -mediated immunity underlie disseminated disease caused by *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) live vaccines. We hypothesized that some patients with osteitis after BCG vaccination may have an impaired IFN-γ immunity. Our aim was to investigate IL-12 and IFN-γ *ex vivo* production stimulated with BCG and BCG+IFN-γ or BCG+IL-12, respectively, in BCG osteitis survivors.

Methods: Fresh blood samples were collected from 132 former BCG osteitis Finnish patients now aged 21-49 years, and IL-12 and IFN-γ were measured in cell cultures with and without stimulation with BCG and with BCG+IFN-γ or BCG+IL-12, respectively. As a pilot study, known disease-causing genes controlling IFN-γ immunity (*IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, NEMO* and *CYBB*) were investigated in 20 selected patients by whole exome sequencing.

Results: By the limit of $<5^{th}$ percentile, *ex vivo* IL-12 concentration and increase in concentration was low in five, and *ex vivo* IFN-γ concentration and increase in concentration was low in six patients (including two samples with both IL-12 and IFN-γ findings). By the limit of $<10^{th}$ percentile, an additional six and four patients were respectively detected (including two samples with both findings). With two exceptions, low concentrations and low increases in concentrations picked-up the same cases. Mutations in known disease-causing IFN-γ-related genes were not found in any of these patients.

Conclusion: These findings call for searching of mutations in new genes governing IFN-γ-dependent immunity to live BCG vaccine.

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. Approximately one third of the world's population is infected with *M. tuberculosis*, but only 10% of infected hosts develop an active disease.[1] Host immunity and variation especially in innate immunity influence the risk to get TB and other mycobacterial diseases.[2,3] After phagocytosis of mycobacteria, macrophages produce different mediators like interleukin-1 (IL-1) which enhance the maturation of dendritic cells, and IL-12 which enhance the maturation of T helper type-1 (Th1) cells, further promoting the production of interferon-gamma (IFN-γ).[4]

The Bacillus Calmette-Guérin (BCG) vaccine has been globally used as a vaccine against TB.[5] Currently, most western countries have stopped universal BCG vaccinations due to a risk of vaccination complications and low TB prevalence. However, severe complications like BCG osteitis have been rare in most populations, for example in Japan only 0.2 cases per 100,000 vaccinations.[6]

BCG vaccination of newborns belonged to the national vaccination program in Finland from 1941 to 2006, and the coverage of the vaccinations was nearly 100%.[7,8] The National Public Health Institute registered systematically invasive complications such as BCG osteitis until 1988.[7-9] According to the national registers, 222 children developed BCG osteitis confirmed by culture of BCG strain, by typical histology and age, or by both during the years 1960-1988.[7,8] The mean age when the first signs of osteitis appeared was 1.5 years (range 0.3-5.7 years), and 96% of the children were vaccinated within the first days after birth and the remaining 4% during the first year of life.[7,8] Between 1971 and 1978, when the Copenhagen BCG strain was in use, the annual incidence of BCG osteitis increased to 36.9 (mean) per 100,000 vaccinated newborns, and

decreased after the change to the Glaxo BCG vaccine to 6.4 (mean) per 100,000 vaccinated newborns[7,8], which still was higher than that reported from other countries.[6]

Human IFN-γ plays an important role in the immune response to BCG vaccine and in the defense against mycobacteria. The understanding of the pathogenesis of mycobacterial diseases has been improved by studies of Mendelian susceptibility to mycobacterial disease (MSMD). It is a rare genetic condition that makes an individual susceptible to weakly virulent mycobacteria, such as BCG[10-14] and atypical mycobacteria. The patients are also vulnerable to the more virulent *M. tuberculosis*[3,13,15] and half have suffered from typhoidal or non-typhoidal salmonellosis.[12-14] MSMD patients are usually otherwise healthy, and their immunity is intact except for a limited defect in innate immunity affecting IFN-γ. Since the first observations in 1996[12,16], nine MSMD causing genes have been discovered.[13] Seven of these genes are autosomal (*IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15* and *IRF8*), and two are X-linked (*NEMO* and *CYBB*). By 2015, 18 different disorders have been defined due to the high level of allelic heterogeneity, and these disorders are linked to the function of the IFN-γ pathway, impairing either the production of IFN-γ or the response to IFN-γ.[13]

The hypothesis of the study was that the patients with severe complications like osteitis after BCG vaccination have a compromised function of the IFN-γ pathway. The aim of the study was to investigate, by using blood cell cultures obtained from the former BCG osteitis patients, IL-12 and IFN-γ *ex vivo* production stimulated with BCG and BCG+IFN-γ or BCG+IL-12, respectively. Fresh whole blood samples were available from 132 of those 222 adults who had suffered from BCG osteitis in infancy.[8,9] The nine known genes of MSMD were investigated by whole exome sequencing (WES) in 20 study subjects as a pilot study.

MATERIALS AND METHODS

The diagnostics of invasive complications caused by BCG vaccinations was centralized in one national laboratory (National Public Health Institute, Helsinki, Finland) from 1960 to 1988, and during these 29 years, altogether 222 BCG osteitis cases were diagnosed in Finland.[8,9] In 2007-2008, a questionnaire and invitation to the study was sent to 203 (91.4%) former BCG osteitis patients with current address available, and 160 (78.8%) replied. The questionnaire included questions on chronic illnesses, long-term use of medicines, repeated or chronic infections, and mycobacterial infections. Nineteen subjects were not contacted; six had moved to overseas, and no residence information was available from 13 subjects with BCG osteitis in early childhood.

Fresh whole blood samples were obtained from 132 of the 160 former BCG osteitis patients who agreed to attend the study. Blood samples were collected into heparinized tubes at laboratories around the country and were immediately (within 12 hours) transported to the Tuberculosis Reference Laboratory, National Institute for Health and Welfare, Turku, Finland.

At the laboratory, the blood samples were diluted 1:2 in RPM 1640 medium (GibcoBRL) supplemented with penistreptomycin at 10000 U/mL (GibcoBRL). The detailed protocol of whole blood culture and stimulation was described previously.[17] In brief, 6 mL of the diluted blood sample was dispensed into four wells (1.5 mL/well) of a 24-well plate (Nunc). They were cultured and stimulated with BCG (4x10E7 cells/0.1mL), BCG and recombinant IFN-γ (50 μL, 165000U/mL), or BCG and recombinant IL-12 (50 μL, 700 ng/mL), respectively. For each sample, whole blood without stimulation was used as a negative control. After 18 hours of incubation, 450 μL supernatant was collected from each well for determination of IL-12 and after 48 hours of incubation, all supernatants left in each well were collected for determination of IFN-γ. The cytokine concentrations were determined by using ELISA kits (Quantikine, R&D systems)

according to the manufacturer's protocol. The detection limit of the test was 0.7 pg/mL for IL-12 and 17.6 pg/mL for IFN-γ.

The differentials of white blood cells were counted in 119/132 (90%) study subjects before the samples were transported, and the IL-12 and IFN-γ concentrations in the cell cultures are expressed per peripheral blood mononuclear cells (PBMCs), including lymphocytes and monocytes. The IL-12 and IFN-γ concentrations (in pg/mL) per PBMCs (1.0 x 10E9/L) were used in the analyses.

Data on PBMCs counts were available in 119, data on stimulated IL-12 concentrations in 125 and data on stimulated IFN-γ concentrations in 131 cases. Calculated concentrations per PBMCs were present in 115 (IL-12) and 118 (IFN-γ) cases, respectively.

Given the implication of IFN-γ pathway in mycobacterial infections, the nine known genes of MSMD (18 known gene defects) were studied in 20 study subjects as a pilot study. The samples were picked-up to include cases with low and high IL-12 concentrations after BCG+IFN-γ stimulation, and low and high increases in stimulated concentration, and those with low and high IFN-γ concentrations after BCG+IL-12 stimulation, and low and high increases in stimulated concentration, respectively. WES was performed by the New York Genome Center using an Illumina HiSeq 2500 machine, with an Agilent 71 Mb SureSelect exome kit. The reads were aligned to the human reference genome with a BWA aligner, and then re-calibrated and annotated with GATK[18], PICARD (http://picard.sourceforge.net/) and ANNOVAR.[19] The variations were further filtered and investigated with our in-house on-line server. The known gene defects of *IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, NEMO* and *CYBB* were examined as described earlier.[13]

Ethics

The study was accepted by the Ethics Committee of the Tampere University Hospital District. A written consent was obtained from the study subjects, including permission to perform genetic studies concerning susceptibility to mycobacterial infections.

Statistical Analysis

The SPSS 19.0 package (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp) was used for statistical analyses. Exploratory data analyses revealed that the IL-12 and IFN-γ concentrations and increases in concentrations were non-normally distributed, and therefore, the results are expressed as medians, 5th, 10th, 25th, 75th, 90th and 95th percentiles and ranges. The PBMC counts (cells x 10E9/L) are expressed as means, standard deviations (SD) and ranges.

RESULTS

Study Subjects and Peripheral Blood Mononuclear Cells

The median age of the 132 former BCG osteitis patients was 33.0 years (range 21-49 years), and 72 (54.5%) were females. None of the 160 study subjects, including those 132 with blood samples for cell cultures available, reported TB, or any other mycobacteriosis than infant BCG osteitis.

Among the 160 patients with blood samples available, 8% reported allergy, 7% asthma, 3% hypothyreosis, 3% psoriasis, 2% rheumatoid arthritis, 2% epilepsy, 2% multiple sclerosis, and less than 1% diabetes, spondylarthritis, stroke, breast cancer or migraine. Seven patients (5%) reported an earlier *Salmonella* infection.

The mean PBMC count (SD, range) was 2.80 x10E9/L (0.89, 0.60-5.49). The figures for lymphocytes were 2.35 (0.81, 0.54-4.80) and for monocytes 0.45 (0.18, 0.06-1.10).

IL-12 and IFN-γ Concentrations With and Without Stimulations

Before BCG stimulation, IL-12 was measurable (>0.7 pg/mL) in 17 (13%) and IFN-γ was measurable (>17.6 pg/mL) in 15 (11%) samples. The respective figures were 84 (64%) and 131 (99%) after stimulation with BCG. Thus, IL-12 concentration was below the detection limit in 48 (36%) cases even after BCG stimulation.

The median IL-12 concentration per PBMCs was 38.1 pg/mL after BCG+IFN-γ stimulation. The median IFN-γ concentration per PBMCs was 16708.7 pg/mL after BCG+IL-12 stimulation (Table 1).

The median increase of the IL-12 concentration per PBMCs was 36.5 pg/mL between BCG alone and BCG+IFN-γ stimulations. The median increase of the IFN-γ concentration per PBMC was 12516.0 pg/mL between BCG alone and BCG+IL-12 stimulations (Table 2).

By the limit of <5th percentile, IL-12 *ex vivo* concentration and increase in concentration was low in five and *ex vivo* IFN-γ concentration and increase in concentration in six cases, two cases with low concentrations and low increases in both IL-12 and IFN-γ as included (Table 3). By the limit of <10th percentile, *ex vivo* IL-12 concentration and increase in concentration was low in additional six and *ex vivo* IFN-γ concentration and increase in concentration in additional four cases, two cases with low concentrations and low increases in both IL-12 and IFN-γ as included (Table 3). With two exceptions, low *ex vivo* concentrations and low increases in concentrations picked-up the same cases. None presented with a low (<10th percentile) stimulated IFN-γ and a high (>90th percentile)

stimulated IL-12 concentration, or with a low (<10th percentile) stimulated IL-12 and a high (>90th percentile) stimulated IFN-γ concentration in cell cultures (data not shown).

The reported current or earlier diseases in low (<5th or 5-10th percentiles) IL-12 or IFN-γ producers are presented in Table 3. There were four subjects with low production of both IL-12 and IFN-γ, and one of them reported asthma, one diabetes, one stroke and one reported no disease (Table 3).

Gene Defects of the IFN-γ Pathway

Known genes of MSMD including *IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, NEMO* and *CYBB* were studied in 20 study subjects by WES. No mutations were found in coding regions of these genes. Seven of them belonged to the groups of low (<10th percentile) stimulated *ex vivo* IL-12 concentration and low increase in concentration (N=4), or low (<10th percentile) stimulated *ex vivo* IFN-γ concentration and low increase in concentration (N=3).

Register for causes of death

No residence information was available from 13 subjects with BCG osteitis in early childhood, but their dates of birth were recorded in the study documents. The register for causes of death (Statistics Finland) charted at a group level the causes of death of all deceased persons who had been born at the same day as the 13 former BCG osteitis patients without any residence information available. Nobody had died of tuberculosis or any other infection.

DISCUSSION

The IFN-γ pathway is crucial for immunity against mycobacteria, including BCG, and to date, 18 disorders of the IFN-γ pathway have been identified.[13] In order to reveal potential cases for defects of the IFN-γ pathway predisposing to complications of BCG vaccination, we analyzed the *ex vivo* levels of IL-12 and IFN-γ in blood cell cultures stimulated with BCG alone and BCG+IFN-γ or BCG+IL-12, respectively, in 132 former BCG osteitis patients. Since reference values are not available, we classified the concentrations and increases in concentrations as low by the limit of 5th percentile or 10th percentile. Low stimulated *ex vivo* IL-12 concentrations and low increases in concentrations revealed the same cases, with only one exception, and low stimulated *ex vivo* IFN-γ concentrations and low increases in concentrations also revealed the same cases, again with only two exceptions. There were four cases (20% of all those with low concentrations or low increases in either IL-12 or IFN-γ) with low stimulated concentrations and low increases in concentrations of both IL-12 and IFN-γ. In the previous study[17], which was done by the same methodology as our study, all 50 healthy BCG-vaccinated controls responded to the stimulation with BCG with increased IFN-γ production and all but two with increased IL-12 production in cell cultures. In line, IFN-γ was measurable in 11% of cases before BCG stimulation but in 99% after BCG stimulation in the present study. The respective figures for IL-12 were 13% and 64%. IL-12 was measurable in all cases after stimulation with BCG + IFN-γ. On average, the stimulated IFN-γ and IL-12 levels were lower in the present study than in the historical controls[17], but as is well-known, the comparisons with historical controls studied in different laboratories are not reliable. Anyway, our results suggest some weakness of the IFN-γ pathway, although we could not reveal any increased infection morbidity or mortality after BCG osteitis was recovered.

An important result of the present study was that mutations in known genes affecting IFN-γ pathway were not found in those 20 cases which were studied, though seven of them presented with low stimulated *ex vivo* IL-12 or IFN-γ concentrations or low increases in concentrations, and two presented with low stimulated *ex vivo* concentrations and low increases of both IL-12 and IFN-γ.

Among 11 Iranian children who presented with invasive complications like cervical lymphadenitis or osteitis after BCG vaccination, 10 (91%) had an impaired *ex vivo* IFN-γ response to BCG+IL-12 stimulation.[20] Evidently, these patients had MSMD, a genetic defect either in IL-12 receptor function or in IFN-γ production, but gene tests were not done. Instead, no significant differences were found in stimulated IL-12p40 and IFN-γ concentrations in cell cultures between 11 patients with non-TB mycobacterial cervical lymphadenitis and healthy controls from Israel.[21] Despite the negative result, the authors considered MSMD possible. In the present study, only 64% of the former BCG osteitis patients had measurable IL-12 concentration after BCG stimulation in cell cultures, but after BCG+IFN-γ stimulation, IL-12 was measurable in all cases. Instead, IFN-γ was measurable in all cell cultures after BCG stimulation. However, the study did not allow an evaluation of the magnitude of these responses due to the lack of appropriate healthy controls or reference values. Despite this, our results suggest that stimulation tests of blood cells may help to select patients for genetic studies, when thus far uncovered gene defects are searched. The currently known 18 gene defects of the IFN-γ pathway in *IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, NEMO* and *CYBB* genes account for only half the known MSMD cases.[13]

The most common MSMD is complete recessive IL-12Rβ1 deficiency, for which BCG disease is the most common infection.[13,22] The clinical phenotype ranges from fatal infection in infancy to asymptomatic course throughout adulthood.[22] Interestingly, BCG vaccination or BCG disease protects against subsequent environmental mycobacteriosis.[22] Multifocal osteitis occurs frequently in partial dominant IFNGR1 and STAT1 diseases.[13,23,24] However, BCG osteitis and multifocal

osteitis are separate clinical entities, and all our BCG osteitis patients presented with only one focus.[8] The cellular phenotype of partial IFNGR1 disease is characterized by a reduced *ex vivo* response to IFN-γ.[22] Likewise, the defect of the cellular IFN-γ response is partial in STAT1 disease, and the outcome of infections including BCG disease has mainly been good.[13] In MSMD patients, BCG infections like other mycobacterioses have diverse manifestations ranging from localized to disseminated cases. Thus, the beneficial prognosis of early-childhood BCG osteitis in the patients of the present cohort does not rule out MSMD.

Evidently, the high BCG osteitis incidence in Finland reflects a selective genetic susceptibility to the disease, and the lack of severe or exceptional infections in later life rules out primary immune deficiencies. Recently, we have documented differences in polymorphisms of genes regulating mannose binding lectin production (*MBL2* gene) and toll-like receptor-2 subfamily function between the subjects of the present early-life BCG osteitis cohort and Finnish population-based controls.[25,26] In this cohort, three patients had the homozygous variant *MBL2* genotype (O/O), and in one of them, IFN-γ concentration was at the 5th percentile level after stimulation with BCG and IL-12. No other evidence of low IFN-γ or IL-12 production was found.

The strength of this study is the large number of BCG osteitis patients from a period of 29 years in a country with high BCG osteitis incidence during the study years.[7-9] At that time, the microbiological diagnostics of BCG complications was centralized, and complications were systematically recorded in one national register. In addition, the homogeneity of the study patients who are all of Finnish origin is an advantage in genetic studies. We were able to document patients with low *ex vivo* stimulated IL-12 and/or IFN-γ production; this finding is commonly linked with susceptibility to mycobacterial disease. MSMD genetics were available for two of four BCG osteitis survivors who had low *ex vivo* production of both IL-12 and IFN-γ. No mutations were identified.

There are some weaknesses in this study. First, we were able to collect fresh blood samples from only 60% of the former BCG osteitis patients. This is mainly because of long distances within the country, and therefore, a selection bias is unlikely. Since references in healthy individuals are not available, the significances of the stimulated IL-12 and IFN-γ responses could not be assessed. The lack of a control group is a clear weakness of the study. On the other hand, to be representative of the population, the control group should be large covering different age groups and different parts of the country. Since the distances in our country are long and the transfer of some samples within 24 hours to the reference laboratory was challenging, we had 25 transfer control samples from healthy persons, but this material is too small and also non-representative to serve as a study control.

In conclusion, known gene defects of the IFN-γ pathway were not found in a sample of 20 adults from Finland with a history of BCG osteitis in infancy. On the other hand, there were marked differences in *ex vivo* IL-12 and IFN-γ concentrations after stimulations, when the whole cohort of 118 subjects were examined. This suggests that there may be defects in the production of IL-12 and/or IFN-γ, causing decreased immunity and increased susceptibility to BCG. Based on current results, further genetic studies even to find novel genetic defects are indicated, and they could first be focused on those with low *ex vivo* IL-12 or IFN-γ concentrations or low increases in concentration.[27] The final goal of the studies on the association between genetic factors and BCG infections is to identify risk groups for severe complications of BCG vaccination.

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TABLE 1. IL-12 and IFN-γ concentrations in cell cultures per PBMC in 115 and 118 adults with BCG osteitis in infancy.

	IL-12 concentration (pg/mL) after BCG+IFN-γ stimulation (N=115)	IFN-γ concentration (pg/mL) after BCG+IL-12 stimulation (N=118)
5th percentile	3.5	2229.4
10th percentile	5.2	4474.2
25th percentile	12.4	8597.8
Median	38.1	16708.7
75th percentile	66.0	31330.9
90th percentile	109.6	45801.5
95th percentile	152.4	59741.3
Range	2.9-274.7	543.0-81402.3

TABLE 2. Increases of IL-12 and IFN-γ concentrations per PBMC after BCG+IFN-γ or BCG+IL-12 stimulations, respectively, compared to concentrations after BCG stimulation alone. The detection limit was used for calculations if the concentration after BCG stimulation was non-measurable.

	Increase in IL-12 concentration (pg/mL) (N=114)	Increase in IFN-γ concentration (pg/mL) (N=110)
5th percentile	3.5	2236.9
10th percentile	5.1	4390.3
25th percentile	11.7	7035.0
Median	36.5	12516.0
75th percentile	61.2	22393.9
90th percentile	108.9	32620.3
95th percentile	147.7	43708.4
Range	-3.0-260.5	990.3-66182.5

TABLE 3. Twenty low IL-12 or IFN-γ producers identified with four criteria based on results after BCG+IFN-γ or BCG+IL-12 stimulations of cell cultures, respectively (the *ex vivo* concentration or increase in concentration of IL-12 or IFN-γ <5th percentile or <10th percentile, respectively).

No	**Sex (m/f), age (years)**	**IL-12 concentration low**	**IL-12 increase low**	**IFN-γ concentration low**	**IFN-γ increase low**
	<5th percentile				
25	f, 45			x	x
64	f, 32	x	x	x	x
65	m, 36*			x	x
66	f, 34			x	x
67	m, 34*	x	x		
72[1]	f, 22	x	x	x	x
74	f, 47			x	x
82	m, 28			x	
128[2]	f, 41*	x	x		
132[3]	f, 34*	x	x		

	5th-10th percentile				
26	m, 33			x	x
59	f, 38*	x	x		
62	m, 35				x
110	f, 28	x	x		
115[4]	f, 37	x	x		
130[5]	f, 35*	x	x	x	x
137[6]	f, 48*	x	x	x	x
141[1,3]	f, 46		x		
142	f, 49			x	x
155	f, 21	x	x		

**IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, NEMO*, and *CYBB* genes were studied in seven cases, and all tests gave a negative result.

Reported diseases: [1]Asthma; [2]Allergy; [3]*Salmonella* infection; [4]Multiple sclerosis; [5]Diabetes; [6]Stroke

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Interleukin-10 gene promoter region polymorphisms are not associated with BCG osteitis in vaccinated infants

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SUMMARY

SETTING: Complications arising from bacille Calmette-Guérin (BCG) vaccination were recorded in a national register in Finland until 1988. In the period 1960–1988, 222 patients suffered from BCG osteitis.

OBJECTIVE: To evaluate whether single nucleotide polymorphisms (SNPs) in the promoter region of the gene encoding interleukin 10 (IL-10) are associated with BCG osteitis after vaccination in neonates.

DESIGN: Blood samples of 132 former BCG osteitis patients now aged 21–49 years were analysed in a controlled study for *IL10 rs*1800896 (–1082G/A), *rs*1800871 (–819C/T), *rs*1800872 (–592C/A) and *rs*1800890 (–3575T/A) polymorphisms.

RESULTS: The frequencies of genotypes of *IL10 rs*1800896, *rs*1800871, *rs*1800872 and *rs*1800890, the frequencies of variant genotypes and the frequencies of major or minor alleles did not differ between patients and controls. Furthermore, the frequencies of the eight possible combinations of the three *IL10* alleles located close to each other (*IL10 rs*1800896, *IL10 rs*1800871 and *IL10 rs*1800872) were surprisingly similar.

CONCLUSION: Our results suggest that polymorphisms of the IL-10 encoding gene do not play a central role in the development of complications due to BCG vaccination, although the *IL10* gene, especially *IL10 rs*1800896 (–1082G/A) polymorphism, is known to be associated with tuberculosis risk in Europeans and North Americans.

KEY WORDS: immunity; genetics; vaccine; complication

IT HAS BEEN SHOWN that the genetic constitution has an influence on tuberculosis (TB) susceptibility in humans.[1] The bacille Calmette-Guérin (BCG) vaccine, which was developed nearly a century ago, provides partial protection against TB, mainly against severe forms such as meningitis in infants;[2] it is still widely used, particularly in the developing world. Most industrialised countries have stopped universal vaccination because of low TB risk and vaccine-induced complications. Localised abscesses and regional lymphadenitis are common, whereas severe complications such as BCG osteitis are rare in most populations. For example, in Japan there were only 0.2 cases per 100 000 vaccinations.[3]

Finnish newborns were BCG-vaccinated against TB from 1941 to 2006,[4] and invasive complications such as BCG osteitis were systematically registered by the National Public Health Institute, Helsinki, Finland, until 1988.[4,5] During the years 1960–1988, 222 children developed BCG osteitis, which was confirmed by culture of BCG strain, by typical histology and age, or both.[4,5] Between 1971 and 1978, the mean annual incidence of BCG osteitis increased to 36.9/100 000 vaccinated newborns, and decreased after the change of the vaccine to 6.4/100 000 vaccinated newborns. Despite this decrease, the incidence of BCG osteitis in Finland remained substantially higher than that reported from other countries.[6]

The human interleukin 10 (IL-10), a protein consisting of 160 amino acids, acts as an important immune regulatory and anti-inflammatory cytokine that downregulates the expression of T-helper 1 (Th1) cytokines through macrophage deactivation and the blocking of interferon-gamma (IFN-γ) release by Th1 lymphocytes.[7] The ability to produce IL-10 varies between individuals due to genetically regulated differences.[8] In previous studies, IL-10 has been associated with many diseases, including TB.[9] The roles of the different *IL10* gene single nucleotide

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polymorphisms (SNPs), as well as IL-10 production, vary depending on the infection and the population studied. The *IL10* gene is highly polymorphic, and point mutations in the proximal promoter region, including *rs*1800896, *rs*1800871 and *rs*1800872, form distinct haplotypes associated with IL-10 production.[10,11]

Susceptibility to TB and responses to BCG vaccination are genetically regulated; however, different genes and polymorphisms seem to play a role in different populations.[12] Our hypothesis was that polymorphisms of the *IL10* gene are associated with the risk of severe complications such as osteitis after BCG vaccination in newborns.

The aim of the study was to evaluate whether the SNPs *rs*1800896 (–1082G/A), *rs*1800871 (–819C/T), *rs*1800872 (–592C/A) and *rs*1800890 (–3575T/A) in the gene encoding IL-10 are associated with BCG osteitis after vaccination as newborn.

STUDY SUBJECTS AND METHODS

The BCG vaccination of infants began in Finland in 1941; from 1951, nearly every newborn received the vaccine. Microbe-specific diagnosis of invasive complications caused by BCG vaccination was centralised in one national laboratory, the National Public Health Institute, Helsinki, Finland, from 1960 to 1988. Over these 29 years, 222 cases of BCG osteitis were diagnosed.[4,5] In 2007–2008, a questionnaire and invitation to participate in this study was sent to 203 former BCG osteitis patients with a current address available; 160 of the 203 (78.8%) replied.

Blood samples were obtained from 132/160 former BCG osteitis patients who agreed to participate in the study. Blood samples were collected at laboratories around the country and were immediately transported to the Tuberculosis Reference Laboratory, National Institute for Health and Welfare, Turku, Finland, where they were frozen at –70°C.

Samples of the control population were obtained from a prospective cohort study, Steps to the Healthy Development and Well-being of Children (STEPS study). The design of the STEPS study and the characteristics of the study population have been published previously.[13] In brief, in the period 2008–2010, 1827 children of Finnish origin in the Hospital District of Southwest Finland were recruited into the study without any selection criteria, and *IL10* genotypes were determined in blood samples collected during study clinic visits at 2 months of age before administration of any vaccines except rotavirus vaccine ($n = 401$; males 53.0%, 53.2% and 53.2% for *rs*1800896, *rs*1800871 and *rs*1800872, and $n = 378$; males 51.9% for *rs*1800890).

In Finland, universal BCG vaccination was discontinued in 2006. Thereafter, vaccinations were focused only on infants at high risk for TB.

Laboratory analyses

Genotyping of the *IL10 rs*1800896 (–1082G/A) SNP was performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Waltham, MA, USA) for both PCR and allelic discrimination, as described earlier.[14] *IL10 rs*1800890 (–3575T/A) genotypes were determined by polymerase chain reaction (PCR), followed by pyrosequencing, as described previously.[15] Primers were designed to recognise the polymorphic site in the *IL10* promoter region (A changes to T).

The detection of *IL10* polymorphism sites *rs*1800871 (–819A/G) and *rs*1800872 (–592T/G) was performed using PCR and sequencing of the amplified region. *IL10 rs*1800871 and *rs*1800872 are in full linkage in Caucasian populations. The SNPs at position –592 and –819 were detected simultaneously in one PCR reaction. The sequences of PCR primers were forward: 5′-TAGGTCTCTGGGCCTTAGTT-3′ and reverse: 5′-AAGGCCAATTTAATCCAAGGTT-3′. The correct PCR product size (440 base pair [bp]) was verified using agarose gel electrophoresis. The forward primer was used also for the sequencing reaction. The SNP at location –1082 was amplified with primers forward: 5′-TCTCCAGCACATAGAATGAAACC-3′ and reverse: 5′-CTTCCCCAGGTAGAGCAACA-3′. The PCR product size was 330 bp. The forward primer was also used for the sequencing reaction. Sequencing reactions were performed at the Institute for Molecular Medicine Finland, Helsinki, Finland. All PCR reactions were performed in the following conditions: initial denaturation at 95°C, denaturation at 95°C for 2 min, annealing at 60°C for 30 s and extension at 72°C for 40 s. After 40 cycles, final denaturation was performed at 72°C for 7 min.

Our test gave the reverse transcription sequence instead of the forward transcription sequence used in most previous publications. To allow for comparisons with other studies, we therefore expressed the alleles G/A (*IL10 rs*1800871) and G/T (*IL10 rs*1800872) as C/T (*IL10 rs*1800871) and C/A (*IL10 rs*1800872), respectively. Genotyping of 401 controls (*rs*1800896, *rs*1800871 and *rs*1800872) from extracted DNA was performed by Sequenom massARRAY iPlex Gold® System (Sequenom Inc, San Diego, CA, USA) at the University of Eastern Finland, Kuopio, Finland.

Ethics

The study was approved by the Ethics Committee of the Tampere University Hospital District, Tampere, Finland. All study subjects provided written consent, including permission to perform genetic studies on susceptibility to mycobacterial infections. The study protocol concerning the genetic control group of children was approved by the Ethics Committee of

the Hospital District of Southwest Finland, Turku, Finland. Written consent was obtained from the children's parents.

Statistical analyses

SPSS, v 19.0 (IBM Corp, Statistical Package for the Social Sciences Statistics for Windows, Armonk, NY, USA) was used for statistical analysis. χ^2 and Fisher's exact tests were used when appropriate, for comparing genotypes and allele frequencies of cases and controls. Allele combinations are presented as percentages and their 95% confidence intervals (95%CIs).

RESULTS

The median age of the 132 former BCG osteitis patients was 33.0 years (range 21–49); 72 (54.5%) were females. All were of Finnish origin. In the questionnaire, the study subjects were asked if they had suffered from TB or any other mycobacteriosis or other severe or uncommon infections after infant BCG osteitis. The 160 subjects who returned the questionnaire, including the 132 with blood samples for SNPs available, did not report any such infections. Of the 132 patients, 8% reported allergy, 7% asthma, 3% hypothyreosis, 3% psoriasis, 2% rheumatoid arthritis, 2% epilepsy, 2% multiple sclerosis and <1% diabetes, spondylarthritis, stroke, breast cancer or migraine. Seven patients (5%) reported an earlier *Salmonella* infection.

In *IL10 rs*1800896 (–1082G/A), the genotype was AA in 43 (33.9%), AG in 56 (44.1%) and GG in 28 (22.0%) cases (vs. 34.4%, 47.1% and 18.5% in controls, $P = 0.655$). The frequency of variant genotype (AG or GG) was 66.1% (vs. 65.6% in controls, $P = 0.908$) and that of the minor allele G 44.1% (vs. 42.0% in controls, $P = 0.560$).

In *IL10 rs*1800871 (–819C/T), the genotype was CC in 79 (61.2%), CT in 42 (32.6%) and TT in 8 (6.2%) cases (vs. 59.1%, 34.4% and 6.5% in controls, $P = 0.911$). The frequency of variant genotype (CT or TT) was 38.8% (vs. 40.9% in controls, $P = 0.667$) and that of the minor allele T 22.5% (vs. 23.7% in controls, $P = 0.690$).

In *IL10 rs*1800872 (–592C/A), the genotype was CC in 79 (61.2%), CA in 42 (32.6%) and AA in 8 (6.2%) cases (vs. 59.1%, 34.4% and 6.5% in controls, $P = 0.911$). The frequency of variant genotype (CA or AA) was 38.8% (vs. 40.9% in controls, $P = 0.667$) and that of the minor allele A 22.5% (vs. 23.7% in controls, $P = 0.690$).

In *IL10 rs*1800890 (–3575 T/A), the genotype was AA in 55 (41.7%), AT in 56 (42.4%) and TT in 21 (15.9%) cases (vs. 44.7%, 44.4% and 10.8% in controls, $P = 0.307$). The frequency of variant genotype (AT or TT) was 58.3% (vs. 55.3% in controls, $P = 0.544$) and that of the minor allele T 37.1% (vs. 33.1% in controls, $P = 0.232$).

Table Allele combinations of *IL10 rs*1800896 (–1082G/A), *IL10 rs*1800871 (–819C/T) and *IL10 rs*1800872 (–592C/A) in 127 former BCG osteitis patients and 400 population-based controls

Combination	Cases *n* (%) (95%CI)	Controls *n* (%) (95%CI)
GCC	84 (22.6) (18.6–27.1)	262 (21.6) (19.4–24.0)
GCA	22 (5.9) (3.9–8.8)	71 (5.9) (4.7–7.3)
GTC	22 (5.9) (3.9–8.8)	71 (5.9) (4.7–7.3)
GTA	22 (5.9) (3.9–8.8)	71 (5.9) (4.7–7.3)
ACC	91 (24.5) (20.4–29.1)	300 (24.8) (22.4–27.3)
ACA	41 (11.0) (8.2–14.6)	137 (11.3) (9.6–13.2)
ATC	41 (11.0) (8.2–14.6)	137 (11.3) (9.6–13.2)
ATA	49 (13.2) (10.1–17.0)	163 (13.4) (11.6–15.5)

IL = interleukin; BCG = bacille Calmette-Guérin; CI = confidence interval.

As the frequencies of genotypes and allele frequencies were surprisingly similar in former BCG osteitis cases and controls, we calculated the frequencies of the eight possible combinations of the three *IL10* SNPs (*IL10 rs*1800896, *IL10 rs*1800871 and *IL10 rs*1800872) located in the same proximal promoter region of the gene. Again, there were no significant differences in the frequencies of these allele combinations between cases and controls (Table).

DISCUSSION

The main result of the study was that the 132 former BCG osteitis patients did not differ in terms of frequencies of polymorphisms *rs*1800896 (–1082G/A), *rs*1800871 (–819C/T), *rs*1800872 (–592C/A) and *rs*1800890 (–3575T/A) of the *IL10* gene from controls representing the Finnish population. The frequencies of the genotypes, allele frequencies, and the frequencies of the eight allele combinations of the *IL10 rs*1800896 (–1082G/A), *rs*1800871 (–819C/T) and *rs*1800872 (–592C/A) located in the same proximal promoter area of the *IL10* gene were surprisingly similar between cases and controls.

The four SNPs of the IL-10 encoding gene studied were not associated with BCG osteitis in infancy. However, it has been shown in previous studies that IL-10 plays a role in various infections, and that the SNPs of the *IL10* gene predispose to, for example, allergic diseases such as atopic dermatitis and wheezing. One study found that three of four *IL10* linkage disequilibrium blocks, including *rs*1800890, and two distal promoter SNPs presented as determi-

nants for reduced T regulatory cell numbers and potentially changed balance in immune regulation in SNP carriers.[16]

In many studies, *IL10* gene polymorphisms have been associated with increased TB susceptibility.[17] One meta-analysis concluded that the –1082G/A polymorphism of the *IL10* gene could be a risk factor for TB in Europeans.[17] In another recent meta-analysis, *IL10* –1082G/A polymorphism was associated with TB risk in Europeans and North Americans; it was also found that *IL10* –819T/C and –592A/C polymorphisms could be risk factors for TB in Asians.[18] In Turkish TB patients, the *IL10* –1082 G allele frequency was significantly more common than in healthy controls.[19] In a Tunisian study, the *IL10* –1082A/G genotype was significantly associated with increased risk of developing extra-pulmonary TB, whereas the –1082A/A genotype seemed to be associated with resistance to pulmonary TB.[20] In a recent controlled study, genotypes of the *IL10* –1082G/A polymorphism were significantly associated with TB.[21] Household contacts of TB patients were at increased risk of developing TB if they had the G/A genotype of *IL10*. There were also significant differences in allele distribution of *IL10* –592A/C in patients with TB compared with healthy controls in the Sudanese population.[22]

Some previous studies have found no association between *IL10* gene polymorphisms and TB. For example, the *IL10* promoter –1082G/A region was not associated with susceptibility or resistance to TB.[23] In Egyptian children, no differences were observed in the frequency of *IL10* –1082 alleles or genotypes between TB patients and controls, but a difference in the frequency of *IL10* –1082 GG genotype was found between patients with pulmonary and extra-pulmonary TB.[24]

In a controlled Australian study, the *IL10* –1082 AA genotype was significantly associated with pulmonary non-tuberculous mycobacterial disease.[25] Distinct *IL10* promoter haplotypes have been associated with the transcriptional activity of IL-10. High, intermediate and low IL-10 production has been associated with the *IL10* –1082/–819/–592 combinations GCC, ACC and ATA, respectively.[10,11,26] High-producer *IL10* GCC haplotype was associated with increased susceptibility to both pulmonary and extra-pulmonary TB.[20]

The innate cytokine response of neonates to BCG varies between populations.[27] For example, children with previous miliary or meningeal TB who received the BCG vaccine did not have a major defect in their cytokine pathways.[28] Allele frequencies of 49 SNPs, including *IL10* genes, were studied in a community-based cohort of 9960 individuals, 98% of whom were Caucasian.[29] The distribution of alleles was similar to the distribution in previous databases (SNP500 Cancer Database, National Cancer Institute; dbSNP Database, National Center for Biotechnology Information, Bethesda, MD, USA), except in African Americans. Several studies have revealed that ethnicity significantly influences the distribution of cytokine gene polymorphisms.[30–32]

The strength of the study is the homogeneity of the study group, as all participants were of Finnish origin, as well as the long time period (29 years) during which all the diagnosed BCG osteitis patients in the country were included. At that time, data on all cases with BCG complications were collected in a national register. The cases were then compared with strictly population-based controls from South-Western Finland.[13] The distribution of *IL10* gene polymorphisms in populations from different parts of the country has not been studied; however, the frequencies of some other polymorphisms, such as those in genes regulating mannose-binding lectin production, were very similar in south-western and east-northern Finnish populations.[33]

The weakness of the study is that not all of the 222 former BCG osteitis patients took part in the study. Questionnaire data were available from 72% and blood samples were collected from 60% of the patients. However, this is mainly because the samples needed to be transported from all over the country. Selection bias is therefore unlikely.

In the future, testing of polymorphisms in genes regulating responses to BCG might be a tool for identifying those at risk for severe complications due to BCG vaccination. Although there is an urgent need for a new TB vaccine, its development will probably be linked to the present BCG vaccine. One study line is investigating the possibility of boosting the immune response to BCG, which no doubt requires more knowledge about the immunity of tuberculous infection and about responses to BCG strains and their components.

In conclusion, the study results indicate that the SNPs *rs*1800896 (–1082G/A), *rs*1800871 (–819C/T), *rs*1800872 (–592C/A) and *rs*1800890 (–3575T/A) of the *IL10* gene are not more common in former BCG osteitis patients than in population-based controls in Finland. This is an interesting finding, as the polymorphisms of the *IL10* gene have been associated with TB susceptibility in previous studies from other parts of the world. Recent studies also suggest that there is a genetic immunity-related predisposition to BCG vaccine complications in Finland.[33,34] The immunological responses in the host against *M. tuberculosis* and *M. bovis* of the BCG vaccine are clearly areas that need more research.

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Conflicts of interest: none declared.

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RESUME

CONTEXTE : Les complications du vaccin bacille Calmette-Guérin (BCG) ont été enregistrées dans un registre national en Finlande jusqu'en 1988. Entre 1960 et 1988, 222 patients ont souffert d'une ostéite due au BCG.

OBJECTIF : Evaluer si les polymorphismes d'un seul nucléotide (SNPs) dans la région promotrice du gène codant pour interleucine 10 (IL-10) étaient associés à l'ostéite BCG après vaccination chez le nouveau-né.

SCHÉMA : Des échantillons de sang de 132 patients ayant eu une ostéite BCG et maintenant âgés de 21–49 ans ont été analysés dans le cadre d'une étude contrôlée pour les polymorphismes *IL10 rs*1800896 (–1082G/A), *rs*1800871 (–819C/T), *rs*1800872 (–592C/A) et *rs*1800890 (–3575T/A).

RÉSULTATS : Les fréquences des génotypes d'IL10 *rs*1800896, *rs*1800871, *rs*1800872 et *rs*1800890, les fréquences des génotypes variantes et les fréquences des allèles majeurs ou mineurs et n'ont pas été différentes entre les patients et les témoins. De même, les fréquences des huit combinaisons possibles des trois allèles *IL10* proches les uns des autres (*IL10 rs*1800896, *IL10 rs*1800871 et *IL10 rs*1800872) ont été étonnamment similaires.

CONCLUSIONS : Nos résultats suggèrent que les polymorphismes des gènes codant pour IL-10 ne jouent aucun rôle central dans le développement des complications du vaccin BCG, bien que le polymorphisme du gène *IL10*, particulièrement *IL10 rs*1800896 (–1082G/A), soit connu pour être associé au risque de tuberculose chez les Européens et les Américains du Nord.

RESUMEN

MARCO DE REFERENCIA: Hasta 1988 se registraban en Finlandia las complicaciones de la vacunación antituberculosa con el bacille Calmette-Guérin (BCG). De 1960 a 1988, 222 pacientes padecieron osteítis causada por el BCG.

OBJETIVO: Examinar si la presencia de polimorfismos de nucleótido único en la región promotora del gen que codifica la interleucina 10 (IL-10) se asocia con aparición de osteítis causada por el BCG después de la vacunación del recién nacido.

MÉTODOS: En un estudio comparativo con testigos se analizaron muestras de sangre de 132 pacientes con antecedente de osteítis por BCG de edad de 21–49 años, con el objeto de buscar los siguientes polimorfismos del gen *IL10*: *rs*1800896 (–1082G/A), *rs*1800871 (–819C/T), *rs*1800872 (–592C/A) y *rs*1800890 (–3575T/A).

RESULTADOS: No se observaron diferencias entre los pacientes y los testigos con respecto a la frecuencia de los genotipos *rs*1800896, *rs*1800871, *rs*1800872 y *rs*1800890 del gen *IL10*, la frecuencia de los genotipos variantes ni de los alelos mayores y menores. Asimismo y por sorpresa, las frecuencias de ocho posibles combinaciones de los tres alelos de *IL10* cercanos entre sí (*IL10 rs*1800896, *IL10 rs*1800871 e *IL10 rs*1800872) fueron equivalentes.

CONCLUSIÓN: Estos resultados indican que los polimorfismos del gen que codifica la IL-10 no cumplen ninguna función primordial en la aparición de las complicaciones de la vacunación antituberculosa, aunque se sabe que el gen *IL10* y sobre todo el polimorfismo *rs*1800896 (–1082G/A) se asocia con el riesgo de padecer tuberculosis en las poblaciones europeas y americanas del norte.