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Rubella vaccine-induced cellular immunity: evidence of associations with polymorphisms in the Toll-like, vitamin A and D receptors, and innate immune response genes

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Abstract

Toll-like, vitamin A and D receptors and other innate proteins participate in various immune functions. We determined whether innate gene-sequence variations are associated with rubella vaccine-induced cytokine immune responses. We genotyped 714 healthy children (11–19 years of age) after two doses of rubella-containing vaccine for 148 candidate SNP markers. Rubella virus-induced cytokines were measured by ELISA. Twenty-two significant associations (range of *P* values 0.002–0.048) were found between SNPs in the vitamin A receptor family (RARA, RARB, TOP2B and RARG), vitamin D receptor and downstream mediator of vitamin D signaling (RXRA) genes and rubella virus-specific (IFN- γ , IL-2, IL-10, TNF- α , and GM-CSF) cytokine immune responses.

A TLR3 gene promoter region SNP (rs5743305, -8441A > T) was associated with rubella-specific GM-CSF secretion. Importantly, SNPs in the TRIM5 gene coding regions, rs3740996 (His43Tyr) and rs10838525 (Gln136Arg), were associated with an allele dose-related secretion of rubella virus-specific TNF- α and IL-2/GM-CSF, respectively, and have been previously shown to have functional consequences regarding the antiviral activity and susceptibility to HIV-1 infection. We identified associations between individual SNPs and haplotypes in, or involving, the RIG-I (DDX58) gene and rubella-specific TNF- α secretion. This is the first paper to present evidence that polymorphisms in the TLR, vitamin A, vitamin D receptor, and innate immunity genes can influence adaptive cytokine responses to rubella vaccination.

Introduction

Innate immunity is recognized to play an important role in the response to viral pathogens. Viral infection or live viral vaccination activates host immune responses through cellular membrane bound proteins and other host sensor molecules, which stimulate various signaling pathways that induce production of cytokines (and chemokines) (Biacchesi et al. 2009). Produced by different cell types, cytokines are essential for the development and orchestration of both innate and adaptive immunity (Smith and Humphries 2009). Therefore, it is important to determine whether cytokine immune responses following live rubella virus vaccine could be influenced by polymorphisms in host innate genes. Evidence for the involvement of host gene polymorphisms has been improved by the identification of polymorphisms in HLA genes that influence humoral and cellular (cytokine) immune responses to rubella vaccine (Ovsyannikova et al. 2009a, b). Not studied in the context of rubella vaccine-induced immunity is the genetic diversity of vitamin A and D receptor and innate immune response genes that might contribute to the heterogeneity of vaccine-induced immunity.

Toll-like receptors (TLRs), which are largely distributed on several immune cells, including macrophages, dendritic cells and lymphocytes; trigger innate immune responses and are involved in host defense against pathogens. Of the 11 human TLRs, those central to antiviral innate immunity include TLR3 (which recognizes viral double-stranded RNA), TLR7 (which recognizes viral single-stranded RNA) and TLR4 (which recognizes envelope components of viruses) that are important for viral recognition (Katze et al. 2008).

Vitamins A and D (and their receptors) mediate the immunoregulatory properties of retinoic acid and 1,25-dihydroxyvitamin D3, respectively, which have hormone-like attributes and influence innate and adaptive immune responses (Mora et al. 2008; Cantorna and Mahon 2005; Geissmann et al. 2003; Villamor and Fawzi 2005). When dietary supplementation with vitamin A is given to children, there is an improved antibody response to some vaccines (Villamor and Fawzi 2005; Rahman et al. 1999; Bahl et al. 2002; Benn et al. 2002). The active form of vitamin D3 acts through binding and the activation of the nuclear vitamin D receptor (VDR) which is expressed by activated B and T lymphocytes (Mora et al. 2008). Recent studies demonstrate that vitamin D deficiency predisposes children to respiratory infections and that VDR gene haplotypes might influence the risk of HIV-1 acquisition (de la Torre et al. 2008). Vitamin D has also been proposed to be a “seasonal stimulus” to explain the seasonality of epidemic influenza (Hope-Simpson 1981; Cannell et al. 2006). Other studies have shown that activated vitamin D stimulates expression of antimicrobial peptides (AMP) in human monocytes and neutrophils, and TLR triggering of a vitamin D-mediated antimicrobial response in humans (Liu et al. 2006; Wang et al. 2004). Thus, it is important to determine whether cytokine immune responses following rubella vaccine are influenced by polymorphisms in vitamin A or D and their receptor genes.

Interferon- α/β production in infected cells is important for resistance to viral infection and can be triggered through the cytoplasmic RNA helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Yoneyama and Fujita 2007; Loo et al. 2008). The cytoplasmic helicase protein RIG-I (also known as DDX58) has been implicated in viral dsRNA and 5'-tri-phosphate (5'-ppp) containing viral RNA recognition and demonstrated to act through the mitochondrial antiviral signaling protein VISA (MAVS/Cardif/IPS-1) (Pichlmair et al. 2006).

Human TRIM5 and TRIM22 are members of a well-conserved tripartite motif protein family. The innate antiviral factor TRIM5 is known to inhibit the replication of some retroviruses, including HIV-1, during its interaction with the viral capsid protein and is characterized by marked amino acid diversity (Speelmon et al. 2006; Goldschmidt et al. 2006). TRIM22 is an E3 ubiquitin ligase and is involved in both innate and adaptive immune responses against pathogens (Eldin et al. 2009).

In this study, we examined the association of polymorphisms in candidate innate immune response genes to rubella vaccine-specific cytokine responses. These genes include the Toll-like receptor (TLR) family proteins (TLR3 and TLR4), vitamin A (retinoic acid) receptor family (RARA, RARB and RARG), modulator of retinoic acid receptor alpha function (type II topoisomerase beta, TOP2B) located downstream of the RARB gene, vitamin D (1,25-dihydroxyvitamin D3) receptor (VDR), downstream mediator of vitamin D signaling (RXRA), retinoic acid-inducible protein I (RIG-I) pathway (DDX58, CASP10 and VISA) antiretroviral TRIM factors (TRIM5 and TRIM22), and apoptotic CASP8. In this paper, we sought to determine whether cytokine immune responses in healthy children following rubella vaccination were influenced by polymorphisms in these immune response candidate genes.

Materials and methods

Study subjects

Our study cohort comprised a combined sample of 738 subjects from 2 independent age-stratified random cohorts of healthy children and young adults from all socioeconomic strata, identified by Minnesota Independent School District 535, in Rochester. As previously described, in 2001–2002, we enrolled 346 healthy adolescents (age 12–18 years) in Rochester, MN, USA (cohort 1) (Ovsyannikova et al. 2004). Three hundred and forty-two parents agreed to allow their adolescents to take part in the current rubella vaccine study. In 2006–2007, we enrolled a new cohort of 396 healthy adolescents and young adults (age 11–19 years) in Rochester, MN (cohort 2). All 738 study subjects (combined cohort) had a written medical record of having received two age-appropriate doses of live measles-mumps-rubella (MMR) vaccine containing the Wistar RA 27/3-strain ($TCID_{50} \geq 1,000$) of rubella virus (Merck Research, West Point, PA, USA) (Ovsyannikova et al. 2009a). The participants lived in a community where no case of rubella infection had been reported during their life-times. While 738 study subjects were enrolled in the study, genotyping data were available for only 714 subjects. A single venipuncture to obtain blood samples for DNA and peripheral blood mononuclear cells (PBMC) was approved by the Mayo Clinic Institutional Review Board (IRB). The Mayo Clinic IRB granted approval for the study, and written informed consent (parental permission and assent from minors) was obtained at the time of enrollment in the study.

Immune assays

Cytokine IL-2 ($n = 713$), IL-4 ($n = 691$), IL-5 ($n = 691$), IL-6 ($n = 713$), IL-10 ($n = 713$), IL-12p40 ($n = 711$), IFN- γ ($n = 713$), TNF- α ($n = 713$), and GM-CSF ($n = 711$) secretion levels in response to rubella virus stimulation (W-Therien strain, a kind gift from Dr. Teryl Frey,

Georgia State University) were determined in PBMC culture supernatants by ELISA. We used pre-optimized conditions for time of incubation and multiplicity of infection (MOI) for each cytokine (Ovsyannikova et al. 2009a). All cytokines were tested at an MOI of 5 with the exception of TNF- α , which was tested at an MOI of 0.05. The optimal length of culture used for the different cytokines was as follows: for IL-12p40 and GM-CSF (18 h); for IL-4, IL-5, IL-6, and IL-10 (24 h); for IFN- γ (2 days), and for TNF- α and IL-2 (8 days). Rubella-specific cytokine responses were quantitatively determined in cell-free supernatants by ELISA following the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA, USA). For all cytokine outcomes, we obtained three rubella virus stimulated measures and three unstimulated measures. Median background levels from unstimulated control cell cultures were subtracted from the median rubella-induced responses to calculate corrected secretion values. Negative corrected values indicate that the unstimulated secretion levels were, on average, higher than the rubella virus stimulated secretion levels.

Where sufficient cells were available, ELISPOT assays were performed for the detection of rubella-specific IFN- γ and IL-10 secreting cells using commercially available kits (Human IFN- γ ELISPOT kit, R&D Systems, Minneapolis, MN, USA and human IL-10 ELISPOT kit, BD Biosciences, San Diego, CA, USA) in samples obtained from 719 and 725, respectively, of the 738 subjects. The assays were performed in PBMC cultures as previously described (Ryan et al. 2005; Ovsyannikova et al. 2009a), following the manufacturer's protocol. The cells were stimulated in triplicate with the live W-Therien strain of rubella virus at a MOI of 2.5 or phytohemagglutinin (5 μ g/ml PHA, Sigma) as a positive control. PBMC cultured in triplicate in the absence of live attenuated rubella virus were used as negative controls in each assay. Spot forming cells (SFC), i.e. rubella-specific cytokine-producing cells were detected 24 h later by scanning and analyzing plates on an ImmunoSpot[®] S4 Pro Analyzer (Cellular Technology Ltd., Cleveland, OH, USA) using ImmunoSpot[®] version 4.0 software (Cellular Technology Ltd.).

TagSNP selection

We selected tagSNPs from innate immune response candidate genes ($n = 14$) belonging to the TLR family (TLR3 and TLR4), vitamin A receptor family (RARA, RARB, TOP2B, and RARG), vitamin D receptor (VDR), downstream mediator of vitamin D signaling (RXRA), RIG-I pathway (DDX58, CASP10 and VISA), antiretroviral TRIM factors (TRIM5 and TRIM22), and apoptotic CASP8. The details of our SNP selection have been described previously (Dhiman et al. 2008b). Briefly, we used a linkage disequilibrium (LD) tagSNPs selection approach (Carlson et al. 2004) to generate a list of SNPs within and 10 kb upstream and downstream of these 14 genes using the Hapmap Phase II (<http://www.hapmap.org>), Seattle SNPs (<http://pga.mbt.washington.edu/>) and NIEHS SNPs (<http://egp.gs.washington.edu/>) as source databases. We included SNPs that had validation data, successful predictive genotyping scores for Illumina Golden-Gate assays, a minor allele frequency (MAF) ≥ 0.05 and a pairwise LD threshold of $r^2 < 0.90$ for Caucasians. We selected 153 potential SNPs in our candidate genes of interest using the ldSelect algorithm. We used the nomenclature described by den Dunnen and Antonarakis (2001) for all genotype variants.

Genotyping methods

Our genotyping methods have been previously described in detail (Dhiman et al. 2007). DNA (250 ng) was extracted from blood drawn from each study participant ($n = 738$). Genomic DNA samples were genotyped for 153 candidate SNPs selected from innate immune response genes using a custom designed 768-plex Illumina GoldenGate[™] assay (Illumina Inc., San Diego, CA, USA) along with SNPs from other gene families of interest (Dhiman et al. 2008b). All the SNPs selected for the custom Illumina panel had design scores >0.4 . A Corriel Trio DNA (mother: NA11875, father: NA10859, daughter: NA10858) and two other genomic DNA

controls were used as standards to review and refine clustering. These controls were genotyped on each plate, which allowed us to assess genotyping concordance of replicate subjects.

Illumina 10% GenCall scores >0.4 and call rates $>90\%$ were used as thresholds for the initial laboratory quality control. The data from genotype calls made by using Bead-Studio 2 software were transferred to SAS for further analysis. Our overall genotyping success rate for the Illumina 768-plex platform and Taqman platform was 94.53%. The study sample success rate was 96.75%. SNP-specific deviation from Hardy–Weinberg Equilibrium (HWE) was tested and we excluded any SNP that displayed violations of HWE ($P < 0.001$). Subject exclusions were made based on DNA quality ($n = 6$), complete genotyping failure on both platforms ($n = 4$) and low call rates below 95% ($n = 14$), leaving 714 subjects in the study.

We used PCR-based TaqMan assays (Applied Biosystems, Foster City, CA, USA) as the secondary platform to genotype SNPs ($n = 6$) that failed genotyping on the Illumina platform. All assays were performed according to the manufacturer's instructions and the results were analyzed on the ABI Prism 7900 using Sequence Detection Software (Applied Biosystems). Of these six SNPs, one also failed by Taqman, and four were excluded because the minor allele frequency was $<5\%$. This resulted in 148 SNPs available for analysis in 714 subjects.

Statistical methods

The purpose of the efforts reported here was to assess associations between genetic variation in candidate SNPs and levels of rubella cellular immune response. The following outcomes were examined: nine measures of rubella virus-specific in vitro cytokine secretion (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IFN- γ , TNF- α , and GM-CSF, each reported in pg/ml), and two measures of cellular immunity via rubella vaccine-induced memory cell frequencies (IFN- γ and IL-10, evaluated as count variables). Assessments of cytokine secretion and ELISPOT measures resulted in six recorded values for each of the outcomes of interest per individual: three prior to stimulation with rubella virus and three post-stimulation. For descriptive purposes, a single response measurement per individual was obtained for each outcome by subtracting the median of the three unstimulated values from the median of the three stimulated values. Data were summarized across individuals using frequencies and percentages for categorical variables, and medians and inter-quartile ranges for continuous variables.

Participants' genotypes were used to estimate allele frequencies for each SNP of interest. We assessed departures from HWE in Caucasian subjects using a Pearson goodness-of-fit test (Weir 1996). Estimates of pair-wise LD based on the r -squared statistic were obtained using Haploview software, version 3.32 (Barrett et al. 2005).

Separate analyses were carried out for each outcome of interest. Repeated measures approaches were implemented for each outcome, simultaneously modeling all six observed measurements. This was achieved by including the genotype variable in the regression model, together with a variable representing stimulation status. The resulting covariate reflecting the genotype-by-stimulation status interaction was then tested for statistical significance. These repeated measures models are similar to paired t tests, where they compare differences between the two stimulation states within each individual among groups of individuals defined by their SNP genotypes. In these models, we allowed for within-subject correlations without imposing any constraints on the nature of the correlations, using an unstructured within-person variance–covariance matrix. Primary tests of association assumed an ordinal (log-additive) SNP effect, based on the number of copies of the minor allele.

To further explore genomic regions containing statistically significant single-SNP effects, 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 (EM) algorithm, similar to the method outlined by Schaid et al. (2002). Haplotype design variables were then created by generating a matrix composed of the possible haplotypes for each person, weighting by the posterior probability of those possible haplotypes per person, and collapsing back to a single row per person. Analyses then proceeded using the repeated measures analyses as described above. Only haplotypes with estimated frequencies of greater than 1% were considered. 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-by-stimulation status interaction term.

All analyses adjusted for the following set of covariates potentially associated with immune response: age at enrollment, race, gender, age at first rubella vaccination, time from second vaccination to enrollment, and cohort status (cohort 1 vs. cohort 2). Due to data skewness, original cytokine secretion and CMI values were replaced with inverse cumulative normal (probit) transformed values in all linear regression models. 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, USA).

Results

Subjects demographics and cytokine immune responses

The demographic and cytokine immune variables of our study subjects have been previously described (Ovsyannikova et al. 2009a). The majority of the study population was white (91%), with 46% being female, and a median age at enrollment of 15 years. The median age at the first and second immunization were 15 months and 11 years, respectively, and the median time between last rubella immunization and sample draw was 5.8 years. Cytokine secretion patterns were skewed toward proinflammatory responses, characterized by higher levels of IL-6 and moderate levels of TNF- α and GM-CSF. Rubella virus-specific IFN- γ and IL-2 (Th1-like) cytokines were detected at a lower level, while IL-4, IL-5, IL-10 (Th2-like) secretion was suppressed, although still detectable in the case of IL-10. Rubella-specific IFN- γ and IL-10 ELISPOT T cell memory responses were barely detectable. We did not assess associations with IL-4, IL-5, IL-12p40 secretion, and ELISPOT responses since these were not detectable in our study.

Associations between SNPs in innate immunity genes and rubella-specific IFN- γ , IL-2 and IL-10 cytokine secretion

We found 30 SNPs significantly associated ($P < 0.05$) with variations in rubella virus-specific IFN- γ , IL-2 and IL-10 secretion levels (Table 1). Specific SNPs in the TLR3, vitamin A (RARB and RARG), RIG-I (DDX58) and TRIM 22 genes were associated with rubella-specific IFN- γ secretion levels. Minor allele variants of two regulatory SNPs (rs6822014, $P = 0.005$ and rs3775296, $P = 0.006$) in the promoter and 5'UTR of the TLR3 gene were associated with an allele dose-related decrease in secreted IFN- γ in response to rubella virus; however, these are not independent associations since these two SNPs are in high pairwise LD with each other ($r^2 = 0.92$). We found six significant SNP associations (range of P values 0.002–0.036) in intronic and UTR (5' and 3') gene regions belonging to the RARB gene (rs1997352 and rs1529672, $r^2 = 0.57$; and rs12636426) and the RARG gene (rs7398676 and rs10783561, $r^2 = 0.67$; and rs3741434) of the vitamin A receptor. Specifically, increased carriage of major allele G for rs12636426 ($P = 0.036$) and minor allele G for rs3741434 (116A > G, $P = 0.020$) located in the intronic and 3'UTR regions of the RARB and RARG genes, respectively, were associated with a dose-related decrease in IFN- γ levels. We also found significant associations (range of P values 0.024–0.038) between minor alleles of four SNPs (rs10813821, rs626214, rs592515, and rs9650702) located in the RIG-I (DDX58) gene and dose-related increases in IFN- γ secretion, although these are not independent associations since the first three SNPs are in LD

with each other (r^2 0.55–0.73). Similarly, minor allele T for 3'UTR SNPs (rs7948996 and rs12285602, r^2 = 0.35) in the TRIM22 gene, also demonstrated a significant allele dose relationship toward a higher IFN- γ response.

We also examined SNP associations with rubella virus-specific IL-2 secretion levels (Table 1). Two of the seven identified significant SNP associations were within genes of the TRIM system. Increased representation of major allele T for a coding-synonymous SNP in the TRIM22 gene (rs2291842) was associated with an allele dose-related decrease in IL-2 levels (P = 0.049). Increased representation of minor allele A for a coding-nonsynonymous SNP in exon 2 of the TRIM5 gene (rs10838525, Gln136Arg) demonstrated significant association with variation in rubella-specific IL-2 levels (P = 0.045). In addition, SNPs in the TLR3 (rs13126816), vitamin A [RARA (rs9303286 and rs12946680, r^2 = 0.85) and RARB (rs7648325)], and RIG-I pathway [VISA (rs4815617)] genes were associated with rubella-specific IL-2 secretion levels.

We found six significant associations (range of P values 0.023–0.044) in UTR or intronic SNPs, all belonging to genes of the vitamin A receptor beta system [RARB (rs1058378, rs1881706, rs12630664, rs1286733, {rs17526942 and rs1286729, r^2 = 0.98})] and three additional SNPs belonging to the RIG-I [VISA (rs7262903)], retinoid X receptor alpha [RXRA (rs3118536)] and downstream of RARB topoisomerase (DNA) II beta [TOP2B (rs72624894)] genes and rubella virus-specific IL-10 secretion levels (Table 1). Increased representation of minor allele A for a coding-nonsynonymous SNP of the VISA gene (rs7262903, Gln198Lys) was associated (P = 0.028) with an allele dose-dependent decrease of IL-10 secretion (Table 1).

We also identified eight RARB haplotypes with frequencies $\geq 1\%$ in our study subjects (Table 2). A haplotype analysis suggested associations between IL-10 secretion and the RARB haplotypes that approached significance (global P value 0.081). The RARB haplotype GAAGGGCC was significantly associated (P = 0.006) with lower (t statistic -2.75) rubella-specific IL-10 secretion, while the AGTAG AGA haplotype had a suggestive association (P = 0.067) with higher (t statistic 1.83) secretory IL-10 levels.

Associations between SNPs in innate immunity genes and rubella-specific TNF- α , GM-CSF and IL-6 cytokine secretion

We found 25 SNPs significantly associated ($P \leq 0.05$) with secreted levels of rubella virus-specific proinflammatory cytokines, TNF- α , GM-CSF and IL-6 (Table 3). SNPs in the VDR, TLR3, DDX58 and TRIM5 genes were associated with rubella-specific TNF- α secretion levels. Two VDR SNPs (rs11568820 and rs7970314, r^2 = 0.92) were associated with variations in rubella-specific TNF- α secretion. The minor allele for rs11568820 located in the promoter region of the VDR gene was associated (P = 0.020) with an allele dose-related decrease in TNF- α secretion levels, an important mediator of immune and inflammatory responses. Further, increased representation of the minor allele of the promoter SNP (rs7970314, P = 0.032) in the VDR gene was also associated with an allele dose-related decrease in TNF- α secretion. A single 3'UTR SNP (rs1914926, P = 0.031) in the TLR3 gene was associated with a minor allele dose-related decrease in rubella virus-specific TNF- α levels. The most striking association was observed between a coding nonsynonymous SNP with known functional significance (rs3740996, His43Tyr) in the TRIM5 gene, associated with an allele dose-related decrease in rubella virus-specific TNF- α secretion levels (P = 0.027).

We found six significant associations (range of P values 0.009–0.040) between intronic and 5' UTR region SNPs located in a LD block with known functional significance, all belonging to the RIG-I gene [DDX58 (rs592515 and rs6476363 and rs3824456, $r^2 \geq 0.74$), (rs3739674 and rs10813829 and rs4633144, r^2 0.16–0.74)] pathway system and variations in TNF- α secretion levels.

Given that several significant SNP associations were within the DDX58 gene region, we performed a focused haplotype analyses. The haploview output for the genotyped SNPs in the DDX58 and their common haplotypes associated with rubella-specific TNF- α secretion are shown in Fig. 1. The global test from our haplotype analysis demonstrated a statistically significant association between DDX58 haplotypes and rubella-specific TNF- α secretion ($P = 0.030$) (Table 4). The most common haplotype AGAA GAAGGG was significantly associated ($P = 0.011$) with lower (t statistic -2.56) rubella specific TNF- α secretion. In addition, the DDX58 haplotype TAGGCCGGGC was associated ($P = 0.034$) with higher (t statistic 2.13) rubella virus-specific TNF- α levels. Similarly, the haplotype TAAGGCACGC had a marginally significant association with higher TNF- α secretion in response to rubella virus stimulation (t statistic 1.85 , $P = 0.065$).

For GM-CSF, other proinflammatory cytokine, we found 12 significant SNP associations with variations in rubella virus-specific secretion and three of them were in coding SNPs, belonging to the innate genes DDX58 (rs10813831, Arg7Cys, $P = 0.015$), TRIM5 (rs10838525, $P = 0.050$) and TRIM22 (rs7935564, $P = 0.027$) (Table 3). Three significant ($P < 0.05$) associations were found between intronic SNPs in the vitamin A receptor [RARB (rs1153600 and rs1286756, $r^2 = 0.70$) and rs6793694] gene and secreted levels of GM-CSF in response to rubella virus stimulation. Three significant associations were also found between 5' region and intronic SNPs in the TLR3 (rs11721827, $P = 0.021$; rs5743305, $P = 0.050$) and TLR4 (rs10983754, $P = 0.048$) genes and secreted levels of GM-CSF. A single intronic SNP (rs6747918, $P = 0.048$) in the CASP8 gene was associated with variation in GM-CSF secretion levels.

Finally, three coding SNPs in the VISA, DDX58 and antiretroviral TRIM22 factor genes were associated with rubella-specific IL-6 secretion levels. Minor allele A for a nonsynonymous SNP (rs7262903, Gly198Lys) from the VISA gene was associated with an allele dose-related decrease ($P = 0.003$) in rubella virus-induced IL-6 secretion. Minor allele A for a nonsynonymous SNP (rs10813831, Arg7Cys,) from the DDX58 gene was associated ($P = 0.050$) with variations in IL-6 secretion levels. Finally, minor allele G for a synonymous SNP (rs2291842, Asp214Asp) in the TRIM22 gene was associated ($P = 0.028$) with variations in IL-6 secretion levels (Table 3).

Gene polymorphisms and evidence for cross-regulation of cytokine secretion patterns

It is widely appreciated that regulation of cytokine secretion patterns may be due to the cross-regulation of different T cell subsets (Mosmann and Coffman 1989; Mosmann 1991). To further investigate the impact of the innate immunity genes in controlling cross-regulation of rubella virus-specific cytokine immune responses, we investigated the potential role of specific SNPs in cross-regulation profiles of related secreted cytokines. Since several Th1/Th/ proinflammatory cytokines have regulatory properties that promote or block the expression of other cytokines, we examined and found evidence of an association between secreted IL-2 (Th1) and GM-CSF (proinflammatory) cytokines with a nonsynonymous TRIM5 SNP rs10838525 (exon 2, Gln136Arg). The TRIM22 synonymous SNP rs2291842 demonstrated significant associations with both IL-2/Th1 ($P = 0.049$) and IL-6/proinflammatory ($P = 0.028$) secretion. The DDX58 nonsynonymous SNP rs10813831 (Arg7Cys) demonstrated significant associations with both GM-CSF ($P = 0.015$) and IL-6 ($P = 0.050$) secretion levels. Regarding the regulation of IL-10/Th2 secretion (Table 1), the minor allele A for a nonsynonymous SNP in the VISA gene (rs7262903, Gln198Lys) was also associated with an allele dose-related decrease in IL-6 (proinflammatory) levels ($P = 0.003$, Table 3). We also found evidence of an association of rubella virus-specific IFN- γ (Th1) secretion levels with an intronic SNP (rs592515, $P = 0.038$) in the DDX58 gene; the same SNP rs592515 was also associated with a major allele dose-related decrease in TNF- α (proinflammatory) secretion levels ($P = 0.009$).

In addition, another intronic DDX58 SNP (rs9650702) associated with an allele dose-related increase in IFN- γ secreted levels ($P = 0.025$), was also significantly associated an allele-dose-related decrease in GM-CSF secretion ($P = 0.023$).

Discussion

Genetic polymorphisms play an important role in rubella vaccine-induced immune responses. The effect of innate immunity gene polymorphisms on cytokine responses induced by rubella vaccination is as expected, heterogeneous. Single nucleotide polymorphisms (SNPs) that effect innate and adaptive immunity play a significant role in the type and direction or bias of host response generated by live viral vaccination.

Data suggest that the capability of some persons to respond to TLR ligands may be impaired by SNPs within the TLR genes, resulting in an altered susceptibility to viral infection and/or viral vaccination (Schroder and Schumann 2005; Dhiman et al. 2008a). Our data provide evidence for associations of polymorphisms in promoter and intronic regions of TLR3 and TLR4 genes with rubella virus-specific cytokine immune responses, such as IFN- γ , IL-2, TNF- α , and GM-CSF. In our study, rs6822014 and rs3775296, both of which are in strong pairwise LD ($r^2 = 0.92$) in the TLR3 gene, appear to be important SNPs significantly associated with lower rubella IFN- γ secretion in an allele dose-related manner. Importantly, we identified a promoter polymorphism (rs5743305, -8441A > T) in the TLR3 gene, which was also associated with rubella virus-induced GM-CSF secretion. The same SNP, rs5743305, has been suggested to be a risk factor for lower immune responses to measles vaccine (Dhiman et al. 2008a). Specifically, the heterozygous variant for rs5743305 located in the 5' region of the TLR3 gene was found to be associated with low antibody and low lymphoproliferative responses to measles vaccination (Dhiman et al. 2008a). This provides enough confidence that rs5743305 in the TLR3 gene may play a role in viral immunity and may be an important factor influencing variations in humoral and cellular immune responses to both measles and rubella vaccines.

We also genotyped our study subjects for known SNPs in the vitamin A receptor family (RARA, RARB, TOP2B and RARG), vitamin D receptor (VDR) and downstream mediator of vitamin D signaling (RXRA) genes. Our data provide evidence for 22 associations of polymorphisms in promoter and intronic regions of vitamin A and vitamin D receptor genes and their downstream mediators of signaling with different measures of rubella-specific cytokine immune responses. Further, we identified individual SNPs and haplotypes in the vitamin A receptor (RARB) gene that appears to influence rubella virus-induced IL-10 secretion levels. In our study, an allele dose-related decrease in rubella virus-specific TNF- α secretion levels was observed with increased representation of the minor alleles for SNPs rs11568820 and rs7970314 (LD, $r^2 = 0.92$) located in the promoter region of the VDR gene. The rs11568820 (G > A) SNP is a known functional Cdx2 polymorphism due to its location in the binding site of transcription factor Cdx2 and is positioned in the promoter region, upstream of exon 1e (Yamamoto et al. 1999). Rs11568820 is a functional SNP affecting VDR transcription; the G allele can diminish VDR transcriptional activity relative to the A allele, but has not been studied in relation to vaccine-induced immunity (Arai et al. 2001). Recently, VDR gene haplotypes for rs11568820-G:rs4516035-A:rs10735810-C:rs1544410-G:rs17878969-L polymorphisms were found to be associated with protection from HIV-1 infection (de la Torre et al. 2008). These observations offer functional biological insights into our findings and suggest that proinflammatory immune responses to viral infection or live viral vaccination are influenced by functional polymorphisms in the VDR gene.

In our study, an allele dose-related decrease in rubella virus-specific GM-CSF/IL-6 responses and an allele dose-dependent increase in rubella TNF- α response was observed with increased

representation of the minor alleles for SNPs rs10813831 (Arg7Cys, located in the CARD domain) and rs3824456, respectively, in the DDX58 (RIG-I) gene. The same coding nonsynonymous DDX58 SNP, rs10813831, and intronic SNP, rs3824456; were recently shown to be associated with susceptibility to type 2 diabetes mellitus (Ling et al. 2007), suggesting a possible functional role for these SNPs or others in high LD with them. It has also been suggested that the IFN- β innate immune response to viral (Newcastle disease and Influenza viruses) infection in human dendritic cells is strongly dependent on the level of RIG-I (DDX58) and can be modified by a functional rs10813831 polymorphism (Hu et al. 2007; Wetmur et al. 2007). Further studies are needed to validate these results and rule out the possibility of false positive associations.

Since a variety of genetic variants may operate together to determine the outcome of vaccine-induced immune responses, it is logical to propose that the observed cytokine secretion effects in our study may be a result of combinations of SNP-defined alleles (i.e. multigenic model). Indeed, both global test and individual haplotype analyses revealed significant associations between DDX58 haplotypes and rubella virus-specific TNF- α secretion. The most common haplotype AGAAGAAGGG was associated with lower rubella-specific TNF- α secretion, while the TAGGCCGGGC haplotype was significantly associated with higher TNF- α secretion. These findings provide further evidence for involvement of genetic variants in the DDX58 (RIG-I) gene in the mechanisms underlying cellular (cytokine) immune responses to rubella vaccine.

The human apoptotic CASP8 gene, whose product is also known as caspase-8, encodes an interleukin-1 β converting enzyme (ICE)-related cysteine protease that is activated by the interaction with several death receptors (Grenet et al. 1999). We identified a SNP (rs6747918, -33635G > A) in the CASP8 promoter that is in complete LD with rs3834129 ($r^2 = 1.00$) and was associated with variations in rubella-specific GM-CSF secretion.

The tripartite interaction motif 5 α (TRIM5) has been recognized to play a role in immunity against retroviral (HIV-1) infection (van Manen et al. 2008; Sewram et al. 2009). Our data provide evidence for associations of polymorphisms in the TRIM5 gene with variations in rubella virus-specific immune responses (TNF- α , GM-CSF and IL-2). We identified two nucleotide polymorphisms in the TRIM5 gene coding regions (rs3740996 and rs10838525) that were associated with an allele dose-related secretion of rubella virus-specific TNF- α and IL-2/GM-CSF cytokines, respectively. As demonstrated in the literature, SNPs rs3740996 (His43Tyr) and rs10838525 (Gln136Arg) located in exon 2 have been reported to have functional consequences regarding the antiviral activity of TRIM5 and susceptibility to HIV-1 infection or disease progression (Sawyer et al. 2006; van Manen et al. 2008; Javanbakht et al. 2006; Goldschmidt et al. 2006). Our data suggest the functional importance of the TRIM5 gene and its genetic variants in rubella vaccine-induced cellular immunity.

From several genetic variants, we were able to demonstrate evidence for cross-regulation of different cytokines, which may be a result of independent effects or may reflect the complex cross-regulation in cytokine networks. IL-10 is a key immunoregulatory cytokine which modulates the production of proinflammatory cytokines (including IL-6), ameliorates excessive Th1 and CD8+ T cell responses and regulates Th2 responses (Couper et al. 2008). In accordance with this, we observed cross-regulation for IL-10 and IL-6 secretion levels by a single nonsynonymous genetic variant rs7262903 in the VISA gene. Similarly, IL-6 is a multifunctional mediator, known to redirect the immune system from innate to adaptive immune response via induction of IL-2-dependent antigen specific T helper and T cytotoxic cell proliferation and differentiation (Jones 2005; Kishimoto 2006). We also found evidence for cross-regulation of IL-6 and IL-2 secretion by a TRIM22 genetic variant (rs2291842). Innate GM-CSF plays a role in IL-6-dependent Th17 cell regulation and is known to enhance

IL-6-dependent survival of antigen-specific CD4⁺ T cells (Sonderegger et al. 2008). Accordingly, we demonstrated evidence for cross-regulation of GM-CSF and IL-6 secretion by a nonsynonymous SNP, rs10813831, in the DDX58 gene. Th1 immune response is directed and typified by the signature cytokine IFN- γ , but often involves TNF, and both cytokines were cross-regulated by a single RIG-I (DDX58) intronic SNP rs592515. These and other cross-regulation patterns observed in our study provide additional evidence that some genetic variants might be involved in the mechanisms underlying heterogenous rubella vaccine immune responses.

The major strength of our study design is the well-characterized homogenous study cohort with documented MMR vaccine coverage and no known wild type rubella virus circulating in the community. Another plus is the use of the LD tagSNP selection approach, which allowed us a high degree of confidence in inferring genotypes/haplotypes and associations for SNPs of interest. The quantitative immune profiling of our study subjects allowed us to look for allele dose-related variations as well as cross-regulation patterns for genetic variants, which increased our confidence in the observed associations. Nevertheless, we are conscious of some limitations to the present study. Our results cannot be extrapolated to other ethnic groups. The multiple testing issue could result in potential false-positive associations. Our analyses examined 148 SNPs across 6 immune measures, resulting in a total of 888 tests. Assuming independent tests of association (an assumption not entirely correct due to the LD structure of the SNPs and the correlated nature of the immune response measures), we would expect 44 associations to be statistically significant by chance alone, at the $P = 0.05$ level. Our study identified 55 significant associations with measures of rubella virus-specific cytokine immune response, which is suggestive that at least some of the observed effects are real. However, as with any statistical association, the study needs to be replicated in an independent cohort to validate the findings. Nevertheless, the observed allele-dose relationships and cytokine cross-regulation pattern, as well as the high biological plausibility of the SNP associations in light of what is already known suggest the validity of our results.

In conclusion, our data show significant associations between polymorphisms in TLR, RIG-I, vitamin A, vitamin D receptor and several other innate immunity genes, and cellular cytokine responses to rubella vaccination. These data regarding association of SNPs with rubella virus-specific cytokine production can be regarded as preliminary, and require replication in an independent cohort. Our findings with SNPs that have been previously found to have a significant functional role is further evidence of the role of these SNPs in infectious disease susceptibility and rubella vaccine-induced immunity. Such vaccine immunogenetic studies provide a starting point for understanding the complex interaction of genetic variants that influence host response to pathogens and vaccines. In turn, understanding genetic variations and their effect on immune response phenotypes will help to inform development of new vaccines and immunotherapies directed against infectious pathogens.

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Fig. 1.

The linkage disequilibrium output for DDX58 SNPs from Haploview. Haplotype block structure of the DDX58 (RIG-I) gene region in the study cohort. Three intronic (rs592515 and rs6476363 and rs3824456, $r^2 \geq 0.16$) and three 5'UTR/intronic (rs3739674 and rs10813829 and rs4633144, $r^2 \geq 0.92$) SNPs from the DDX58 gene were associated with TNF- α secretion. The LD block structure was analyzed using Haploview software, version 3.32. The r^2 color scheme is: white ($r^2 = 0$), shades of gray ($0 < r^2 < 1$), black ($r^2 = 1$)

Table 1
Associations between SNPs in innate immunity genes and rubella-specific IFN- γ , IL-2 and IL-10 cytokine secretion

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
IFN- γ						
RARB	rs1997352	Intron	CC	399	8.5 (3.2, 25.6)	0.002
			CA	244	10.4 (3, 23.4)	
			AA	48	4.2 (0.4, 12.8)	
TLR3	rs6822014	Promoter	AA	459	9.0 (3.1, 28.0)	0.005
			AG	207	8.3 (2.6, 18.0)	
			GG	26	6.6 (1.8, 11.1)	
TLR3	rs3775296	5'UTR	CC	464	9.0 (3.0, 28.2)	0.006
			CA	202	8.3 (3.0, 18.0)	
			AA	26	7.8 (1.8, 11.5)	
RARG	rs3741434	3'UTR	AA	526	9.0 (3.2, 25.6)	0.020
			AG	153	8.3 (2.2, 20.5)	
			GG	13	3.8 (2.6, 10.7)	
DDX58	rs10813821	Intron	GG	243	7.0 (2.3, 21.9)	0.024
			GA	322	8.4 (3.4, 23.3)	
			AA	127	11.6 (4.3, 27.5)	
DDX58	rs9650702	Intron	GG	436	7.9 (2.8, 22.0)	0.025
			GA	229	9.9 (3.6, 29.9)	
			AA	27	14.7 (4.0, 24.0)	
RARG	rs7398676	Promoter	GG	217	9.9 (3.4, 28.4)	0.026
			GT	323	7.9 (3.0, 23.2)	
			TT	150	8.7 (2.1, 21.7)	
TRIM22	rs7948996	3'intergenic	CC	425	7.8 (2.9, 21.7)	0.034
			CA	221	10.7 (2.8, 25.6)	
			AA	46	14.0 (4.5, 42.5)	
RARG	rs10783561	Promoter	TT	215	8.7 (2.2, 21.7)	0.034
			TA	328	7.8 (3.1, 23.5)	
			AA	147	11.6 (3.6, 28.4)	
RARB	rs1529672	Intron	CC	470	8.6 (3.1, 24.1)	0.036

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
DDX58	rs626214	Promoter	CA	200	10.2 (3.0, 22.0)	0.036
			AA	22	2.3 (−0.2, 13.1)	
			AA	192	6.7 (1.9, 21.7)	
			AC	334	8.9 (3.5, 22.4)	
			CC	165	10.3 (3.5, 27.5)	
RARB	rs12636426	Intron	GG	589	7.9 (2.9, 23.4)	0.036
			GC	95	10.6 (3.7, 23.7)	
			CC	8	16.5 (8.0, 126.4)	
			AA	226	6.5 (2.1, 21.3)	
			AT	226	9.9 (3.6, 28.4)	
DDX58	rs592515	Intron	TT	225	9.0 (3.6, 23.4)	0.044
			GG	569	8.3 (3.0, 22.4)	
			GA	111	11.5 (2.6, 32.4)	
			AA	12	21.3 (6.5, 46.3)	
IL-2						
RARA	rs9303286	Intron	GG	540	18.1 (8.0, 32.4)	0.026
			GC	136	16.3 (4.5, 27.3)	
			CC	14	14.4 (9.0, 24.5)	
VISA	rs4815617	Promoter	GG	557	17.1 (7.7, 29.3)	0.028
			GA	126	20.4 (8.1, 34.7)	
			AA	9	16.9 (8.4, 38.5)	
			GG	397	17.2 (7.6, 29.4)	
			GA	258	17.2 (7.9, 32.5)	
TLR3	rs13126816	Intron	AA	37	19.6 (9.2, 34.1)	0.032
			CC	522	18.2 (8.0, 32.0)	
			CG	153	15.6 (5.4, 27.3)	
RARA	rs12946680	Intron	GG	17	12.1 (9.0, 24.5)	0.040
			GG	271	19.2 (9.1, 32.8)	
			GA	348	15.1 (5.8, 29.0)	
			AA	73	19.1 (8.3, 33.2)	
			GG	224	15.9 (7.9, 30.8)	
TRIM5	rs10838525	Coding	GA	376	17.5 (6.9, 27.6)	0.045
RARB	rs7648325	5'intergenic	GA	224	15.9 (7.9, 30.8)	0.047
			GA	376	17.5 (6.9, 27.6)	

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
TRIM22	rs2291842	Coding synonymous, Asp214Asp	AA	92	22.8 (9.0, 37.0)	0.049
			AA	465	17.2 (7.0, 30.3)	
			AG	201	17.7 (8.3, 30.5)	
			GG	26	21.5 (10.7, 37.8)	
IL-10						
TOP2B	rs7624894	Intron	AA	547	4.2 (2.3, 6.9)	0.023
			AG	135	4.1 (2, 6.2)	
RARB	rs1058378	3'UTR	GG	10	6.0 (2.9, 15.9)	0.023
			AA	545	4.2 (2.4, 6.9)	
			AC	136	4.1 (1.9, 6.2)	
			CC	10	6.0 (2.9, 15.9)	
RXRA	rs3118536	Intron	CC	465	4.3 (2.3, 7.0)	0.024
			CA	208	4.0 (2.0, 6.1)	
			AA	19	3.4 (2.9, 6.7)	
			CC	488	4.5 (2.5, 6.6)	
VISA	rs7262903	Coding nonsynonymous, Gln198Lys	CA	190	3.8 (1.7, 7.0)	
RARB	rs1881706	Intron	AA	13	2.4 (1.4, 5.8)	0.029
			GG	331	4.3 (2.4, 7.0)	
			GA	292	4.2 (2.0, 6.4)	
			AA	68	3.9 (2.6, 6.2)	
RARB	rs12630664	Promoter	GG	602	4.2 (2.2, 6.7)	0.031
			GA	87	4.6 (2.5, 6.6)	
			AA	3	3.0 (−5.3, 10.1)	
			AA	258	4.0 (1.8, 6.6)	
RARB	rs1286733	Intron	AG	338	4.3 (2.4, 6.9)	0.041
			GG	96	4.5 (2.4, 6.6)	
			GG	539	4.1 (2.0, 6.6)	
			GA	141	4.6 (2.8, 7.1)	
RARB	rs17526942	Intron	AA	12	5.3 (4.9, 5.7)	0.043
			GG	541	4.1 (2.0, 6.6)	
			GA	139	4.6 (2.8, 7.1)	
			GA			

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
<hr/>						
A alanine, G guanine, C cytosine, T thymine						

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

^bIQR, interquartile range, values are in pg/ml measured by ELISA

^cOne degree-of-freedom ordinal *P* value from the repeated measures regression analysis adjusting for age at enrollment, gender, race, age at first MMR, time from second MMR to enrollment, and cohort status. Only statistically significant associations (*P* < 0.05) are presented

Table 2

Vitamin A receptor (RARB) haplotype associations with rubella virus-specific IL-10 cytokine secretion

RARB Haplotype ^a	Frequency	Test statistic (haplotype <i>t</i> statistic)	Allele <i>P</i> value ^b	Global <i>P</i> value
GAAGGGCA	0.506	−0.13	0.899	0.081
GAAGGGCC	0.063	−2.75	0.006	
GATGGGGC	0.031	−0.96	0.338	
GGTGGGCA	0.054	−0.95	0.342	
GGTGGAGA	0.051	0.36	0.723	
GGTGAGGA	0.148	1.18	0.240	
AGTAGAGA	0.106	1.83	0.067	
AGTAGAGC	0.013	1.16	0.246	

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

^aRARB genetic variants from left to right: rs1286729, rs1286733, rs1286735, rs17526942, rs3773438, rs1656463, rs1730218, rs1058378

^bOne degree-of-freedom ordinal *P* value from the repeated measures regression analysis adjusting for age at enrollment, gender, race, age at first MMR, time from second MMR to enrollment, and cohort status

Table 3

Associations between SNPs in innate immunity genes and rubella-specific TNF- α , GM-CSF and IL-6 cytokine secretion

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
TNF- α						
DDX58	rs592515	Intron	AA	226	22.5 (-13.1, 76.4)	0.009
			AT	226	26.0 (-5.4, 82.1)	
			TT	225	41.9 (-2.0, 100.0)	
DDX58	rs6476363	Intron	AA	235	41.9 (-2.0, 100.0)	0.009
			AG	312	25.1 (-6.8, 81.1)	
			GG	145	21.6 (-13.8, 85.3)	
DDX58	rs3739674	5'UTR	CC	271	39.2 (-2.0, 100.0)	0.012
			CG	312	25.4 (-8.6, 77.5)	
			GG	105	26.2 (-9.2, 93.9)	
DDX58	rs10813829	Intron	CC	276	39.5 (-2.0, 99.5)	0.013
			CA	310	24.8 (-10.0, 74.0)	
			AA	106	25.6 (-9.2, 109.3)	
DDX58	rs4633144	Intron	GG	277	38.7 (-2.2, 99.0)	0.020
			GA	305	25.9 (-7.6, 79.3)	
			AA	110	19.4 (-13.4, 93.9)	
VDR	rs11568820	Promoter	GG	428	33.3 (-2.5, 93.0)	0.020
			GA	231	25.4 (-14.9, 84.9)	
			AA	33	21.4 (-11.9, 65.9)	
TRIM5	rs3740996	Coding nonsynonymous, His43Tyr	GG	553	34.7 (-3.6, 95.6)	0.027
			GA	131	16.2 (-15.1, 65.9)	
			AA	8	-13.8 (-37.5, 61.5)	
TLR3	rs1914926	3'intergenic	GG	418	25.5 (-10.9, 82.1)	0.031
			GC	225	36.8 (-0.4, 108.2)	
			CC	49	24.7 (-7.0, 70.9)	
VDR	rs7970314	Promoter	AA	414	32.3 (-3.1, 89.8)	0.032
			AG	240	28.8 (-10.8, 95.5)	
			GG	38	17.7 (-13.1, 65.9)	
DDX58	rs3824456	Intron	GG	411	26.2 (-10.7, 89.8)	0.040

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
GM-CSF			GC	243	33.1 (-4.7, 88.7)	
			CC	37	50.4 (13, 100.2)	
DDX58	rs10813831	Coding nonsynonymous, Arg7Cys	GG	387	28.5 (23.9, 32.8)	0.015
			GA	262	27.7 (23.2, 32.2)	
			AA	39	25.8 (21.7, 30.3)	
			AA	492	27.8 (23.4, 32.0)	
TLR3	rs11721827	Intron	AC	179	28.6 (23.9, 33.6)	0.021
			CC	19	30.3 (25.4, 35.3)	
			GG	436	28.5 (23.9, 32.8)	
			GA	227	27.4 (22.8, 32.0)	
DDX58	rs9650702	Intron	AA	27	26.0 (22.1, 30.3)	0.026
			GG	294	28.0 (23.3, 32.9)	
			GA	308	28.3 (24.0, 31.9)	
			AA	88	26.0 (22.1, 31.6)	
RARB	rs1153600	Intron	AA	227	27.5 (22.5, 31.4)	0.027
			AG	331	28.4 (24.4, 32.7)	
			GG	132	28.1 (23.3, 33.7)	
			GG	232	28.0 (22.9, 32.6)	
RARB	rs1286756	Intron	GA	323	28.2 (24.0, 32.6)	0.031
			AA	135	27.4 (22.9, 32.2)	
			AA	218	28.0 (23.9, 33.3)	
			AC	348	28.2 (23.7, 32.7)	
TRIM22	rs12294511	Intron	CC	124	27.7 (22.3, 31.0)	0.041
			GG	261	29.4 (24.7, 33.0)	
			GA	338	27.6 (23.6, 32.5)	
			AA	91	24.9 (21.7, 31.4)	
RARB	rs6793694	Intron	GG	607	28.2 (23.4, 32.8)	0.048
			GA	81	26.4 (23.2, 30.5)	
			AA	2	29.5 (24.6, 34.3)	
			AA	173	28.7 (23.8, 32.5)	
TLR4	rs10983754	Promoter	AG	351	27.8 (23.5, 32.8)	0.048
CASP8	rs6747918	Promoter				0.048

Gene/secreted cytokine	SNP ID	Location	Genotype	N ^a	Median level, pg/ml (IQR) ^b	P value ^c
TRIM5	rs10838525	Coding nonsynonymous, Gln136Arg	GG	165	28.0 (23.3, 32.2)	0.050
			GG	269	28.4 (23.9, 33.3)	
			GA	348	27.7 (23.3, 32.2)	
			AA	73	27.4 (22.9, 30.7)	
TLR3	rs5743305	Promoter	AA	276	28.3 (24.0, 33.1)	0.050
			AT	308	27.8 (22.9, 31.8)	
			TT	106	28.1 (24.0, 32.7)	
IL-6						
VISA	rs7262903	Coding nonsynonymous, Gln198Lys	CC	488	3,683.5 (3,142.0, 4,062.5)	0.003
			CA	190	3,641.5 (3,124.8, 4,095.1)	
			AA	13	3,381.8 (3,369.1, 3,701.1)	
TRIM22	rs2291842	Coding synonymous, Asp214Asp	AA	465	3,671.5 (3,160.1, 4,033.3)	0.028
			AG	201	3,732.8 (3,099.0, 4,126.2)	
			GG	26	3,569.0 (3,115.1, 4,071.3)	
DDX58	rs10813831	Coding nonsynonymous, Arg7Cys	GG	387	3,679.5 (3,160.0, 4,105.7)	0.050
			GA	263	3,684.3 (3,099.0, 4,061.2)	
			AA	40	3,615.6 (3,057.4, 3,910.9)	

A alanine, G guanine, C cytosine, T thymine

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

^bIQR, interquartile range, values are in pg/ml measured by ELISA

^cOne degree-of-freedom ordinal P value from the repeated measures regression analysis adjusting for age at enrollment, gender, race, age at first MMR, time from second MMR to enrollment, and cohort status. Only statistically significant associations ($P < 0.05$) are presented

Table 4DDX58 (RIG-I) haplotype associations with rubella virus-specific TNF- α cytokine secretion

DDX58 haplotype ^a	Frequency	Test statistic (haplotype <i>t</i> statistic)	Allele <i>P</i> value ^b	Global <i>P</i> value
AAGGCCGGGC	0.065	-0.47	0.636	0.030
AGAGGCGGAC	0.043	-1.18	0.237	
AGAAGAAGGG	0.370	-2.56	0.011	
TAAGGCACGC	0.213	1.85	0.065	
TAAGGCGGAC	0.200	0.08	0.939	
TAGGCCGGGC	0.070	2.13	0.034	

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

^aDDX58 genetic variants from left to right: rs592515, rs6476363, rs669260, rs4633144, rs13300238, rs10813829, rs11795343, rs3824456, rs10813831, rs3739674

^bOne degree-of-freedom ordinal *P* value from the repeated measures regression analysis adjusting for age at enrollment, gender, race, age at first MMR, time from second MMR to enrollment, and cohort status