

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

Inna G. Ovsyannikova · Iana H. Haralambieva ·  
Robert A. Vierkant · V. Shane Pankratz ·  
Robert M. Jacobson · Gregory A. Poland

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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 ACGGCGAGAAAAGAGAAG AGAGAGAA ( $p = 0.01$ ) 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- $\gamma$  Elispot responses (38 vs. 26 spot-forming cells per  $2 \times 10^5$  PBMCs,  $p = 0.00002$ ). We observed an

additional 12 associations ( $p < 0.01$ ) between coding (nonsynonymous and synonymous) polymorphisms within the TLRs (TLR2, 7, and 8), IKBKE, TICAM1, NFKBIA, IRAK2, and KIAA1542 genes and variations in measles-specific IL-2, IL-6, IFN- $\alpha$ , IFN- $\gamma$ , IFN- $\lambda$ -1, and TNF- $\alpha$  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.

## 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)

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I. G. Ovsyannikova · I. H. Haralambieva · R. M. Jacobson ·  
G. A. Poland (✉)  
Mayo Vaccine Research Group, Mayo Clinic,  
Guggenheim 611C, 200 1st Street S.W.,  
Rochester, MN 55905, USA  
e-mail: poland.gregory@mayo.edu

R. A. Vierkant · V. S. Pankratz  
Department of Health Sciences Research,  
Mayo Clinic, Rochester, MN 55905, USA

R. M. Jacobson · G. A. Poland  
Department of Pediatric and Adolescent Medicine,  
Mayo Clinic, Rochester, MN 55905, USA

I. G. Ovsyannikova · R. M. Jacobson · G. A. Poland  
Program in Translational Immunovirology and Biodefense,  
Mayo Clinic, Rochester, MN 55905, USA

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 TLR2–6 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 (TLR3, 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 11–19 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 11–22 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

Measles-specific neutralizing antibody levels were quantified using a high throughput fluorescence-based plaque reduction microneutralization assay (PRMN), as described

(Haralambieva et al. 2008). Heat-inactivated sera were diluted fourfold 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 doze,  $ND_{50}$ ) 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  $ND_{50}$  values to be transformed into mIU/ml (Haralambieva et al. 2008).

### IFN- $\gamma$ Elispot

IFN- $\gamma$  Elispot responses were assessed using kits from R&D Systems (Minneapolis, MN), as described (Ryan et al. 2005). Seven wells were plated with  $2 \times 10^5$  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  $\mu$ 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 for cytokines

Enzyme-linked immunosorbent assays (ELISAs) were performed to measure the level of seven [IL-2 ( $n = 739$ ), IL-6 ( $n = 737$ ), IL-10 ( $n = 740$ ), IFN $\alpha$  ( $n = 734$ ), IFN- $\gamma$  ( $n = 737$ ), IFN $\lambda$ -1 ( $n = 738$ ), and TNF- $\alpha$  ( $n = 732$ )] 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  $2 \times 10^5$  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 h; IL-6, MOI = 1.0, 72 h; IL-10, MOI = 0.5, 48 h; IFN- $\alpha$ , MOI = 1.0, 24 h; IFN- $\gamma$ , MOI = 1.0, 72 h; IFN $\lambda$ -1, MOI = 1.0, 72 h; TNF- $\alpha$ , MOI = 1.0, 24 h. Cytokines IL-2, IL-6, IL-10, IFN- $\gamma$ , and TNF- $\alpha$  were measured

using kits from BD Biosciences (San Jose, CA), TNF- $\alpha$  was measured using kits from Mabtech (Cincinnati, OH), and IFN $\lambda$ -1 was measured using kits from R&D Systems. Cytokine-specific ICCs ranged from 0.65 (IL-2, unstimulated values) to 0.94 (IFN- $\alpha$  and IL-6, stimulated values).

### TagSNP selection

A panel of SNPs from TLRs (TLR2–9) 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 TAB 2), TLR adaptor molecule 1 (TRIF or TICAM1), and KIAA1542 (IRF7 downstream molecule)] formed the basis of this study. SNPs within each gene and 5 kb 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)  $\geq 0.05$  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; Haralambi-eva 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- $\alpha$ , IFN- $\gamma$ , IFN $\lambda$ -1, and TNF- $\alpha$ , each reported in units of pg/ml); a measure of CMI via IFN- $\gamma$  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 pair-wise linkage LD based on the  $r^2$  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 interquartile 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- $\gamma$  Elispot counts for spot-forming cells (SFC per

$2 \times 10^5$  cells) were 36 (IQR 12; 69). Further, median values for MV-specific IL-2, IL-6, IL-10, IFN- $\alpha$ , IFN- $\gamma$ , IFN- $\lambda$ -1 and TNF- $\alpha$  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 ACGGCGAGAAAAGA GAAGAGAGAGAA was associated with lower ( $t$  statistic  $-2.52$ ,  $p = 0.012$ ) measles-specific antibodies, while another common haplotype GCAGGAGGCGATGGGGA AGGAAGGCG demonstrated suggestive association with higher ( $t$  statistic 1.71,  $p = 0.087$ ) antibody levels.

**Table 1** Associations between SNPs located in coding and regulatory regions in TLR genes and their associated intracellular signaling molecules and MV-specific antibody responses

Gene	SNP ID	Location/function	Genotype <sup>a</sup>	N <sup>a</sup>	Median antibody titer mIU/ml (IQR) <sup>b</sup>	p value <sup>c</sup>
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)	
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 total of 385 SNPs were examined; only those found to be statistically significant ( $p \leq 0.01$ ) in coding and regulatory regions were included in the table

SNP rs16906053 in a combined cohort displayed violations of Hardy–Weinberg equilibrium ( $p = 2.84E^{-10}$ )

A adenine, C cytosine, G guanine, T thymine

<sup>a</sup> Values are presented as homozygous major allele/heterozygous/homozygous minor allele

<sup>b</sup> IQR interquartile range, values are median levels in mIU/ml as measured by plaque reduction microneutralization assay

<sup>c</sup> Test 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

#### Associations between SNPs in TLR with associated intracellular signaling molecules and IFN- $\gamma$ Elispot response

Overall, we found seven SNPs located in coding and regulatory regions significantly associated ( $p < 0.01$ ) with variations in MV-specific IFN- $\gamma$  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- $\gamma$  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- $\gamma$  Elispot responses (38 vs. 26 SFC per  $2 \times 10^5$  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- $\gamma$  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- $\gamma$  Elispot responses after measles vaccination.

**Table 2** MAP3K7 haplotype associations with MV-specific antibody responses

MAP3K7 Haplotype <sup>a</sup>	Frequency	Back-transformed mean (mIU/ml)	Back-transformed 95% CI (mIU/ml)	Test statistic (haplotype <i>t</i> statistic)	Allele <i>p</i> value <sup>b</sup>	Global <i>p</i> value
ACGGCGAGAAAAGAGAAGAGAGAGAA	0.248	777.6	(711.0; 850.6)	-2.52	<b>0.012</b>	0.082
GCAGGAGGCGATGGGGAAGGAAGGCG	0.274	875.2	(804.0; 952.7)	1.71	0.087	
GGAACAACCAATAGAAGGGGGAAGAA	0.018	812.6	(587.9; 1123.1)	-0.21	0.830	
GGAACAACCAGTAGAAGGGGAAAGAA	0.273	861.3	(790.4; 938.6)	1.36	0.173	
GGAGCGAGCAATGAGAAGGGAAAGAA	0.062	787.7	(651.8; 952.0)	-0.55	0.585	

Common mitogen-activated protein kinase kinase kinase 7 (MAP3K7) haplotypes and association analysis with measles-specific antibody levels. 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 ( $p \leq 0.05$ ) are highlighted in bold

<sup>a</sup> The 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, rs205343, rs157688, rs1145736, rs16883216, rs157434, rs3799897, rs282071, rs9359896, rs4455639, rs282065

<sup>b</sup> One 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 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- $\alpha$ , IFN- $\gamma$ , IFN- $\lambda$ -1 and TNF- $\alpha$  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 0.0003–0.002) 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 five significant SNP associations with variations in measles-specific IFN- $\alpha$  secretion in promoter SNPs belonging to the genes NFKBIA (rs2233409,  $p = 0.001$ , rs3138052,  $p = 0.002$ , rs1050851/coding,  $p = 0.004$  and rs3138053,  $p = 0.008$ ;  $r^2 \leq 0.97$ ) and TLR4 (rs10759932,  $p = 0.001$ ). Of note, coding SNP rs1050851 in the NFKBIA (MAD3) gene was associated (734 vs. 530 pg/ml,  $p = 0.004$ ) with IFN- $\alpha$  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 two significant associations between promoter region SNPs belonging to the IRAK3 (rs11465990,  $p = 0.001$  and rs11176082,  $p = 0.005$ ) signaling pathway and IFN- $\alpha$  secretion in an allele dose-dependent manner.

SNP associations with MV-specific IFN- $\gamma$  secretion were also examined. Increased representation of major

**Table 3** Associations between SNPs located in coding and regulatory regions in TLR genes and their associated intracellular signaling molecules and MV-specific IFN- $\gamma$  Elispot responses

Gene	SNP ID	Location/function	Genotype <sup>a</sup>	N <sup>a</sup>	Median SFC per $2 \times 10^5$ PBMCs (IQR) <sup>b</sup>	<i>p</i> value <sup>c</sup>
Combined cohort of subjects						
KIAA1542	rs702966	3'UTR	CC	365	38 (16; 77)	2.2884E <sup>-05</sup>
			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)	
			GG	18	35 (15; 51)	
NFKB1	rs7674640	Flanking 3'UTR	AA	40	26 (5; 53)	0.0059
			AG	39	19 (3; 43)	
			GG	8	22 (4; 35)	

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

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

A adenine, C cytosine, G guanine, T thymine, SFC spot-forming cells

<sup>a</sup> Values are presented as homozygous major allele/heterozygous/homozygous minor allele

<sup>b</sup> IQR interquartile range, values are median levels in SFC per  $2 \times 10^5$  PBMC as measured by IFN- $\gamma$  Elispot

<sup>c</sup> 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

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- $\gamma$  secretion in an allele dose-dependent manner. The Caucasian subgroup revealed two significant associations (range of *p* values 0.002–0.009 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 one additional promoter SNP (rs1786704,  $p = 0.009$ ) belonging to the TIRAP gene with measles-specific IFN- $\gamma$  secretion. Additionally, the Caucasian subgroup demonstrated one signifi-

cant association between a coding SNP in the KIAA1542 (rs11246212, Val1448Ala,  $p = 0.009$ ) gene and secreted of IFN- $\gamma$  in an allele dose-dependent manner.

Four significant SNP associations with variations in measles-specific IFN- $\lambda$ -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 two significant associations in 5'UTR SNPs, belonging to TLR7 (rs2897827,  $p = 0.005$ ) and NFKBIA

**Table 4** Associations between SNPs located in coding and regulatory regions in TLR genes and their associated intracellular signaling molecules and MV-specific cytokine immune responses

Secreted cytokine	Gene	SNP ID	Location/function	Genotype <sup>a</sup>	N <sup>a</sup>	Median, pg/ml (IQR) <sup>b</sup>	p value <sup>c</sup>
IL-2	<i>Combined cohort of subjects</i>						
	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)	
	<i>Caucasian subgroup</i>						
	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)	
	<i>African-American subgroup</i>						
	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)		
			GG	1	30.6 (30.6; 30.6)		
TLR6	rs6815827	Flanking 3'UTR	GG	77	29.1 (15.4; 51.0)	0.0041	
			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	<i>Combined cohort of subjects</i>						
	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	rs6995910	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)	
	<i>Caucasian subgroup</i>						
	None						
<i>African-American subgroup</i>							
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)		



**Table 4** continued

Secreted cytokine	Gene	SNP ID	Location/function	Genotype <sup>a</sup>	N <sup>a</sup>	Median, pg/ml (IQR) <sup>b</sup>	p value <sup>c</sup>
IL-10	<i>Combined cohort of subjects</i>						
	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)	
	<i>Caucasian subgroup</i>						
	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)	
	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)		
<i>African-American subgroup</i>							
None							
IFN- $\alpha$	<i>Combined cohort of subjects</i>						
	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)	
				GG	59	681.7 (365.8; 1,259)	
	NFKBIA	rs1050851	Coding Ala102Ala	GG	486	530.4 (251.2; 975.0)	0.0043
				GA	208	581.5 (284.6; 1,096)	
AA				40	734.4 (427.7; 1,278)		

**Table 4** continued

Secreted cytokine	Gene	SNP ID	Location/function	Genotype <sup>a</sup>	N <sup>a</sup>	Median, pg/ml (IQR) <sup>b</sup>	p value <sup>c</sup>	
IFN- $\gamma$	NFKBIA	rs3138053	Flanking 5'UTR	AA	410	546.0 (254.1; 1,002)	0.0084	
				AG	262	543.0 (280.9; 1,014)		
				GG	62	658.6 (328.2; 1,244)		
	<i>Caucasian subgroup</i>							
	NFKBIA	rs3138052	Flanking 5'UTR	AA	312	588.5 (277.6; 1,026)	0.0075	
				AG	218	581.5 (281.9; 1,101)		
				GG	50	720.3 (365.8; 1,259)		
	<i>African-American subgroup</i>							
	IRAK3	rs11465990	Flanking 3'UTR	GG	86	402.3 (212.2; 609.0)	0.0010	
				GC	2	1,931.3 (1,770.8; 2,092)		
				CC	0	–		
	IRAK3	rs11176082	Flanking 5'UTR	GG	53	321.8 (202.2; 551.7)	0.0048	
GA				34	479.0 (258.0; 678.1)			
AA				1	805.2 (805.2; 805.2)			
<i>Combined cohort of subjects</i>								
TIRAP	rs1786704	Flanking 3'UTR	AA	460	71.8 (37.9; 126.2)	0.0004		
			AG	244	60.6 (32.8; 101.4)			
			GG	32	36.3 (15.8; 87.7)			
TLR4	rs10759930	Flanking 5'UTR	GG	302	70.1 (35.7; 136)	0.0010		
			GA	320	65.6 (34.8; 112.9)			
			AA	113	68.3 (32.5; 114.1)			
IRAK2	rs9854688	Coding Leu503Ile	CC	713	66.5 (34.4; 119.7)	0.0058		
			CA	23	70.1 (57.3; 121.1)			
			AA	1	84.6 (84.6; 84.6)			
<i>Caucasian subgroup</i>								
TLR4	rs10759930	Flanking 5'UTR	GG	221	75.0 (37.0; 147.1)	0.0019		
			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)			
KIAA1542	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)			
<i>African-American subgroup</i>								
None								
IFN $\lambda$ -1	<i>Combined cohort of subjects</i>							
	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)		
				AA	113	46.1 (17.6; 79.1)		
	TLR4	rs16906053	Flanking 5'UTR	AA	700	36.2 (14.9; 76.3)	0.0085	
				AG	29	16.5 (5.4; 33.9)		
GG				9	14.0 (–295.7; 22.0)			

**Table 4** continued

Secreted cytokine	Gene	SNP ID	Location/function	Genotype <sup>a</sup>	N <sup>a</sup>	Median, pg/ml (IQR) <sup>b</sup>	p value <sup>c</sup>	
<i>Caucasian subgroup</i>								
	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)		
	NFKBIA	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)		
<i>African-American subgroup</i>								
	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)		
TNF- $\alpha$	<i>Combined cohort of subjects</i>							
	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	
				AG	9	11.3 (6.2; 15.4)		
				GG	7	9.8 (5.0; 36.6)		
	TLR7	rs5741881	Coding Leu1029Leu	AA	710	13.8 (9.3; 18.8)	0.0096	
				AG	22	8.8 (4.6; 14.0)		
				GG	0	–		
	<i>Caucasian subgroup</i>							
	None							
	<i>African-American subgroup</i>							
		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	–		
	NFKBIA	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 total of 385 SNPs were examined; only those found to be statistically significant in coding and regulatory regions ( $p \leq 0.001$ ) 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- $\alpha$  in the African-American subgroup ( $p = 1$ ), rs5744077/IFN $\lambda$ -1 ( $p = 1$ ) and rs16906053/IFN $\lambda$ -1 ( $p = 2.84E^{-10}$ ) in the combined cohort, rs5744081/TNF- $\alpha$  ( $p = 2.40E^{-05}$ ), rs5979763/TNF- $\alpha$  ( $p = 2.40E^{-05}$ ), and rs5741881/TNF- $\alpha$  ( $p = 1$ ) in the combined cohort, and rs11465990/TNF- $\alpha$  ( $p = 1$ ) in the African-American subgroup displayed violations of Hardy–Weinberg equilibrium

A adenine, C cytosine, G guanine, T thymine

<sup>a</sup> Values are presented as homozygous major allele/heterozygous/homozygous minor allele

<sup>b</sup> IQR interquartile range, values are median levels in pg/ml as measured by ELISA

<sup>c</sup> 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

(rs3138050,  $p = 0.007$ ) and measles-specific IFN $\lambda$ -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 $\lambda$ -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- $\alpha$  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- $\alpha$  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 (TLR2–9) in antiviral immunity (Bieback et al. 2002; Matsumoto et al. 2004; Schlender et al. 2005). Other studies established an increased expression of TLR2, 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 $\alpha/\beta$ , IL-6, IFN- $\alpha/\beta$  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 TLR2, 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 $\kappa$ 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 TLR2–4 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 (NF $\kappa$ B) and two kinase pathways: the MAPK cascade that promotes activation of mitogen-activated protein kinase 8 (MAPK8) and the I $\kappa$ B 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 I $\kappa$ B 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 TLR2, 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- $\gamma$  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 Tschoop 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- $\alpha/\beta$ ) 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- $\gamma$  Elispot responses.

We found 58 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- $\alpha$ , IFN- $\gamma$ , IFN- $\lambda$ -1 and TNF- $\alpha$ ) cytokine responses. TLR-virus interactions are triggered by transmembrane TLRs on the cellular surface (TLR2, 4) and within endosomal and endoplasmic reticulum intracellular compartments (TLR3, 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, Ikk $\alpha/\beta/\gamma$ , 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- $\beta$  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- $\alpha$ . Similarly, the TLR4 genetic variant rs10759932

was associated with an allele dose-related secretion of IL-10, IFN- $\alpha$  and IFN- $\gamma$ . Another TLR4 polymorphism (rs10759930) demonstrated an association with secretion of IL-2, IFN- $\gamma$  and IFN- $\lambda$ -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- $\alpha$  and TNF- $\alpha$  levels in the combined cohort of subjects and in African-American subjects, respectively. Evidence was also found for the possible regulation of IFN- $\gamma$  Elispot response and IFN- $\lambda$ -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- $\gamma$  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- $\gamma$  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 (TLR2, 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; Vandebroek and Goris 2003).

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**Conflict of interest** 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-Permanente.

## References

- Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265
- Barton GM (2007) Viral recognition by Toll-like receptors. *Semin Immunol* 19:33–40
- Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, Wagner H, Kirschning CJ, ter Meulen V, Schneider-Schaulies S (2002) Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J Virol* 76:8729–8736
- Boehme KW, Compton T (2004) Innate sensing of viruses by Toll-like receptors. *J Virol* 78:7867–7873
- Bowie AG, Hago IR (2005) The role of Toll-like receptors in the host response to viruses. *Mol Immunol* 42:859–867
- Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA (2004) Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 74:106–120
- Chiron D, Bekeredjian-Ding I, Pellat-Deceunynck C, Bataille R, Jegou G (2008) Toll-like receptors: lessons to learn from normal and malignant human B cells. *Blood* 112:2205–2213
- Dhiman N, Ovsyannikova IG, Vierkant RA, Ryan JE, Pankratz VS, Jacobson RM, Poland GA (2008) Associations between SNPs in toll-like receptors and related intracellular signaling molecules and immune responses to measles vaccine: preliminary results. *Vaccine* 26:1731–1736
- Dhiman N, Haralambieva IH, Kennedy RB, Vierkant RA, O'Byrne MM, Ovsyannikova IG, Jacobson RM, Poland GA (2010) SNP/haplotype associations in cytokine and cytokine receptor genes and immunity to rubella vaccine. *Immunogenetics* 62:197–210
- Finberg RW, Wang JP, Kurt-Jones EA (2007) Toll-like receptors and viruses. *Rev Med Virol* 17:35–43
- Gordon D, Finch SJ (2005) Factors affecting statistical power in the detection of genetic association. *J Clin Invest* 115:1408–1418
- Hahm B, Cho JH, Oldstone MB (2006) Measles virus-dendritic cell interaction via SLAM inhibits innate immunity: Selective signaling through TLR4 but not other TLRs mediates suppression of IL-12 synthesis. *Virology* 358:251–257
- Haralambieva IH, Ovsyannikova IG, Vierkant RA, Poland GA (2008) Development of a novel efficient fluorescence-based plaque reduction microneutralization assay for measles immunity. *Clin Vaccine Immunol* 15:1054–1059
- Haralambieva IH, Dhiman N, Ovsyannikova IG, Vierkant RA, Pankratz VS, Jacobson RM, Poland GA (2010) 2'-5'-Oligoadenylate synthetase single-nucleotide polymorphisms and haplotypes are associated with variations in immune responses to rubella vaccine. *Hum Immunol* 71:383–391
- Harley JB, Alarcon-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, Moser KL, Tsao BP, Vyse TJ, Langefeld CD, Nath SK, Guthridge JM, Cobb BL, Mirel DB, Marion MC, Williams AH, Divers J, Wang W, Frank SG, Namjou B, Gabriel SB, Lee AT, Gregersen PK, Behrens TW, Taylor KE, Fernando M, Zidovetzki R, Gaffney PM, Edberg JC, Rioux JD, Ojwang JO, James JA, Merrill JT, Gilkeson GS, Seldin MF, Yin H, Baechler EC, Li QZ, Wakeland EK, Bruner GR, Kaufman KM, Kelly JA (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXX, KIAA1542 and other loci. *Nat Genet* 40:204–210
- Hattersley AT, McCarthy MI (2005) What makes a good genetic association study? *Lancet* 366:1315–1323
- Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* 117:5965–5973
- Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K (2002) A comprehensive review of genetic association studies. *Genet Med* 4:45–61
- Hunt R, Sauna ZE, Ambudkar SV, Gottesman MM, Kimchi-Sarfaty C (2009) Silent (synonymous) SNPs: should we care about them? *Methods Mol Biol* 578:23–39
- Kishida S, Sanjo H, Akira S, Matsumoto K, Ninomiya-Tsuji J (2005) TAK1-binding protein 2 facilitates ubiquitination of TRAF6 and assembly of TRAF6 with IKK in the IL-1 signaling pathway. *Genes Cells* 10:447–454
- Matsumoto M, Funami K, Oshiumi H, Seya T (2004) Toll-like receptor 3: a link between toll-like receptor, interferon and viruses. *Microbiol Immunol* 48:147–154
- Meylan E, Tschopp J (2006) Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell* 22:561–569

- Motsinger AA, Haas DW, Hulgan T, Ritchie MD (2007) Human genomic association studies: a primer for the infectious diseases specialist. *J Infect Dis* 195:1737–1744
- Murabayashi N, Kurita-Taniguchi M, Ayata M, Matsumoto M, Ogura H, Seya T (2002) Susceptibility of human dendritic cells (DCs) to measles virus (MV) depends on their activation stages in conjunction with the level of CDw150: role of Toll stimulators in DC maturation and MV amplification. *Microbes Infect* 4:785–794
- Ovsyannikova IG, Dhiman N, Jacobson RM, Vierkant RA, Pankratz VS, Poland GA (2007) HLA homozygosity does not adversely affect measles vaccine-induced cytokine responses. *Virology* 364:87–94
- Ovsyannikova IG, Dhiman N, Haralambieva IH, Vierkant RA, O’Byrne MM, Jacobson RM, Poland GA (2010) Rubella vaccine-induced cellular immunity: evidence of associations with polymorphisms in the Toll-like, vitamin A and D receptors, and innate immune response genes. *Hum Genet* 127:207–221
- Poland GA, Ovsyannikova IG, Jacobson RM, Smith DI (2007) Heterogeneity in vaccine immune response: the role of immunogenetics and the emerging field of vaccinomics. *Clin Pharmacol Ther* 82:653–664
- Poland GA, Ovsyannikova IG, Jacobson RM (2008) Vaccine immunogenetics: bedside to bench to population. *Vaccine* 26:6183–6188
- Rassa JC, Ross SR (2003) Viruses and Toll-like receptors. *Microbes Infect* 5:961–968
- Ryan JE, Ovsyannikova IG, Poland GA (2005) Detection of measles virus-specific IFN-gamma-secreting T-cells by ELISPOT. In: Kalyuzhny AE (ed) *Handbook of ELISPOT: methods and protocols*. Humana Press Inc, Totowa, pp 207–217
- Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA (2002) Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 70:425–434
- Schlender J, Hornung V, Finke S, Gunthner-Biller M, Marozin S, Brzozka K, Moghim S, Endres S, Hartmann G, Conzelmann KK (2005) Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. *J Virol* 79:5507–5515
- Tanabe M, Kurita-Taniguchi M, Takeuchi K, Takeda M, Ayata M, Ogura H, Matsumoto M, Seya T (2003) Mechanism of up-regulation of human Toll-like receptor 3 secondary to infection of measles virus-attenuated strains. *Biochem Biophys Res Commun* 311:39–48
- tenOever BR, Servant MJ, Grandvaux N, Lin R, Hiscott J (2002) Recognition of the measles virus nucleocapsid as a mechanism of IRF-3 activation. *J Virol* 76:3659–3669
- Vandenbroeck K, Goris A (2003) Cytokine gene polymorphisms in multifactorial diseases: gateways to novel targets for immunotherapy? *Trends Pharmacol Sci* 24:284–289
- Weir BS (1996) *Genetic data analysis II: methods for discrete population genetic data*. Sinauer Associates, Inc., Sunderland, pp 98–99
- Yu Y, Ge N, Xie M, Sun W, Burlingame S, Pass AK, Nuchtern JG, Zhang D, Fu S, Schneider MD, Fan J, Yang J (2008) Phosphorylation of Thr-178 and Thr-184 in the TAK1 T-loop is required for interleukin (IL)-1-mediated optimal NFkappaB and AP-1 activation as well as IL-6 gene expression. *J Biol Chem* 283:24497–24505