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Association of *Toll-like receptor 2* rs111200466 polymorphism with low serum levels of IL-33 in early childhood

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TLR2 is one of 10 human TLRs, which plays an important role in the recognition of pathogens and activation of the innate immunity *via* NF-κB pathway. NF-κB activation induces the expression of various pro-inflammatory genes. This study examines the effect of *TLR2* polymorphisms on the production of blood pro-inflammatory cytokines in healthy Finnish children. One hundred forty-six children who participated in a prospective observational birth cohort study in Turku, Finland, were included. DNA samples were analysed by PCR-based sequencing for two common *TLR2* polymorphisms (rs5743708 Arg753Gln; rs111200466–196 to –174del). Serum concentrations of IL-33, IL-31, IL-17A and IL-17F were measured by multiplex immunoassay and sST2 by ELISA in children at the age of 13 months. Children with variant type of *TLR2* rs111200466 (ins/del or del/del) had significantly lower level of serum IL-33 (median, 0.00 pg/mL; IQR 0.00–17.60) than those with ins/ins type of *TLR2* (19.81 pg/mL; IQR 0.00–51.78) ($p = 0.0001$). Almost all study subjects had serum concentrations of IL-17A, IL-17F and IL-31 below the detection limit and therefore not included in the final analyses. No differences in levels of above four cytokines and sST2 were found between *TLR2* rs5743708 genotypes (GG and GA). Our results indicated that the *TLR2* rs111200466 deletion was associated with a low level of serum IL-33, suggesting that the polymorphism may impair the production of IL-33.

Key words: TLR2; polymorphism; IL-33; rs111200466; cytokine.

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Toll-like receptors (TLRs) are pattern recognition receptors (PRRs), which play a crucial role to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (1). One of the most studied TLRs is TLR2, which acts as a heterodimer with TLR1 or TLR6 and detect diverse PAMPs from bacteria, fungi and parasites (2). Stimulation of TLR2 with its ligand initiates a MyD88-dependent intracellular downstream signalling pathway which leads to the activation of nuclear factor-κB (NF-κB) or the mitogen-activated protein kinases (MAPKs), to modulate gene transcription and further induces the production of inflammatory cytokines and type 1 interferons (IFNs) (2).

Polymorphisms in the genes encoding human TLRs can have functional effects on many infectious (3) and autoimmune (4) diseases. One of the most studied single nucleotide polymorphism (SNP) of *TLR2* is rs5743708. This SNP consists of a G to A substitution, which causes the replacement of Arginine by Glutamine (Arg753Gln) in the C-terminal end of TLR2. Based on the earlier studies, it seems that this variation does not affect the ability of TLR2 to respond to its ligands, but it might result in reduced TLR2-dependent signalling (5).

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Several studies have shown its association with increased risk to the development of tuberculosis and septic shock (6, 7). Another variation of interest is recently found 23-bp deletion −196 to −174 (rs111200466) located in the promoter area of *TLR2* gene. This polymorphism has been shown to reduce *TLR2* gene transcription activity (8, 9).

Interleukin (IL)- 33 is a tissue-derived nuclear cytokine of the IL-1 family and it has a crucial role to regulate T helper (Th) 2 immunity *via* suppressor of tumorigenicity 2 protein (ST2) (10). Recently, there has been renewed interest in the role of IL-33/ST2 axis in airway hyperresponsiveness and eosinophilic inflammation (11, 12), which are known to play a key role in the development of asthma.

Another molecule that has recently attracted interest is IL-31, which plays an important role in several diseases, like atopic dermatitis and allergic pathologies. IL-33 together with pruritogenic cytokine IL-31 is involved in the inflammation axis IL-31/IL-33, which is shown to be a potential inflammatory pathway in allergic and inflammatory diseases (13).

Asthma is a common chronic disease affecting both children and adults. Previous studies have shown that about 50% of asthma cases are classic Th2-type, including early-onset asthma, which is the most common phenotype during childhood. Another common type of asthma is so-called non-type 2 immunity asthma where Th1 and Th17 have a putative role and thought to be followed on activation of airway epithelial cells by bacteria, viruses or reactive oxygen species like hydrogen peroxide (14). IL-17-driven inflammatory pathway is shown to increase airway hyperresponsiveness, and elevated levels of IL-17A and IL-17F have been found in bronchial biopsies of patients with severe asthma and neutrophilic airway inflammation (14).

The prospective observational birth cohort study, Steps to the Healthy Development and Well-being of Children (STEPS) was launched in Southwest Finland in 2008, aims to search for the precursors and causes of problems in children's health and well-being (15). Within the STEPS Study project, we have focused on investigating factors predisposing to asthma in childhood. Recently, we found that children who developed asthma at age of 7 years had significantly lower levels of serum sST2 at age of 13 months than those who did not develop asthma (16). Despite that, elevated levels of serum IL-33 have been reported in children and adults with asthma (10, 17); in the STEPS study cohort, we did not observe any differences in serum IL-33 levels in children who later developed asthma and those who did not (16). Hence, we aimed to explore the role of *TLR2* polymorphisms on production of asthma-related cytokines in the same cohort.

MATERIALS AND METHODS

Study design and sample collection

For this study, we analysed material collected from STEPS study (15), where 923 children participated in the follow-up group for respiratory tract infections. These children were followed intensively from birth to 2 years of age and after that with the annual questionnaires and collection of health registry data. Nasopharyngeal (NP) and serum samples were collected at scheduled participant visits at age 2, 13 and 24 months. In this study, we included all children (n = 146) whose 13-month serum samples and follow-up data were available. Blood samples for genetic analyses were collected at the age 2-month visit and serum samples at the age 13-month visit (16). All sera in two aliquots have been stored at −20°C and were only thawed once before used in this study.

The STEPS study was approved by the Ministry of Social Affairs and Health and the Ethics Committee of the Hospital District of Southwest Finland (27 February 2007) and was found ethically acceptable by the Ministry of Social Affairs and Health (STM 1575/2008, STM 1838/2009) and the Ethics Committee of the Hospital District of Southwest Finland (19.2.2008 §63, 15.4.2008 §134, 19.4.2011 §113). All participants or parents of participating children gave their written informed consent.

Genetic analyses and cytokine measurements

Genomic DNA was extracted from peripheral blood samples (18). *TLR2* rs5743618 was determined previously by pyrosequencing (18). PCR-based method described originally by Tahara et al. was used for identification and differentiation of 23 bp insertion or deletion at −196 to −174 of promoter of *TLR2* rs111200466 (19). The Platinum Taq DNA enzyme (Thermo Fisher Scientific, Waltham, MA, USA) with following PCR parameters: denaturation 2 min at 92 °C, 40 cycles at 94 °C for 30 s, at 58°C for 40 s and 72 °C for 60 s was used. The PCR products were visualized by electrophoresis on a 2.0% agarose gel and staining with Midori Green (NIPPON Genetics, Tokyo, Japan). A single band at 286 bp was judged as wild type; a single 264-bp band was judged as homozygous variant, whereas the heterozygous variant had two bands of 286 and 264 bp as described by Tahara et al. (19). Six randomly selected samples were confirmed by Sanger sequencing.

Serum IL-33, IL-31, IL-17A and IL-17F were determined by multiplex immunoassay (Bio-PLex 200, Bio-Rad Laboratories, Hercules, CA, USA) with MILLIPLEX Th17 Kit (Merck & Co., Kenilworth, NJ, USA) and serum sST2 with the ELISA kit (Elabscience Biotechnology, Wuhan, China). The detection limits of measured cytokines and sST2 were 3.1 pg/mL and 190 pg/mL, respectively.

Statistical analysis

Statistical analyses were performed using SPSS software, version 28.0 (IBM Corp. in Armonk, NY). The non-normally distributed data were compared by Mann–Whitney *U*-test. In the haplotype analyses, the Bonferroni correction was used for determining adjusted p-values (p_a). Deviations from the Hardy–Weinberg equilibrium (HWE)

were studied with the chi-squared test. Two-tailed $p < 0.05$ was considered significant.

RESULTS

The detailed characteristics of the study subjects are shown in Table 1. The frequency of *TLR2* rs5743708 heterozygous variant GA was 7.6%, and no homozygous variant AA was detected. The HWE was p = 0.637. The frequencies of *TLR2 rs111200466* heterozygous (ins/del) and homozygous (del/del) genotypes were 21.6% and 2.4%, and the HWE was p = 0.521.

The serum concentration of IL-33 varied from 0.00 to 2608 pg/mL, with a median of 12.4 pg/mL (IQR 0.00–44.50). The serum concentration of sST2 varied from 85 to 13,626 pg/mL, with a median of 4567 pg/mL (IQR 2714–6111). No correlation was observed between serum concentrations of IL-33 and sST2. Nearly all study subjects had serum concentrations of IL-17A, IL-17F and IL-31 below the detection limit (3.1 pg/mL) and therefore not included in the final analyses. Serum concentrations of four cytokines and sST2 in 146 study subjects and their relation to *TLR2* polymorphisms are presented in Table 2.

Table 1. Characteristics of the study subjects

Characteristics	Total, n = 146 (%)
Sex	
Female	69 (52.7)
Male	77 (47.3)
Mode of delivery	
Vaginal	121 (82.9)
Caesarean section	25 (17.1)
Breastfed over 2 months	122 (83.6)
Missing data (% from total n)	5 (3.4)
Atopy at age of 13 months	23 (15.8)
Missing data (% from total n)	8 (5.5)
Presence of older siblings at time of birth	60 (41.1)
TLR2 genotype	
rs5743618[1]	
GG	133 (92.4)
AG	11 (7.6)
AA	0 (0.0)
rs111200466[2]	
ins/ins	95 (76.0)
ins/del	27 (21.6)
del/del	3 (2.4)
Serum cytokine levels (pg/mL)	**Median (IQR)**
IL-33	12.4 (0.00–44.5)
IL-31[3]	0.05 (0.00–0.18)
IL-17A[3]	0.00 (0.00–1.46)
IL-17F[3]	0.03 (0.00–0.12)
sST2	4567 (2714–6111)

Data are presented as numbers (n) of children and percentages (%).
[1]DNA samples were not available for two subjects.
[2]DNA samples were not available for 21 samples.
[3]The detection limit of assay was 3.1 pg/mL and presented concentrations are estimated values.

When the genotypes of study subjects were compared with serum IL-33 levels, we found that children who carry *TLR2* rs111200466 variants (ins/del or del/del) had significantly lower levels of serum IL-33 (0.00 pg/mL; Q1, Q3 0.00, 17.60) than those who carry ins/ins type of *TLR2* (19.81 pg/mL; IQR 0.00–51.78) (p = 0.0001) (Fig. 1).

Similar association was not observed between *TLR2* rs111200466 genotypes and levels of serum sST2 (Table 2). No significant differences were found between *TLR2* rs5743708 genotypes (GG and GA) and the studied serum cytokines or sST2 levels.

As seen in Fig. 2, the haplotype analysis with the *TLR2* rs111200466 and *TLR2* rs5743708 supported the finding that *TLR2* rs111200466 polymorphism was associated with low serum levels of IL-33 in early childhood. Subjects with ins/ins of *TLR2* rs111200466 and GG genotype of *TLR2* rs5743708 (haplotype A) had approximately the same levels serum IL-33 (19.81 pg/mL; IQR 3.10–51.11) as ins/ins – GA carriers (haplotype C) (25.21 pg/mL; IQR 5.81–72.35) (p_a = 0.999) (Fig. 2). Subjects with *TLR2* rs111200466 deletion (ins/del or del/del) and *TLR2 rs5743708* GG genotype (haplotype B) had a significantly lower levels of IL-33 (0.00 pg/mL; IQR 0.00–10.10 pg/mL) as compared to subjects with ins/ins – GG (haplotype A) (p_a = 0.001) and ins/ins – GA (haplotype C) (p_a = 0.039).

DISCUSSION

In this current study, we found that children with *TLR2* rs111200466 variant genotypes (ins/del or del/del) had significantly lower levels of serum IL-33 at age of 13 months compared with children with wild genotype (ins/ins). In contrast to IL-33, all other studied cytokines were under the detection limit with the exception of few subjects. However, the trend of differences between cytokine levels was similar between children with variant and wild genotypes when compared the estimated values of the IL-17A, IL-17F and IL-31. Similar association was not observed in these children between *TLR2* rs111200466 genotypes and levels of serum sST2. The results of this study did not show that *TLR2* rs5743708 genotypes (GG and GA) would have an effect on serum cytokines or sST2 levels.

TLR2 is one of most studied human TLR, and it recognizes diverse ligands of pathogens. The activation of TLR2 signalling pathway has been shown

Table 2. Serum concentrations of four cytokines and sST2 in 121 healthy Finnish children at age of 13 months and their relation to the TLR2 polymorphisms

Cytokine	*TLR2 rs111200466*		p-value	*TLR2 rs5743708*		p-value
	Median (IQR) pg/mL			Median (IQR) pg/mL		
	Ins/ins (n = 94)	Ins/del or del/del (n = 28)		AA (n = 109)	GA (n = 11)	
IL-33	19.91 (0.00–52.56)	0.00 (0.00–17.53)	0.001[1]	14.59 (0.00–47.07)	27.05 (6.29–91.83)	0.103
IL-31[2]	0.09 (0.00–0.28)	0.00 (0.00–0.06)	0.002	0.05 (0.00–0.21)	0.1 (0.03–0.45)	0.143
IL-17A[2]	0.64 (0.00–2.30)	00.00 (0.00–0.26)	0.001	0.19 (0.00–1.64)	1.46 (0.00–2.40)	0.138
IL-17F[2]	0.07 (0.00–0.17)	0.00 (0.00–0.07)	0.011	0.03 (0.00–0.12)	0.15 (0.02–0.23)	0.057
sST2	4412.50 (2735–6215)	3975.00 (2222–5355)	0.334	4240.00 (2650–6045)	4390.00 (2475–5337)	0.838

[1]Data were analysed by Mann–Whitney *U*-test and two-tailed p < 0.05 was considered significant.
[2]The detection limit of assay was 3.1 pg/mL and presented concentrations are estimated values.

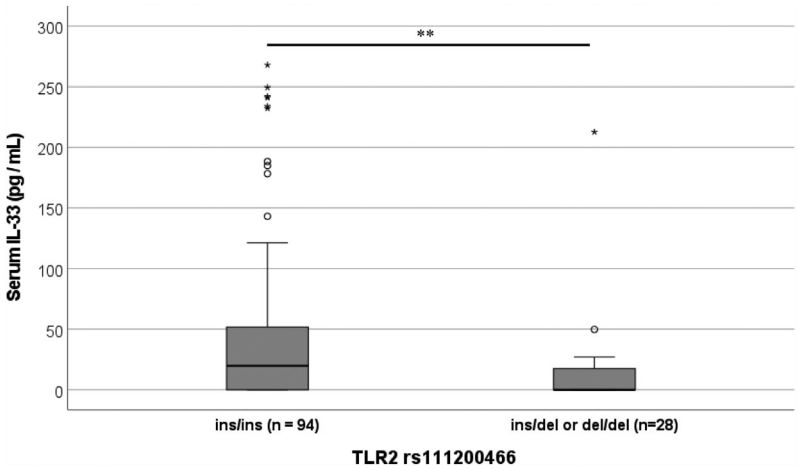

Fig. 1. Comparison of serum IL-33 at 13 months of age between children with and without *TLR2* rs111200466 deletion. The children with the *TLR2* deletion had significantly lower level of serum IL-33 (0.00 pg/mL; IQR 0.00–17.60) than those with ins/ins type of TLR2 (19.81 pg/mL; IQR 0.00–51.78) (p = 0.0001**). Data were analysed by Mann–Whitney *U*-test and two-tailed $p < 0.05$ was considered significant. Outlier values that are more than 1.5 × IQR below Q1 or above Q3 are represented by circles (°) and outlier values that are more than 3.0 × IQR below Q1 or above Q3 are represented by asterisks (*).

increase the production of several cytokines like IL-13 (20) and IL-33 (21). Polymorphisms of *TLR2* gene have many functional effects on several infectious (3) and autoimmune diseases (4).

TLR2 rs5743708 is well studied nonsynonymous polymorphism of *TLR2* gene, which has shown to reduce TLR2-mediated signalling and affect the alternative susceptibility to different infectious disease (3). Within this study, we could not find any associations between the polymorphism and serum levels of IL-33 or sST2. Our finding was in line with a previous study carried out among Finnish adults with BCG osteitis in infancy (22), in which no association was observed between *TLR2* rs5743708 genotypes and serum concentrations of 11 studied cytokines including IL-17A and IL-17F. It seems that even though this polymorphism reduces TLR2 signalling it appears to have no effect on cytokine production in healthy children.

Another studied polymorphism was a 23-bp deletion in promoter area of *TLR2* rs111200466. The frequency of different genotypes observed in this study (Table 2) is nearly equal to those reported in Genome 1000 project for Finnish population.

To date, little is known about the function of *TLR2* rs111200466 polymorphism. Two recent studies with colorectal cancer tissues showed that individuals with variant genotypes del/del or ins/del have higher mRNA expression of *TLR2* gene than those with ins/ins genotype (8, 9). Greene et al. showed that human monocytes-derived macrophages from subjects with ins/del genotype had significantly diminished TLR2 levels compared with those from subjects with ins/ins genotype when stimulated with the TLR2 ligand Pam3cys (23).

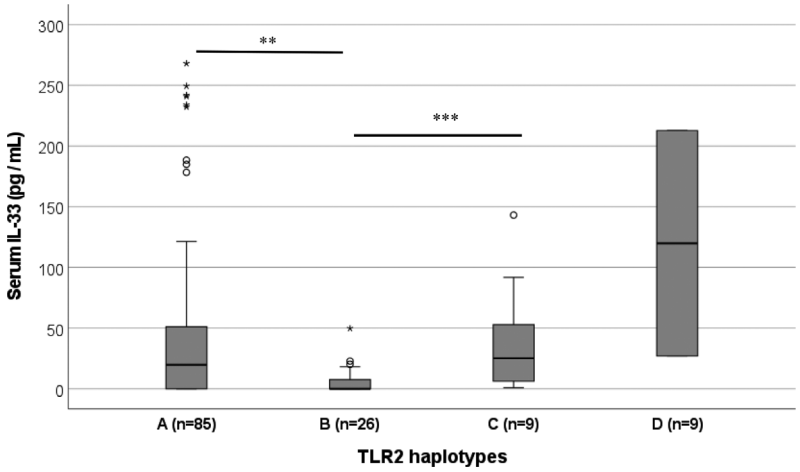

Fig. 2. Comparison of serum IL-33 at 13 months and *TLR2* haplotypes *TLR2* rs111200466 and rs5743618. Haplotypes include A = ins/ins and AA, B = ins/del or del/del and AA, C = ins/ins and GA and D = Ins/del or del/del and AG. Data were analysed by Mann–Whitney U-test and two-tailed $p < 0.05$ was considered significant. The significance values (p_a) have been adjusted by the Bonferroni correction of multiple tests. **p_a = 0.001 and ***p_a = 0.039. Outlier values that are more than 1.5 × IQR below Q1 or above Q3 are represented by circles (°) and outlier values that are more than 3.0 × IQR below Q1 or above Q3 are represented by asterisks (*).

Moreover, Noguchi *et al*. reported that the constructs with *TLR2* rs111200466 del have reduced luciferase activity compared with the ins/ins type constructs (21). Altogether, these findings indicate that the polymorphism of *TLR2* rs111200466 influences expression of TLR2. Our study further indicated that the *TLR2* rs111200466 polymorphism can influence the production of certain cytokines after its activation.

IL-33 is a multifunctional tissue-derived nuclear cytokine from the IL-1 family and is abundantly expressed in endothelial cells, epithelial cells and fibroblast-like cells, during homeostasis and inflammation. Together with sST2, it regulates Th2-type immunity (10). Recent studies have highlighted their role in the development of asthma in humans and animal models (12, 24).

It seems that especially perinatal activation of IL-33 pathway is crucial for development of asthma at young age. De Kleer et al. found that during the alveolar period, young children have highly increased production of the epithelial cytokine IL-33, which boosts the function of group 2 innate lymphoid cells (ILC2s) and further promote tissue remodelling, repair and homeostasis (25). The promotion of Th2 immunity reduces the threshold to allergens and increases the risk for childhood asthma. In addition, it has been shown that numbers of ILC2 and eosinophils directly depend on IL-33 signalling (26), which indicates that genetic factors that regulates IL-33 secretion may play a crucial role in the development of asthma (27).

It is well known that colonization of certain bacteria in nasopharynx (28), lower respiratory tract infections (29), together with environmental and genetic factors increases the risk for childhood asthma (30). With this same STEPS study cohort, we have previously shown that children who developed asthma at age of 7 years were more often colonized with gram negative bacteria such as *Moraxella catarrhalis* or *Haemophilus influenza* in early childhood (31). In the study that included all the children enrolled in the STEPS study, Toivonen et al. found that recurrent acute respiratory infections (ARIs) and wheezing illnesses, hospitalization for wheezing and wheezing caused by either rhinovirus (RV) or respiratory syncytial virus (RSV) at first 2 years of life increased risk for childhood asthma (32).

RV is one of the most prevalent human pathogens that usually causes mild upper respiratory tract symptoms (33), while RSV is one of the main causes of bronchiolitis in infants and small children (34). Both RV and RSV infections seem to increase the production of IL-33 mediated by TLR2 and TLR3 signalling pathway (35). Recently, Zhang et al. showed that infants with acute bronchiolitis caused by RSV, had significantly elevated levels of IL-33 and their Th1/Th2 ratio was imbalanced (36). However, previously Zhang et al. studied infants diagnosed with severe RSV bronchiolitis and they did not found differences in sputum IL-33 levels in infants who later developed recurrent wheezing than in those who did not (37).

In our previous study, with the same STEPS Study cohort, we did not find the relationship between asthma development at 7 years and serum IL-33 concentration at age of 13 months. However, low serum soluble ST2 level in early childhood seems to be associated with the risk for asthma at 7 years of age (16).

The number of study subjects is low, which is a limitation of the study and may partly explain why we did not observe significant difference in serum IL-33 levels between children who later developed asthma and those who did not. Therefore, further studies with the larger number of subjects are needed to evaluate the relationship between serum IL-33 levels and *TLR2* deletion rs111200466 and their relation to the development of childhood asthma. Another limitation is the long-term storage of serum samples studied, which might affect low concentrations of certain serum cytokines. It should be kept in mind that these study subjects were healthy children when the blood samples were collected.

In conclusion, our findings showed that *TLR2* rs111200466 polymorphism has an effect on serum IL-33 levels in early childhood, suggesting that children with the polymorphism may have impaired production of IL-33 and thus contribute to the protection of asthma development. To our knowledge, this is the first time to describe the correlation between *TLR2* rs111200466 polymorphisms and serum cytokine levels.

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