Published in final edited form as:

Hum Genet. 2011 October ; 130(4): 547–561. doi:10.1007/s00439-011-0977-x.

The role of polymorphisms in Toll-like receptors and their associated intracellular signaling genes in measles vaccine immunity

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Abstract

Toll-like receptors (TLRs) and their intracellular signaling molecules play an important role in innate immunity. In this study, we examined associations between polymorphisms in TLR family genes and measles vaccine-specific immune responses. We genotyped 764 subjects (11–22 years old) after two doses of measles vaccine for TLR signaling SNP markers (n = 454). The major alleles of coding SNPs in the TLR2 (rs3804100) and TLR4 (rs5030710) genes were associated with a dose-related increase (660 vs. 892 mIU/ml, p = 0.002) and a dose-related decrease (2,209 vs. 830 mIU/ml, p = 0.001) in measles-specific antibodies, respectively. A significant association was found between lower measles antibody levels and the haplotype ACGGCGAGAAAAGAGAAGAGAGAGAA (p = 0.01) in the MAP3K7 gene. Furthermore, the minor allele of a SNP (rs702966) of the KIAA1542 (IRF7) gene was associated with a dose-related decrease in IFN- γ Elispot responses (38 vs. 26 spot-forming cells per 2×10^5 PBMCs, p = 0.00002). We observed an additional 12 associations (p < 0.01) between coding (nonsynonymous and synonymous) polymorphisms within the TLRs (TLR 2, 7, and 8), IKBKE, TICAM1, NFKBIA, IRAK2, and KIAA1542 genes and variations in measles-specific IL-2, IL-6, IFN- α , IFN- γ , IFN λ -1, and TNF- α secretion levels. Our data demonstrate that polymorphisms in TLR and other related immune response signaling molecules have significant effects on measles vaccine-associated immune responses. These data help to establish the genetic foundation for immune response variation in response to measles immunization and provide important insights for the rational development of new measles vaccines.

Keywords

TLRs; Immunogenetics; Measles vaccine; Antibodies; Cytokines; Immune response

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Disclosures

Dr. Poland is the chair of a safety evaluation committee for novel non-measles vaccines undergoing clinical studies by Merck Research Laboratories. Dr. Jacobson serves on a Safety Review Committee for a post-licensure study of Gardasil for Kaiser-Permanent.

Introduction

In recent years, the contribution of TLRs and their associated intracellular signaling molecules to anti-viral immunity have been well documented (Barton 2007; Boehme and Compton 2004; Rassa and Ross 2003). Paramyxovirus (measles), poxvirus, herpesvirus and retrovirus families activate human T-cells via TLRs to elicit anti-viral innate immune responses (Bieback et al. 2002; Boehme and Compton 2004; Bowie and Haga, 2005; Murabayashi et al. 2002). Pathogen recognition likely plays a significant role in determining the outcome of the host response, including vaccine-induced immune responses (Hahm et al. 2006). In particular, viral detection by host TLRs can be controlled by genetic polymorphisms in the interaction regions between viruses and receptors.

Many studies have confirmed that genetic factors are involved in vaccine-induced immunity, including measles (Poland et al. 2008). For example, polymorphisms in human immune response genes influence inter-individual variations in humoral and cell-mediated immune (CMI) responses to measles vaccine, including HLA class I, class II and non-HLA genes (Poland et al. 2007). Our preliminary studies demonstrated significant associations between SNPs in TLR 2–6 and downstream associated signaling molecule genes (MD2 and MyD88) and variations in immune responses to measles vaccine (Dhiman et al. 2008). The role of TLRs and measles virus (MV) receptors are closely connected, with CD46 and SLAM serving as MV binding/entry receptors, and TLRs (TLR 3, 7 and 9) serving as pattern recognition sensor receptors that stimulate cytokine production and subsequently adaptive immunity (Boehme and Compton 2004; Murabayashi et al. 2002). Several SNPs located in the coding and the regulatory regions of the TLRs and their associated intracellular signaling genes have been analyzed, but no consistent association between these allelic variants and immune responses to measles have been detected to date (Dhiman et al. 2008). To examine novel polymorphisms, we performed an independent study with a new cohort of subjects. We hypothesized that SNPs within the TLRs and their downstream intracellular signaling molecules are associated with variations in humoral and cellular immune responses to measles vaccine.

Materials and methods

Study cohorts

Our study cohort comprised a combined sample of 816 subjects from 2 independent age-stratified random cohorts of healthy schoolchildren and young adults from all socioeconomic strata in Rochester, MN. In December 2006–August 2007, we enrolled 440 healthy children (age 11 to 19 years, cohort 1). A detailed description of study cohort 1 has been published elsewhere (Ovsyannikova et al. 2010; Dhiman et al. 2010; Haralambieva et al. 2010). Three hundred ninety-six parents allowed their children to take part in the current study, and from these 388 children we obtained a blood sample. In November 2008–September 2009, we enrolled an additional 376 healthy children and young adults (age 11 to 22 years, cohort 2). All 764 participants had documentation of having received two doses of measles-mumps-rubella (MMR, Merck) vaccine. The Institutional Review Board of Mayo Clinic approved the study, and we obtained permission through written informed consent from the parents of all children who participated in the study, as well as assent from age-appropriate children.

Plaque reduction microneutralization assay (PRMN)

Measles-specific neutralizing antibody levels were quantified using a high throughput fluorescence-based PRMN, as described (Haralambieva et al. 2008). Heat-inactivated sera were diluted four-fold from 1:4 to 1:4,096 (6 replicates for each dilution) in Opti-MEM

(Gibco, Invitrogen), mixed with an equal volume of low passage challenge virus MVEGFP and incubated for 1 h at 37°C. A standard inoculum of challenge virus was used in Opti-MEM at a dilution adjusted to yield 20–60 plaque forming units per well in the control wells with virus only. Serum/virus mixtures were transferred to a new 96-well plate and mixed with an equal volume of Vero cells in DMEM with 10% FBS (HyClone). The fluorescent green plaques (syncytia) were scanned and counted on an automated Olympus IX71 fluorescent microscope using the Image-Pro Plus Software version 6.3 (MediaCybernetics). The 50% end point titer (neutralizing dose, ND₅₀) was calculated using Karber's method. The use of the 3rd WHO international anti-measles standard (3,000 mIU/ml, NIBSC code no. 97/648) enabled quantitative ND₅₀ values to be transformed into mIU/ml (Haralambieva et al. 2008).

IFN- γ Elispot

IFN- γ Elispot responses were assessed using kits from R&D Systems (Minneapolis, MN), as described (Ryan et al. 2005). Seven wells were plated with 2×10^5 PBMCs per subject: 3 wells were supplemented with MV at an MOI of 0.5, 3 wells were supplemented with culture medium to serve as negative controls, and 1 well was supplemented with 5 μ g/ml of PHA. Plates were read with an ImmunoSpot-S4 ProAnalyzer (CTL, Cleveland, OH). The intraclass correlation coefficients (ICCs) comparing the multiple observations per subject were 0.94 for the stimulated values, and 0.85 for the unstimulated values, indicating reasonably high levels of measurement reliability.

Enzyme-linked immunosorbent assay (ELISA) for cytokines

ELISAs were performed to measure the level of seven [IL-2 (n = 739), IL-6 (n = 737), IL-10 (n = 740), IFN α (n = 734), IFN- γ (n = 737), IFN λ -1 (n = 738), and TNF- α (n = 732)] cytokines secreted by PBMCs following *in vitro* stimulation with MV, as described (Ovsyannikova et al. 2007). Eleven wells on three 96-well round bottom plates were plated with 2×10^5 cells/well in RPMI with 5% FCS. Five wells were supplemented with MV, 5 wells were supplemented with RPMI containing 5% FCS to serve as negative controls, and 1 well was supplemented with PHA. The MOI and incubation time for each cytokine were as follows: IL-2, MOI = 0.5, 48 hours; IL-6, MOI = 1.0, 72 hours; IL-10, MOI = 0.5, 48 hours; IFN- α , MOI = 1.0, 24 hours; IFN- γ , MOI = 1.0, 72 hours; IFN λ -1, MOI = 1.0, 72 hours; TNF- α , MOI = 1.0, 24 hours. Cytokines IL-2, IL-6, IL-10, IFN- γ , and TNF- α were measured using kits from BD Biosciences (San Jose, CA), TNF- α was measured using kits from Mabtech (Cincinnati, OH), and IFN λ -1 was measured using kits from R&D Systems. Cytokine-specific ICCs ranged from 0.65 (IL-2, unstimulated values) to 0.94 (IFN- α and IL-6, stimulated values).

TagSNP selection

A panel of SNPs from TLRs (TLR 2-9) and their associated intracellular signaling molecules [myeloid differentiation protein-2 (LY96 or MD2), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (NFKB1 or NFkB), TNF receptor-associated factor 6 (TRAF6), myeloid differentiation primary response gene (MYD88), CD14 molecule, interleukin-1 receptor-associated kinase (IRAK1-4), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA or MAD3), inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (IKBKE or IkK-i), toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), conserved helix-loop-helix ubiquitous kinase (CHUK or IkK-a), TANK-binding kinase 1 (TBK1), jun oncogene (JUN), mitogen-activated protein kinase kinase kinase 7 (MAP3K7 or TAK1), mitogen-activated protein kinase kinase kinase 7 interacting protein 2 (MAP3K7IP2 or TAB2), TLR adaptor molecule 1 (TRIF or TICAM1), and KIAA1542 (IRF7 downstream

molecule)] formed the basis of this study. SNPs within each gene and 5kb upstream and downstream, were selected based on the linkage disequilibrium (LD) tagSNP selection algorithm (Carlson et al. 2004) from the Hapmap Phase II (<http://www.hapmap.org>), Seattle SNPs (<http://pga.mbt.washington.edu/>) and NIEHS SNPs (<http://egp.gs.washington.edu/>). For each gene, we selected tagSNPs with a minor allele frequency (MAF) ≥ 0.05 and successful Illumina predictive genotyping scores based on a pairwise LD threshold of $r^2 \geq 0.90$ in both the Caucasian and African public source samples using ldSelect (Carlson et al. 2004).

Genotyping methods

Four hundred fifty-four SNPs from the candidate genes ($n = 26$) were included in the two custom Illumina GoldenGate SNP panels (San Diego, CA) for 1,536 and 768 SNPs. Of the 454 SNPs considered, 25 failed the laboratory QA because of failure to amplify, poor clustering, or low call rates. An additional 44 SNPs were excluded due to low minor allele frequencies ($MAF < 0.05$), yielding a total of 385 SNPs available for analysis. Genotype concordance of the duplicated subjects was 100%, and no Mendelian errors were noted in the Coriel CEPH trios. Nineteen of the 764 eligible subject's samples failed because of insufficient/inadequate DNA quality, genotyping failure or low call rates, leaving 745 subjects for analysis.

Statistical analysis

The statistical methods described herein are similar to those carried out for our previous genetic association publications (Ovsyannikova et al. 2010; Dhiman et al. 2010; Haralambieva et al. 2010). Briefly, we assessed associations between genetic variation in candidate SNPs from TLRs and genes coding their associated intracellular signaling molecules, and measures of measles vaccine immunity. We examined seven measures of MV-specific cytokine secretion (IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN λ -1, and TNF- α , each reported in units of pg/ml); a measure of CMI via IFN- γ PBMC responses (evaluated as a count variable using Elispot); and levels of measles-specific antibodies (measured in mIU/ml). Assessments of cytokine secretion and CMI resulted in multiple recorded values per outcome, both prior to stimulation with MV and post-stimulation. For descriptive purposes, a single response measurement per individual was obtained for each outcome by subtracting the median of the unstimulated values from the median of the stimulated values. Assessments of antibody levels resulted in only one recorded value per individual. These summary measures were descriptively summarized across individuals using frequencies and percentages for categorical variables, and medians and inter-quartile ranges for continuous variables.

Observed genotypes were used to estimate allele frequencies for each SNP and departures from Hardy-Weinberg equilibrium (HWE) were assessed using either a Pearson goodness-of-fit test or, for SNPs with a $MAF < 5\%$, a Fisher exact test (Weir 1996). Estimates of pairwise linkage LD based on the r -squared statistic were obtained using Haploview software, version 3.32 (Barrett et al. 2005).

SNP associations with immune response outcomes were individually evaluated using linear regression models. Simple linear regression was used for MV antibody levels, which had only one measured value per individual. Repeated measures approaches were implemented for the cytokine secretion and Elispot variables, simultaneously modeling the multiple observed measurements. This was achieved by including the genotype variable in the regression model, together with a variable representing stimulation status, and testing the statistical significance of the corresponding genotype-by-stimulation status interaction term. In these models, we allowed for within-subject correlations without imposing any

constraints on their structure within a person. Primary tests of association assumed an ordinal (log-additive) SNP effect.

To further explore genomic regions containing statistically significant single-SNP effects for one or more outcomes of interest, we performed post-hoc haplotype analyses. Posterior probabilities of all possible haplotypes for an individual, conditional on the observed genotypes, were estimated using an expectation-maximization algorithm, similar to the method outlined by Schaid *et al.* (2002). This information was used to define haplotype design variables that reflected the number of each of the haplotypes that were expected to be carried by each subject. Analyses were performed on these haplotype design variables using the simple least squares regression approach for antibody levels and the repeated measures approach for the cytokine secretion described above. Because of the imprecision involved in estimating the effects of low-frequency haplotypes, we considered only those occurring with an estimated frequency of greater than 1%. Differences in immune response among all common haplotypes were first assessed simultaneously via a global test. Following these global tests, we examined individual haplotype effects in the spirit of Fisher's protected least significant difference test; individual associations were not considered statistically significant in the absence of global significance. Due to phase ambiguity, haplotype-specific medians and inter-quartile ranges could not be calculated. Thus, descriptive summaries were represented using the t-statistics corresponding to the haplotype main effect term for antibody levels or the haplotype-by-stimulation status interaction term for cytokine secretion.

All association analyses adjusted for age at enrollment, race, gender, age at first measles vaccination, age at second measles vaccination, and cohort status (cohort 1 vs. cohort 2) to account for their potential impact on the measured immune responses. Data transformations were used to correct for data skewness in all linear regression models. An inverse normal transformation was used for all cytokine secretion and Elispot outcome variables, and a log transformation was used for the antibody response measure. All statistical tests were two-sided and, unless otherwise indicated, all analyses were carried out using the SAS software system (SAS Institute, Inc., Cary, NC).

Results

Subjects demographics and immune responses

Eighty percent of the study subjects were Caucasians ($n = 598$, 80.3%), 11.9% ($n = 89$) were African-Americans, with 44% ($n = 328$) female, and a median age at enrollment of 15 (IQR 13; 17) years. The median age at the first and second immunization were 15 (IQR 15; 16) months and 5 (IQR 4; 11) years, respectively, and the median time between last measles immunization and sample draw was 7.5 (IQR 5.6; 9.2) years. Median values (25th, 75th percentiles) for MV neutralizing antibodies in the subjects were 846 mIU/ml (IQR 418; 1,772). Median values for MV-specific IFN- γ Elispot counts for spot-forming cells (SFC per 2×10^5 cells) were 36 (IQR 12; 69). Further, median values for MV-specific IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN- λ -1 and TNF- α cytokine secretions were 38 (IQR 21; 64) pg/ml, 355 (IQR 248; 461) pg/ml, 18 (IQR 11; 28) pg/ml, 551 (IQR 273; 1,025) pg/ml, 67 (IQR 35; 120) pg/ml, 34 (IQR 14; 74) pg/ml, and 14 (IQR 9; 19) pg/ml, respectively.

Associations between SNPs in TLR with associated intracellular signaling molecules and antibody response

Genotyping data were analyzed for the combined cohort of subjects ($n = 745$) and separately for Caucasians ($n = 598$). We also performed a secondary analysis of data collected for the African-American subgroup ($n = 89$). Overall, 16 SNPs were significantly associated ($p <$

0.01) with variations in MV-induced neutralizing antibodies. Six of these SNPs were positioned in the coding and regulatory regions of the TLR2, TLR4, MAP3K7 (TAK1), and TRAF6 genes (Table 1). In the combined cohort of subjects major allele variants AA of two coding synonymous SNPs (rs5030710 and rs3804100) of the TLR4 and TLR2 genes were associated with an allele dose-related decrease (2,209 vs. 830 mIU/ml, $p = 0.001$) and an allele dose-related increase (660 vs. 892 mIU/ml, $p = 0.002$) in measles-specific antibody response, respectively. We found significant associations between two promoter SNPs (rs711264, 970 vs. 613 mIU/ml, $p = 0.001$ and rs806287, 919 vs. 612 mIU/ml, $p = 0.005$; $r^2 = 0.99$) located in the MAP3K7 gene on chromosome 6q16.1 and dose-related decreases in measles antibodies. As expected, in the Caucasian and African-American subgroups we found associations for the promoter and coding SNPs in the MAP3K7 and TLR4 genes (rs711264 and rs5030710), respectively. Specifically, increased carriage of minor allele A for rs711264 located in the promoter region of the MAP3K7 gene, was associated with a dose-related decrease in antibodies (974 vs. 612 mIU/ml, $p = 0.006$) in the Caucasian subgroup. Major allele variant A for a coding SNP (rs5030710; 624 vs. 1,729 mIU/ml, $p = 0.009$) in the TLR4 gene was associated with an allele dose-related increase in antibodies in the African-American subgroup.

Further, five MAP3K7 haplotypes with frequencies $\geq 1\%$ in the combined cohort of subjects were identified (Table 2). A haplotype analysis revealed marginally significant associations between measles antibodies and the MAP3K7 haplotypes (global p -value 0.082). Specifically, the common MAP3K7 haplotype ACGGCGAGAAAAGAGAAGAGAGAGAA was associated with lower (t-statistic -2.52, $p = 0.012$) measles-specific antibodies, while another common haplotype GCAGGAGGCGATGGGAAGGAAGGCG demonstrated suggestive association with higher (t-statistic 1.71, $p = 0.087$) antibody levels.

Associations between SNPs in TLR with associated intracellular signaling molecules and IFN- γ Elispot response

Overall, we found 7 SNPs located in coding and regulatory regions significantly associated ($p < 0.01$) with variations in MV-specific IFN- γ Elispot responses (Table 3). Three significant associations were found with polymorphisms in the KIAA1542 (IRF7) gene region, TLR7 and IKBKE genes and measles-specific IFN- γ Elispot responses in the combined cohort. Specifically, minor allele G for a promoter SNP (rs702966) of the KIAA1542 gene was associated with a dose-related decrease in IFN- γ Elispot responses (38 vs. 26 SFC per 2×10^5 PBMC, $p = 0.00002$). Analysis in the Caucasian subgroup revealed two significant associations between promoter SNPs (rs2760501, $p = 0.001$ and rs2984915, $p = 0.004$; $r^2 = 0.93$) belonging to the JUN (AP-1) gene on chromosome 1p32-p31 and an allele dose-related decrease in IFN- γ Elispot response to MV. In our secondary analysis of the African-American subjects, we identified two potential associations between promoter SNPs belonging to the MAP3K7 (TAK1) and NFKB1 (NFkB) genes (rs157693, $p = 0.001$ and rs7674640, $p = 0.006$) and IFN- γ Elispot responses after measles vaccination.

Associations between SNPs in TLR with associated intracellular signaling molecules and secreted cytokines

Fifty-eight statistically significant associations ($p < 0.01$) with variations in measles-specific IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN λ -1 and TNF- α secretions were observed (Table 4). In a combined cohort of subjects, increased carriage of major allele G for nonsynonymous rs3748022, Pro713Leu ($p = 0.005$) and minor allele G for rs1786704 ($p = 0.009$) located in the coding and 3'UTR regions of the IKBKE (IkK-i) and TIRAP genes, respectively, were associated with a dose-related decrease in IL-2. In the Caucasian subgroup we observed reproduced associations, already identified in the combined cohort for the coding and

3'UTR SNPs in the IKBKE and TIRAP genes (rs3748022, $p = 0.002$ and rs1786704, $p = 0.005$), respectively. In the African-American subgroup, the heterozygous variant for coding synonymous SNP (rs7255265, 12A>G, $p = 0.009$) in the TICAM1 (TRIF) gene resulted in significantly lower measles-specific IL-2 production as compared to the homozygous variants.

Analysis in the combined cohort of subjects revealed two significant associations between coding and 3'UTR SNPs (rs5744081, 1312C>A, $p = 0.003$ and rs5979763, $p = 0.003$; $r^2 = 1$) belonging to the TLR8 gene and an allele dose-related decrease in IL-6 secretion. Minor allele A for a promoter SNP (rs6995910, $p = 0.008$) from the LY96 (MD2) gene was associated with variations in IL-6 secretion. None of these SNPs demonstrated associations with IL-6 response in the subgroup analysis for Caucasian subjects, whereas TLR3 promoter SNP (rs5743309, 330 vs. 230 pg/ml, $p = 0.009$) was found to be associated with a decrease in IL-6 in an allele dose-dependent manner.

Four significant associations ($p < 0.004$) were observed between promoter region SNPs located in the TLR4 (rs7044464 and rs7856729, $r^2 = 1$), TLR6 (rs6815827) and IKBKE (IkK-I, rs6540431) genes, respectively, and variations in IL-10 secretion in the combined cohort. Four additional associations were observed (range of p -values 0.0003 to 0.002) between 3'UTR region SNPs located in a LD block, all belonging to the TLR3 (rs4862633 and rs10025405, $r^2 = 0.96$) and (rs1519312 and rs4608848, $r^2 = 0.91$) genes, and variations in IL-10 secretion in the Caucasian subgroup. The Caucasian subgroup demonstrated significant associations (already identified in the combined cohort) for two promoter SNPs in the TLR4 gene (rs7044464 and rs7856729, $p = 0.0008$, $r^2 = 1$) with MV-specific IL-10 (Table 4).

The combined cohort demonstrated 5 significant SNP associations with variations in measles-specific IFN- α secretion in promoter SNPs belonging to the genes NFKBIA (rs2233409, $p = 0.001$, rs3138052, $p = 0.002$, rs1050851/coding, $p = 0.004$ and rs3138053, $p = 0.008$; $r^2 = 0.97$) and TLR4 (rs10759932, $p = 0.001$). Of note, coding SNP rs1050851 in the NFKBIA (MAD3) gene was associated (734 vs. 530 ng/ml, $p = 0.004$) with IFN- α production in an allele dose-dependent manner. The Caucasian subgroup demonstrated an association (already identified in the combined cohort) for the 5'UTR SNP of the NFKBIA gene (rs3138052, $p = 0.007$). The African-American subgroup demonstrated 2 significant associations between promoter region SNPs belonging to the IRAK3 (rs11465990, $p = 0.001$ and rs11176082, $p = 0.005$) signaling pathway and IFN- α secretion in an allele dose-dependent manner.

SNP associations with MV-specific IFN- γ secretion were also examined. Increased representation of major allele C for a coding SNP of the IRAK2 gene (rs9854688, 66 vs. 70 pg/ml, $p = 0.006$) demonstrated a significant association with MV-specific IFN- γ secretion in an allele dose-dependent manner. The Caucasian subgroup revealed 2 significant associations (range of p -values 0.002 to 0.009 and already identified in the combined cohort) in promoter SNPs, belonging to genes of the TLR4 (rs10759930, $p = 0.002$ and rs10759932, $p = 0.004$) and 1 additional promoter SNP (rs1786704, $p = 0.009$) belonging to the TIRAP gene with measles-specific IFN- γ secretion. Additionally, the Caucasian subgroup demonstrated one significant association between a coding SNP in the KIAA1542 (rs11246212, Val1448Ala, $p = 0.009$) gene and secreted of IFN- γ in an allele dose-dependent manner.

Four significant SNP associations with variations in measles-specific IFN- λ -1 secretion were found in the combined cohort of subjects. Two of them were with coding SNPs, belonging to the TLR genes: TLR2 (synonymous rs3804099, 597T>C, $p = 0.002$) and TLR8

(rs5744077, 28A>G, $p = 0.003$). Analysis of the Caucasian subgroup revealed 2 significant associations in 5'UTR SNPs, belonging to TLR7 (rs2897827, $p = 0.005$) and NFKBIA (rs3138050, $p = 0.007$) and measles-specific IFN λ -1. A secondary analysis of the African-American subgroup revealed significant associations between minor alleles of three promoter SNPs located in the JUN (rs2984915, $p = 0.003$), NFKB1 (rs980455, $p = 0.003$) and TLR4 (rs7869402, $p = 0.009$) genes and variations in IFN λ -1 secretion.

Finally, in the combined cohort, two coding SNPs in the TLR7 (rs5741881, $p = 0.009$) and TLR8 (rs5744081, $p = 0.003$) genes were associated with variations in measles-specific TNF- α secretion. In our secondary analysis of the African-American subjects, we found associations between two coding SNPs (rs1050851, Gly94Ser, $p = 0.004$ and rs2292151, 4756719G>A, $p = 0.005$) located in the NFKBIA (MAD3) and TICAM1 genes, respectively, and TNF- α production.

Discussion

Associations between humoral and CMI responses to measles vaccine with polymorphisms of TLRs (i.e. pattern recognition proteins) and adapter molecules that interact with the TLRs, including intracellular signal transducing proteins were examined in this study. Multiple studies support the role of TLRs (TLR 2-9) in antiviral immunity (Bieback et al. 2002; Matsumoto et al. 2004; Schlender et al. 2005). Other studies established an increased expression of TLR 2, 3 and 4 following infection of dendritic cells (DCs) with both the wild-type and Edmonston vaccine strains of MV (Hahm et al. 2006; Murabayashi et al. 2002; Poland et al. 2007; Tanabe et al. 2003) inducing activation of TLR-downstream signaling pathways, such as IL-1 α/β , IL-6, IFN- α/β and IL-12p40, and upregulation of its own viral binding receptor-SLAM (Bieback et al. 2002; Murabayashi et al. 2002). Measles nucleoprotein has also been shown to stimulate TLR-associated signaling protein IRF-3, leading to IFN production (tenOever et al. 2002).

Previously, we reported that variations in immune response to MV are associated with polymorphisms in the TLR 2, 3, 4, 6, 7, 8 and other molecules implicated in TLR-downstream signaling pathways. None of the 11 identified SNP associations in our smaller pilot study of 190 subjects (Dhiman et al. 2008) were reproduced in this larger cohort of 764 subjects.

In this study, we observed that rs3804100 in the TLR2 gene on chromosome 4q32 appears to be an important coding SNP and is significantly associated with higher (1.5-fold for carriers of the AA genotype) measles neutralizing antibodies in an allele dose-related manner. Conversely, lower (3-fold for carriers of the AA genotype) antibody levels were associated with the major allele of rs5030710, suggesting a role for TLR4 in the antibody response to MV. Although these are synonymous SNPs they may affect mRNA splicing, stability, RNA structure or protein folding (Hunt et al. 2009). TLR2 is able to detect PAMPs from a variety of infectious agents, including viruses. The associations between TLR2 (rs3804100) and TLR4 (rs5030710) coding polymorphisms and measles neutralizing antibodies are important since upregulation of these TLRs after infection with Edmonston strains have been previously reported (Hahm et al. 2006; Murabayashi et al. 2002). TLR2, which signals via the NF κ B activation pathway, is highly expressed on human B-cells, whereas TLR3 and TLR4 signal through an adapter molecule (TRIF), which is not expressed in B-cells (Chiron et al. 2008). We identified an individual promoter SNP (rs5743309) in the TLR3 gene that appears to influence measles-specific IL-6 secretion levels in an allele dose-dependent manner in African-Americans. We also found four significant associations between SNPs (rs4862633 and rs10025405, $r^2 = 0.96$; and

rs1519312 and rs4608848, $r^2 = 0.91$) belonging to the TLR3 gene and variations in IL-10 secretion in the Caucasian subgroup.

Our data, biologic plausibility, and the previously discussed studies all point to the importance of the TLR-initiated immune response pathway. Understanding the functional role of polymorphisms of TLR 2-4 genes and their ligands could contribute to the design of novel therapeutic and diagnostic approaches employing TLR agonists, TLR antibodies and soluble TLRs in vaccine development. Our data provide evidence for associations of polymorphisms in promoter regions of MAP3K7 (rs711264 and rs806287) and TRAF6 (rs331449) genes with measles antibodies in an allele dose-dependent manner. An allele dose-related decrease (1.5-fold) in measles antibody was observed with increased representation of the minor alleles for MAP3K7 gene SNPs rs711264 and rs806287. MAP3K7 or TAK1 is a part of the serine-threonine protein kinase group and is involved in proinflammatory cytokine signaling. TAK1 activates nuclear factor kappa B (NFkB) and two kinase pathways: the MAPK cascade that promotes activation of mitogen-activated protein kinase 8 (MAPK8) and the Ikb kinase cascade that controls the transcription of genes with anti-viral activity (Yu et al. 2008). It has also been demonstrated that TRAF6 is a signal protein that stimulates Ikb kinase (IKK) and MAPK8 (JNK) in response to the proinflammatory cytokine IL-1 (Kishida et al. 2005). However, data on TRAF6 protein activity against MV in humans are lacking. We speculate that polymorphisms in the MAP3K7 and TRAF6 genes may contribute to MV-induced humoral immunity signaling through an as yet unclear mechanism.

We have also identified significant associations between CMI responses to MV and SNPs in TLR 2, 3, 4, 6, 7, 8 and TLR ligands and their signaling molecules, such as KIAA1542, IKBKE, JUN, MAP3K7, NFKB1, TICAM, NFKBIA, TIRAP, IRAK, MyD88, and Ly96 genes. Study cohort and subgroup cohort analyses show significant associations with SNPs in the KIAA1542, TLR7, IKBKE, JUN, and NFKB1 genes and IFN- γ Elispot responses to MV. In this regard, several pathways that support type I IFN production and the initiation of anti-viral IFN response include the TLR7, IKBKE, NFKB1, and IRF-mediated pathways (Meylan and Tschopp 2006). The KIAA1542 (previously known as PHRF1) gene is located on chromosome 11p15 downstream in the signaling TLR/IRF7 pathway, and is a key transcription factor in the type I IFN (IFN- α/β) anti-viral response (Harley et al. 2008). Analysis of our combined cohort demonstrates a strong association ($p = 2.28 \times 10^{-5}$) between promoter SNP rs702966 located in the KIAA1542 gene on chromosome 11p15.5 and IFN- γ Elispot responses.

We found fifty-eight associations ($p < 0.01$) between coding and promoter SNPs in the TLR genes and their associated intracellular signaling molecules and MV-specific (IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN- λ -1 and TNF- α) cytokine responses. TLR-virus interactions are triggered by trans-membrane TLRs on the cellular surface (TLR 2, 4) and within endosomal and endoplasmic reticulum intracellular compartments (TLR 3, 7, 8, 9) leading to activation of type I IFN and cytokine secretion through IRF and NFkB (NFKB1) (Finberg et al. 2007). TLRs have the C-terminal signal transduction domain TIR that is important for stimulation of common downstream adapter molecules (MyD88, TIRAP and TRIF) and activation of signaling pathways (IRAK, Ikb $\alpha/\beta/\gamma$, and TRAF6) (Finberg et al. 2007). We identified two coding SNPs in the nuclear factor kB (NFkB) pathway gene-*IKBKE* (rs3748022) and in the TLR3-mediated IFN- β induction pathway gene-*TICAM1* (rs7255265) that were associated with variations in IL-2 secretion. Despite its role in NFkB-mediated signal transduction, no studies assessing response to measles vaccine appear to have assessed host genetic variation in the IKBKE signaling pathway. Further study of the potential functional consequences of these SNPs might begin by examining the effect of the G or A allele on IKBKE and TICAM1 signaling pathways.

In considering the effect of genotype on phenotype, understanding the mechanistic importance of these identified SNPs is important in understanding outcomes from population-based immunogenetic vaccine studies. Thus, it is critical to determine the functional effect of a given gene variant, and to determine the possible resulting “downstream” immunologic consequences of that effect. Promising findings in our study worth further study include polymorphisms in the coding (rs5744081) and promoter (rs5979763) regions of the TLR8 gene, associated with an allele dose-related secretion of inflammatory IL-6 and TNF- α . Similarly, the TLR4 genetic variant rs10759932 was associated with an allele dose-related secretion of IL-10, IFN- α and IFN- γ . Another TLR4 polymorphism (rs10759930) demonstrated an association with secretion of IL-2, IFN- γ and IFN λ -1, an indication that the relationship between TLR4 and MV is important for the development of vaccine-specific immunity. This finding suggests that the TLR ligands and/or cytokines involved in the TLR4 and TLR8 signaling network may be factors modulating cellular (cytokine) immune responses to measles. Additionally, we identified a coding SNP (rs1050851) in the NFKBIA gene on chromosome 14q13 that appears to influence secretion of both IFN- α and TNF- α levels in the combined cohort of subjects and in African-American subjects, respectively. Evidence was also found for the possible regulation of IFN- γ Elispot response and IFN λ -1 secretion by a promoter SNP, rs2984915, in the AP-1 transcription factor gene known to regulate gene expression following stimulation with various stimuli, including cytokines and viral infections (Hess et al. 2004). Finally, a promoter SNP rs1786704 in the TIRAP gene demonstrated associations with both IL-2 and IFN- γ secretion in an allele dose-dependent manner in both our combined cohort and in the Caucasian subgroup. It is unclear whether polymorphisms in these genes directly affect cytokine secretion since significant associations have also been found with genetic variants in other immune response and/or neighbor genes, such as HLA, cytokine and cytokine receptor, OAS1, ADAR1, RNASEL, and RIG-1 genes.

Our study design is informed by expert reviews on conducting studies examining complex genetic traits (Gordon and Finch 2005; Motsinger et al. 2007) paying attention to concerns regarding the observation of chance associations related to the assignment of statistical significance when making hundreds to thousands of comparisons (Hattersley and McCarthy 2005; Hirschhorn et al. 2002). We examined associations between many genetic polymorphisms and multiple measures of measles immune response. In our assessments, we observed 30% more p-values below 0.01 than would be expected by chance (95% CI: 11% – 55%). Therefore, we expect that a good number of the associations reported here reflect true immune associations. However, additional studies are required to confirm our findings through replication, and identify which of these associations hold up in independent data sets.

There are some limitations to our study. We are studying schoolchildren and young adults (n = 764) who received their measles vaccination several years before participation in this study, and our neutralizing antibody, IFN- γ Elispot and secreted cytokine assays may not accurately reflect the level of immunity induced immediately following measles vaccine. Such levels do, however, reflect current levels of MV immunity and protection. We did not confirm the associations we reported in a smaller cohort of subjects (Dhiman et al. 2008). This may be a reflection that the earlier findings were false positives, identified in a small study set (n = 190). The current study was much larger, and had a much broader scope, and correspondingly higher power to detect true associations. In fact, the strength of some of the associations identified in this study suggests that some of these SNPs are appropriate candidates for replication.

In summary, our study provides evidence that variations in humoral and CMI responses to measles vaccine are influenced by genetic polymorphisms in the TLR genes (TLR 2, 4, 6, 7,

8) including TLR3, a receptor with a documented role in measles immunity, and their associated intracellular signaling molecules. These polymorphisms in TLR and other innate immune response signaling receptors have significant effects on measles vaccine-associated immune responses, giving confidence that these findings are true-positives. Additional studies should identify the actual causal polymorphisms via fine mapping and determine their role in inter-individual variability in response to measles vaccine. Such vaccine immunogenetic studies may also elucidate general principles for understanding the immune response to live viral vaccines. This is required for directed development of new vaccines against measles by ascertaining the genetic basis for vaccine-induced immune response variation (Poland et al. 2007; Vandenbroeck and Goris 2003).

Acknowledgments

We thank the Mayo Clinic Vaccine Research Group staff and subjects who participated in our studies. We thank Megan M. O'Byrne and Matthew J. Phan for their help with this manuscript. This work was supported by NIH grants AI 33144, AI 48793 and 5UL1RR024150-03 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health, and the NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH.

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Associations between SNPs located in coding and regulatory regions in TLR genes and their associated intracellular signaling molecules and MV-specific antibody responses

Table 1

Gene	SNP ID	Location/ function	Genotype ^a	N ^a	Median antibody titer mIU/ml (IQR) ^b	P value ^c
Combined cohort of subjects						
TLR4	rs5030710	coding Ser105Ser	AA	722	830 (418; 1,727)	0.0012
			AG	19	2,209 (477; 2,981)	
			GG	1	5,095 (5,095; 5,095)	
MAP3K7	rs711264	flanking 3'UTR	GG	384	970 (465; 1,845)	0.0013
			GA	290	777 (401; 1,646)	
			AA	59	613 (329; 1,334)	
TLR2	rs3804100	coding Ser450Ser	AA	652	892 (437; 1,829)	0.0018
			AG	90	660 (361; 1,478)	
			GG	2	381 (174; 587)	
TLR4	rs16906053	flanking 5'UTR	AA	705	828 (416; 1,715)	0.0046
			AG	29	1,810 (531; 3,730)	
			GG	10	1,828 (462; 2,556)	
MAP3K7	rs806287	flanking 3'UTR	AA	400	919 (440; 1,835)	0.0051
			AG	287	806 (402; 1,718)	
			GG	56	612 (339; 1,264)	
TRAF6	rs331449	flanking 5'UTR	AA	715	826 (418; 1,729)	0.0071
			AG	28	1,704 (492; 2,833)	
			GG	1	2,272 (2,272; 2,272)	
Caucasian subgroup						
MAP3K7	rs711264	flanking 3'UTR	GG	293	974 (440; 1,850)	0.0064
			GA	242	822 (402; 1,638)	
			AA	52	612 (356; 1,264)	

Gene	SNP ID	Location/ function	Genotype ^a	N ^a	Median antibody titer mIU/ml (IQR) ^b	P value ^c
African-American subgroup						
TLR4	rs5030710	coding Ser105Ser	AA	73	624 (251; 1,832)	0.0099
			AG	15	1,729 (477; 2,811)	
			GG	1	5,095 (5,095; 5,095)	

A-Adenine, C-Cytosine, G-Guanine, T-Thymine

A total of 385 SNPs were examined; only those found to be statistically significant (p = 0.01) in coding and regulatory regions were included in the table.

SNP rs16906053 in a combined cohort displayed violations of Hardy-Weinberg equilibrium (p = 2.84E-10).

^aValues are presented as homozygous major allele/heterozygous/homozygous minor allele.

^bIQR, interquartile range, values are median levels in mIU/ml as measured by plaque reduction microneutralization assay.

^cTest for trend p value from the linear regression analysis adjusting for age, gender, race and age of immunization. The p values are adjusted for age, gender, race (when necessary), age at 1st and 2nd MMR and cohort status (cohort 1 vs. cohort 2) using analysis of covariance methodology.

Table 2

MAP3K7 haplotype associations with MV-specific antibody responses

MAP3K7 Haplotype ^a	Frequen cy	Back- transformed mean (mIU/ml)	Back- transformed 95% CI (mIU/ml)	Test statistic (haplotype t-statistic)	Allele P value ^b	Global P value
ACGGCGAGAAAGAGAGAGAGAA	0.248	777.6	(711.0; 850.6)	-2.52	0.012	0.082
GCAGGAGGCGATGGGGAGGAGGCG	0.274	875.2	(804.0; 952.7)	1.71	0.087	
GGAAACAACCAATAGAAAGGGGAGAA	0.018	812.6	(587.9; 1123.1)	-0.21	0.830	
GGAAACAACCAATAGAAAGGGGAGAA	0.273	861.3	(790.4; 938.6)	1.36	0.173	
GGAGCGAGCAATGAGAGGGGAGAA	0.062	787.7	(651.8; 952.0)	-0.55	0.585	

Common mitogen-activated protein kinase kinase 7 (MAP3K7) haplotypes and association analysis with measles-specific antibody levels.

^aThe string of 26 SNP nucleotides represented in the MAP3K7 haplotypes are from left to right: rs711264, rs157693, rs806287, rs157705, rs157709, rs791062, rs205347, rs462572, rs806284, rs205351, rs967779, rs1145728, rs157682, rs1145729, rs205348, rs157687, rs157688, rs1145736, rs16883216, rs157434, rs3799897, rs282071, rs9359896, rs4455639, rs282065.

Haplotype effects are estimated using the haplotype t-statistic, which reflects the direction and relative magnitude of the estimated haplotypic effect on the antibody measure. Allele p-values compare individual haplotypes to all other haplotypes combined. Statistically significant p values (p < 0.05) are highlighted in bold.

^bOne degree-of-freedom ordinal p-value from regression analysis adjusting for age, gender, race, age at 1st and 2nd MMR and cohort status (cohort 1 vs. cohort 2).

Associations between SNPs located in coding and regulatory regions in TLR genes and their associated intracellular signaling molecules and MV-specific IFN- γ Elispot responses

Table 3

Gene	SNP ID	Location/ function	Genotype ^a	N ^a	Median SFC per 2 × 10 ⁵ PBMCs (IQR) ^b	P value ^c
Combined cohort of subjects						
KIAA1542	rs702966	3'UTR	CC	365	38 (16; 77)	2.2884E-05
			CG	276	32 (12; 61)	
			GG	61	26 (8; 52)	
TLR7	rs179007	flanking 3'UTR	AA	509	34 (12; 64)	0.0074
			AG	95	36 (9; 78)	
			GG	103	40 (16; 80)	
IKBKE	rs6540431	flanking 5'UTR	CC	617	35 (13; 69)	0.0112
			CG	87	37 (10; 70)	
			GG	3	53 (0; 137)	
Caucasian subgroup						
JUN	rs2760501	flanking 5'UTR	AA	493	37 (16; 73)	0.0010
			AC	66	25 (7; 51)	
			CC	3	15 (4; 98)	
JUN	rs2984915	flanking 5'UTR	GG	497	37 (16; 73)	0.0045
			GA	63	26 (7; 51)	
			AA	3	15 (4; 98)	
IKBKE	rs6540431	flanking 5'UTR	CC	480	36 (15; 71)	0.0156
			CG	81	33 (9; 68)	
			GG	2	95 (53; 137)	
African-American subgroup						
MAP3K7	rs157693	flanking 3'UTR	CC	28	7 (1; 37)	0.0014
			CG	41	27 (6; 49)	

Gene	SNP ID	Location/ function	Genotype ^a	N ^a	Median SFC per 2 × 10 ⁵ PBMCs (IQR) ^b	P value ^c
NFKB1	rs7674640	flanking 3'UTR	GG	18	35 (15; 51)	0.0059
			AA	40	26 (5; 53)	
			AG	39	19 (3; 43)	
			GG	8	22 (4; 35)	

A-Adenine, C-Cytosine, G-Guanine, T-Thymine

SFC- spot-forming cells

A total of 385 SNPs were examined; only those found to be statistically significant (p < 0.01) in coding and regulatory regions were included in the table.

SNP rs179007 in a combined cohort displayed violations of Hardy-Weinberg equilibrium (p = 0).

^aValues are presented as homozygous major allele/heterozygous/homozygous minor allele.

^bIQR, interquartile range, values are median levels in SFC per 2×10⁵ PBMC as measured by IFN-γ Elispot.

^cTest for trend p value from the repeated measures linear regression analysis adjusting for age, gender, race and age of immunization. The p values are adjusted for age, gender, race (when necessary), age at 1st and 2nd MMR and cohort status (cohort 1 vs. cohort 2) using analysis of covariance methodology.

Associations between SNPs located in coding and regulatory regions in TLR genes and their associated intracellular signaling molecules and MV-specific cytokine immune responses

Table 4

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^a	N ^a	Median, pg/ml (IQR) ^b	P value ^c
IL-2	Combined cohort of subjects						
	IKBKE	rs3748022	coding Pro713Leu	GG	477	36.0 (19.7; 61.0)	0.0055
				GA	231	38.6 (20.5; 66.7)	
				AA	30	50.3 (39.4; 74.2)	
	TIRAP	rs1786704	flanking 3'UTR	AA	460	39.7 (21.1; 65.3)	0.0091
				AG	246	36.6 (18.6; 62.0)	
				GG	32	31.1 (15.4; 59.3)	
	Caucasian subgroup						
	IKBKE	rs3748022	coding Pro713Leu	GG	367	40.2 (21.1; 64.2)	0.0019
				GA	201	41.7 (21.6; 69.0)	
				AA	25	58.8 (41.9; 74.2)	
	TLR4	rs10759930	flanking 5'UTR	GG	222	42.3 (23.5; 73.3)	0.0021
GA				274	39.7 (21.0; 62.2)		
AA				96	44.9 (21.7; 67.2)		
TIRAP	rs1786704	flanking 3'UTR	AA	373	42.5 (23.3; 67.9)	0.0047	
			AG	199	39.7 (21.2; 66.0)		
			GG	21	34.6 (18.1; 64.4)		
African-American subgroup							
IRAK2	rs6779158	flanking 5'UTR	AA	79	31.0 (15.2; 52.8)	0.0010	
			AG	9	16.8 (3.3; 18.5)		
			GG	0	---		
IRAK2	rs6767796	flanking 5'UTR	AA	67	31.0 (15.8; 50.6)	0.0019	
			AG	20	16.2 (3.1; 43.9)		

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^d	N ^a	Median, pg/ml (IQR) ^b	P value ^c
	TLR6	rs6815827	flanking 3'UTR	GG	1	30.6 (30.6; 30.6)	0.0041
				GG	77	29.1 (15.4; 51.0)	
				GA	11	18.1 (3.3; 31.7)	
				GG	0	---	
	JUN	rs2760496	flanking 3'UTR	AA	60	29.0 (15.6; 50.8)	0.0054
				AC	22	29.7 (11.2; 47.5)	
				CC	6	14.4 (12.0; 43.9)	
	MYD88	rs7744	3'UTR	AA	76	29.8 (15.3; 50.8)	0.0067
				AG	11	22.2 (0.7; 39.9)	
				GG	1	46.6 (46.6; 46.6)	
	TICAM1	rs7255265	coding Thr4Thr	GG	77	29.1 (15.2; 50.6)	0.0098
				GA	9	18.5 (11.3; 30.6)	
				AA	2	20.7 (-6.0; 47.5)	
IL-6	Combined cohort of subjects						
	TLR8	rs5744081	coding Arg438Arg	CC	721	356.2 (248.4; 462.4)	0.0025
				CA	9	278.6 (224.9; 308.2)	
				AA	7	277.7 (124.0; 370.6)	
	TLR8	rs5979763	3'UTR	AA	720	356.3 (248.2; 463.0)	0.0027
				AG	9	278.6 (224.9; 308.2)	
				GG	7	277.7 (124.0; 370.6)	
	LY96	rs695910	flanking 3'UTR	CC	449	360.3 (254.4; 469.5)	0.0083
				CA	247	340.2 (220.3; 452.9)	
				AA	41	385.0 (276.7; 441.3)	
Caucasian subgroup							
None							

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^d	N ^a	Median, pg/ml (IQR) ^b	P value ^c	
IL-10	TLR3	rs5743309	5'UTR	CC	77	330.2 (238.7; 440.6)	0.0093	
				CA	9	287.6 (269.8; 358.9)		
				AA	2	229.8 (18.4; 441.3)		
	Combined cohort of subjects							
	TLR4	rs7044464	flanking 3'UTR	TT	507	17.0 (11.0; 27.0)	0.0012	
				TA	206	19.3 (12.0; 29.7)		
				AA	26	20.6 (14.5; 39.3)		
	TLR4	rs7856729	flanking 3'UTR	CC	499	17.1 (11.1; 27.0)	0.0013	
				CA	210	19.3 (12.0; 29.7)		
				AA	31	19.6 (8.9; 39.3)		
TLR6	rs6815827	flanking 3'UTR	GG	719	18.1 (11.3; 28.2)	0.0016		
			GA	20	17.0 (7.5; 34.8)			
			AA	1	4.5 (4.5; 4.5)			
IKBKE	rs6540431	flanking 5'UTR	CC	648	17.5 (10.7; 27.5)	0.0042		
			CG	88	22.5 (12.6; 32.5)			
			GG	4	17.1 (14.7; 27.9)			
Caucasian subgroup								
TLR3	rs4862633	flanking 3'UTR	GG	357	16.7 (10.8; 28.1)	0.0003		
			GA	194	21.6 (13.4; 28.5)			
			AA	39	24.5 (16.3; 40.1)			
TLR3	rs10025405	flanking 3'UTR	AA	195	22.1 (13.3; 33.5)	0.0006		
			AG	264	17.3 (11.8; 26.8)			
			GG	133	17.2 (10.7; 28.1)			
TLR4	rs7044464	flanking 3'UTR	TT	408	17.8 (11.7; 27.7)	0.0008		
			TA	163	20.1 (13.4; 29.7)			
			AA	22	20.6 (14.5; 39.3)			

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^d	N ^a	Median, pg/ml (IQR) ^b	P value ^c
IFN-α	TLR4	rs7856729	flanking 3'UTR	CC	407	17.9 (11.8; 27.7)	0.0008
				CA	164	19.8 (13.4; 29.7)	
				AA	23	20.2 (11.3; 39.3)	
	TLR3	rs1519312	flanking 3'UTR	AA	217	15.9 (10.4; 28.9)	0.0015
				AG	303	19.4 (12.7; 27.8)	
				GG	74	20.6 (13.0; 34.4)	
	TLR3	rs4608848	flanking 3'UTR	AA	218	16.1 (10.4; 28.9)	0.0020
				AG	303	19.3 (12.6; 27.8)	
				GG	73	20.8 (13.3; 34.4)	
	TIRAP	rs8177376	3'UTR	AA	346	17.5 (11.8; 27.8)	0.0034
				AC	214	21.1 (12.5; 29.4)	
				CC	33	24.1 (13.1; 29.1)	
TLR4	rs10759932	flanking 5'UTR	AA	446	18.6 (11.9; 28.0)	0.0035	
			AG	139	19.4 (13.2; 31.2)		
			GG	8	30.2 (10.3; 55.8)		
African-American subgroup							
None							
Combined cohort of subjects							
NFKBIA	rs2233409	flanking 5'UTR	GG	458	538.8 (248.5; 1,018)	0.0011	
			GA	226	577.5 (281.9; 1,012)		
			AA	50	699.9 (404.4; 1,248)		
	TLR4	rs10759932	flanking 5'UTR	AA	546	544.0 (254.1; 1,007)	0.0013
				AG	172	593.8 (340.8; 1,141)	
				GG	15	681.7 (306.7; 1,126)	
NFKBIA	rs3138052	flanking 5'UTR	AA	406	550.3 (254.1; 1,002)	0.0024	
			AG	258	540.2 (274.6; 1,012)		

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^d	N ^a	Median, pg/ml (IQR) ^b	P value ^c
				GG	59	681.7 (365.8; 1,259)	
				GG	486	530.4 (251.2; 975.0)	
	NFKB1A	rs1050851	coding Ala102Ala	GA	208	581.5 (284.6; 1,096)	0.0043
				AA	40	734.4 (427.7; 1,278)	
				AA	410	546.0 (254.1; 1,002)	
	NFKB1A	rs3138053	flanking 5'UTR	AG	262	543.0 (280.9; 1,014)	0.0084
				GG	62	658.6 (328.2; 1,244)	
				Caucasian subgroup			
				AA	312	588.5 (277.6; 1,026)	
	NFKB1A	rs3138052	flanking 5'UTR	AG	218	581.5 (281.9; 1,101)	0.0075
				GG	50	720.3 (365.8; 1,259)	
				African-American subgroup			
				GG	86	402.3 (212.2; 609.0)	
	IRAK3	rs11465990	flanking 3'UTR	GC	2	1,931.3 (1,770.8; 2,092)	0.0010
				CC	0	---	
				GG	53	321.8 (202.2; 551.7)	
	IRAK3	rs11176082	flanking 5'UTR	GA	34	479.0 (258.0; 678.1)	0.0048
				AA	1	805.2 (805.2; 805.2)	
IFN- γ				Combined cohort of subjects			
				AA	460	71.8 (37.9; 126.2)	
	TIRAP	rs1786704	flanking 3'UTR	AG	244	60.6 (32.8; 101.4)	0.0004
				GG	32	36.3 (15.8; 87.7)	
				GG	302	70.1 (35.7; 136)	
	TLR4	rs10759930	flanking 5'UTR	GA	320	65.6 (34.8; 112.9)	0.0010
				AA	113	68.3 (32.5; 114.1)	
	IRAK2	rs9854688	coding Leu503Ile	CC	713	66.5 (34.4; 119.7)	0.0058

Secreted cytokine	Gene	SNP ID	Location/function	Genotype ^a	N ^a	Median, pg/ml (IQR) ^b	P value ^c				
IFNλ-1	TLR4	rs10759930	flanking 5'UTR	CA	23	70.1 (57.3; 121.1)	0.0019				
				AA	1	84.6 (84.6; 84.6)					
				Caucasian subgroup							
				GG	221	75.0 (37.0; 147.1)					
				GA	274	65.9 (37.6; 113.6)					
				AA	95	60.3 (31.9; 118.8)					
	TLR4	rs10759932	flanking 5'UTR	AA	444	67.6 (35.2; 118.5)	0.0036				
				AG	139	71.8 (37.0; 145.7)					
				GG	8	89.9 (50.3; 237.8)					
				TIRAP	rs1786704	flanking 3'UTR		AA	373	73.9 (38.0; 127.6)	0.0091
								AG	197	61.5 (34.9; 108.8)	
GG	21	33.8 (13.8; 79.1)									
KIAA15 42	rs11246212	coding Val1448Ala	GG				457	67.3 (35.2; 121.0)	0.0092		
			GA				118	76.0 (37.0; 123.3)			
			AA	10	91.8 (51.5; 194.2)						
			African-American subgroup								
			None								
			Combined cohort of subjects								
TLR2	rs3804099	coding Asn199Asn	AA	232	42.4 (16.5; 84.2)	0.0021					
			AG	333	32.2 (14.9; 73.0)						
			GG	170	27.4 (12.5; 59.3)						
			TLR8	rs5744077	coding Met10Val		AA	727	34.5 (14.3; 75.1)	0.0029	
							AG	5	-2.7 (-295.7; 5.4)		
GG	6	25.8 (11.0; 42.3)									
TLR4	rs10759930	flanking 5'UTR				GG	303	29.0 (10.6; 67.2)	0.0059		
						GA	320	38.4 (17.0; 75.8)			

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^d	N ^a	Median, pg/ml (IQR) ^b	P value ^c	
TNF-α	TLR4	rs16906053	flanking 5'UTR	AA	113	46.1 (17.6; 79.1)	0.0085	
				AA	700	36.2 (14.9; 76.3)		
				AG	29	16.5 (5.4; 33.9)		
				GG	9	14.0 (−295.7; 22.0)		
	Caucasian subgroup							
	TLR7	rs2897827	flanking 5'UTR	GG	522	37.9 (16.6; 76.1)	0.0049	
				GA	41	50.5 (23.2; 77.6)		
				AA	28	68.5 (17.0; 115.1)		
	NFKB1A	rs3138050	flanking 5'UTR	GG	368	33.1 (15.0; 72.5)	0.0066	
				GA	190	50.0 (19.9; 83.0)		
AA				34	45.9 (17.6; 81.6)			
African-American subgroup								
JUN	rs2984915	flanking 5'UTR	AA	23	16.6 (−2.8; 30.2)	0.0027		
			AG	44	18.2 (2.8; 40.6)			
			GG	21	19.9 (9.0; 29.0)			
NFKB1	rs980455	flanking 5'UTR	AA	28	17.5 (1.4; 29.9)	0.0035		
			AG	40	15.5 (2.9; 31.8)			
			GG	20	21.4 (8.4; 66.2)			
TLR4	rs7869402	3'UTR	GG	49	16.8 (2.8; 29.6)	0.0088		
			GA	35	20.1 (5.4; 84.1)			
			AA	4	7.8 (−14.1; 43.2)			
Combined cohort of subjects								
TLR8	rs5744081	coding Arg438Arg	CC	716	13.6 (9.3; 18.8)	0.0032		
			CA	9	11.3 (6.2; 15.4)			
			AA	7	9.8 (5.0; 36.6)			
TLR8	rs5979763	3'UTR	AA	715	13.7 (9.3; 18.8)	0.0034		

Secreted cytokine	Gene	SNP ID	Location/ function	Genotype ^a	N ^a	Median, pg/ml (IQR) ^b	P value ^c
TLR7	rs5741881		coding Leu1029Leu	AG	9	11.3 (6.2; 15.4)	0.0096
				GG	7	9.8 (5.0; 36.6)	
				AA	710	13.8 (9.3; 18.8)	
				AG	22	8.8 (4.6; 14.0)	
				GG	0	---	
Caucasian subgroup							
None							
African-American subgroup							
IRAK3	rs11465990		flanking 3'UTR	GG	86	11.6 (6.4; 17.0)	0.0003
				GC	2	47.8 (8.0; 87.7)	
				CC	0	---	
NFKB1A	rs1050851		coding Ala102Ala	GG	80	11.9 (6.7; 17.8)	0.0042
				GA	7	10.1 (3.7; 11.6)	
				AA	1	87.7 (87.7; 87.7)	
TICAM1	rs2292151		coding Asp557Asp	GG	53	11.9 (7.4; 20.1)	0.0055
				GA	32	11.5 (6.3; 15.1)	
				AA	3	8.6 (2.7; 87.7)	
TICAM1	rs4807651		flanking 5'UTR	AA	50	12.1 (7.4; 22.0)	0.0058
				AG	34	11.5 (6.4; 15.0)	
				GG	4	8.5 (5.6; 48.2)	

A-Adenine, C-Cytosine, G-Guanine, T-Thymine

A total of 385 SNPs were examined; only those found to be statistically significant in coding and regulatory regions (p = 0.001) were included in the table.

SNPs rs5744081/IL-6 (p = 2.40E⁻⁰⁵) and rs5979763/IL-6 (p = 2.40E⁻⁰⁵) in the combined cohort, rs11465990/IFN-α in the African-American subgroup (p = 1), rs5744077/IFN-λ-1 (p = 1) and rs16906053/IFN-λ-1 (p = 2.84E⁻¹⁰) in the combined cohort, rs5744081/TNF-α (p = 2.40E⁻⁰⁵), rs5979763/TNF-α (p = 2.40E⁻⁰⁵), and rs5741881/TNF-α (p = 1) in the combined cohort, and rs11465990/TNF-α (p = 1) in the African-American subgroup displayed violations of Hardy-Weinberg equilibrium.

^aValues are presented as homozygous major allele/heterozygous/homozygous minor allele.

^b IQR, interquartile range, values are median levels in pg/ml as measured by ELISA.

^c Test for trend p value from the repeated measures linear regression analysis adjusting for age, gender, race and age of immunization. The p-values are adjusted for age, gender, race (when necessary), age at 1st and 2nd MMR and cohort status (cohort 1 vs. cohort 2) using analysis of covariance methodology.