Published in final edited form as:

Vaccine. 2009 May 26; 27(25-26): 3359–3366. doi:10.1016/j.vaccine.2009.01.079.

Influence of host genetic variation on rubella-specific T cell cytokine responses following rubella vaccination

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Abstract

The variability of immune response modulated by immune response gene polymorphisms is a significant factor in the protective effect of vaccines. We studied the association between cellular (cytokine) immunity and HLA genes among 738 schoolchildren (396 males and 342 females) between the ages of 11 and 19 years, who received two doses of rubella vaccine (Merck). Cytokine secretion levels in response to rubella virus stimulation were determined in PBMC cultures by ELISA. Cell supernatants were assayed for Th1 (IFN- γ , IL-2, and IL-12p40), Th2 (IL-4, IL-5, and IL-10), and innate/proinflammatory (TNF- α , GM-CSF, and IL-6) cytokines. We found a strong association between multiple alleles of the HLA-DQA1 (global p -value 0.022) and HLA-DQB1 (global p -value 0.007) loci and variations in rubella-specific IL-2 cytokine secretion. Additionally, the relationships between alleles of the HLA-A (global p -value 0.058), HLA-B (global p -value 0.035), and HLA-C (global p -value 0.023) loci and TNF- α secretion suggest the importance of HLA class I molecules in innate/inflammatory immune response. Better characterization of these genetic profiles could help to predict immune responses at the individual and population level, provide data on mechanisms of immune response development, and further inform vaccine development and vaccination policies.

Keywords

Rubella vaccine; HLA alleles; Cellular responses; Cytokines; ELISA; ELISPOT

1. Introduction

Evidence suggests that the humoral and cellular immune responses to rubella virus vaccine are genetically determined [1,2]. Multiple genes linked, in part, to the human leukocyte antigen (HLA) system are determinative of the host response to rubella viral antigens by antibody production, lymphocyte proliferation and cytokine production. HLA genes located at chromosome 6p21.3 are the most polymorphic within the human genome [3]. The variability of immune responses modulated by HLA and other genes is a significant factor in the

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Financial disclosure: Dr. Poland is the chair of a DMSB for novel non-rubella vaccines undergoing clinical studies by Merck Research Laboratories.

development of the protective effect of rubella vaccine. We previously performed a twins study to estimate the heritability of antibody responses to rubella vaccine, and demonstrated that, holding environmental factors constant, almost 46% of the variation in antibody response to rubella vaccine was attributable to genetics [4,5]. Several of our studies have already explored associations between HLA polymorphisms and variations in humoral (IgG antibody) and cellular (lymphocyte proliferation, IFN- γ and IL-10 secretion) immune response to live attenuated viral rubella vaccine [1,2,6]. Associations of genetic factors, such as HLA genes, with measures of rubella vaccine-induced immunity have been documented for several HLA loci, but these reported relationships have been less consistent for class I than for class II alleles [1,2]. While both humoral and cell-mediated responses are important for immunity to rubella and protection against disease [7], cell-mediated immunity (CMI) and the contribution of HLA polymorphisms to the T cell cytokine status after two doses of rubella vaccination have been less well studied. To address the fundamental question of whether HLA polymorphisms are associated with variations in cellular immune response to rubella vaccine, HLA typing was first completed in a previously recruited cohort of the 342 healthy schoolchildren, age 12–18 years, after two doses of rubella vaccine (cohort 1). We then examined associations between HLA alleles and measures of rubella vaccine-induced cellular (cytokine and frequency of cytokine-secreting cells) immunity in an additional new cohort of 396 healthy children, age 11–19 years (cohort 2), which was then combined with the 342 previously recruited subjects from the first cohort. Here we report on associations of individual HLA class I and class II alleles with measures of cell-mediated cytokine immune responses in these subjects.

2. Materials and methods

2.1. Study subjects

As previously described, between December 2001 and August 2002 we enrolled 346 healthy children (age 12–18 years) identified through the Minnesota Independent School District 535 registration rolls in Rochester, MN (cohort 1) [1]. Three hundred forty-two parents agreed to allow their children to take part in the current rubella vaccine study, and from these 342 children we obtained a blood sample. In addition, in December 2006–August 2007, we enrolled an additional 396 healthy children and young adults (age 11–19 years) in Rochester, Minnesota (cohort 2). All 738 participants had documentation of having received two doses of measles–mumps–rubella (MMR) vaccine containing the attenuated RA27/3 Wistar strain of rubella virus (Merck). No known circulating rubella virus in the community was observed since the earliest year of birth for any subject. The Institutional Review Board of the Mayo Clinic approved the study, and written informed consent was obtained from the parents of all children who participated in the study, as well as written assent from age-appropriate children.

2.2. Cellular immune assays

Whole blood was collected from each subject in BD Vacutainer® CPT™ cell preparation tubes containing sodium citrate and peripheral blood mononuclear cells (PBMC) were isolated immediately after collection following the manufacturer's protocol (BD, Franklin Lakes, NJ). Isolated PBMC were re-suspended at a concentration of 1×10^7 cells/ml in GIBCO® RPMI 1640 containing L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 20% heat-inactivated fetal calf serum (FCS, HyClone, Logan, UT) and 10% DMSO (Protide Pharmaceuticals, Inc., Lake Zurich, IL). After cryopreservation, PBMC were thawed as previously described [8,9] for use in cellular immunity assays.

ELISPOT assays were performed for the detection of rubella-specific IFN- γ and IL-10 secreting T 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). The assays were performed in PBMC cultures as previously described [10,11].

Unfractionated PBMC were added, in triplicate, at a final concentration of 2×10^5 cells/well, and were stimulated with a multiplicity of infection (MOI) of 2.5 with live W-Therien strain of rubella virus (a gift from Dr. Teryl Frey, Georgia State University) and incubated for 24 h at 37 °C. Stimulation of PBMC with 5 µg/ml of phytohemagglutinin (PHA, Sigma), also measured in triplicate, was used as a positive control. PBMC cultured in the absence of rubella virus were used as negative controls in each assay. After development of the plates according to the manufacturer's protocol, the plates were scanned and analyzed on an ImmunoSpot® S4 Pro Analyzer (Cellular Technology Ltd., Cleveland, OH, USA) using ImmunoSpot® version 4.0 software (Cellular Technology Ltd.).

Cytokine (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IFN- γ , TNF- α and GM-CSF) secretion levels in response to rubella virus stimulation (W-Therien strain) were determined in PBMC culture supernatants by ELISA. Optimization experiments were performed as we have previously described [12] to determine the length in culture and rubella virus MOI that resulted in maximum secretion of each cytokine. Table 1 outlines the optimal cell culture conditions for infection of PBMC for detection of rubella-specific secretion of each cytokine. PBMC (2×10^5 cells/well) were cultured in triplicate in round bottom tissue culture plates in the presence of the optimal amount of live rubella virus as indicated in Table 1. As a negative control, PBMC were also cultured in triplicate in RPMI 1640 media containing 5% FCS (HyClone), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and 1 mM sodium pyruvate (Mediatech, Inc. Manassas, VA). Stimulation of PBMC with 5 µg/ml of PHA (Sigma) was used as a positive control for each subject. For the detection of secreted IL-4, PBMC were cultured in the presence of 2 µg/ml of monoclonal anti-human IL-4R antibody (R&D Systems) as we have previously described [13]. Cell-free supernatants were removed at the pre-determined optimal time point between 24 h and 8 days (Table 1). Rubella-specific cytokine responses were quantitatively determined by ELISA following the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA, USA). 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 stimulated secretion levels.

2.3. Molecular genotyping

Cohort 1 genotyping was performed several years ago, but genotyping methods were the same for both cohorts. Genomic DNA was extracted from fresh heparinized blood samples by conventional techniques using the Puregene® extraction kit (Gentra Systems). Classical HLA-A, -B and -C alleles typing was performed using High Resolution SSP (sequence-specific primer) A, B, and C Unitray® typing kits, respectively. The SSP products consisted of panels of primer mixes, where each primer mix contained one or more specific primer pairs, i.e. the allele- and/or group-specific primers, as well as a control primer pair matching non-allelic sequences in the samples. The control primer pair was used as an internal polymerase chain reaction (PCR) control to verify the efficiency of the PCR amplifications. Any ambiguities were resolved using the Forensic Analytical sequencing kit and AmbiSolv™ when needed (Invitrogen).

Class II HLA typing was performed with high resolution DRB1 SSP, DQA SSP, DQB1 SSP, DPA1 SSP, and DPB1 SSP Unitray® typing kits with the entire locus on a single tray (Invitrogen). PCR was followed by AmbiSolv™ when needed and analyzed using MatchTools software. All PCR amplifications were carried out on an ABI 377 and analyzed using MatchTools software. All reactions were run with negative controls and every 50th PCR reaction was repeated for quality control.

2.4. Statistical methods

The purpose of the efforts reported here was to assess associations between genetic variation in the HLA loci and variations in 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 units of pg/ml), and two measures of CMI via rubella vaccine-induced memory T cell frequencies (IFN- γ and IL-10, evaluated as count variables). Assessments of cytokine secretion and CMI 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, including HLA alleles, and medians and inter-quartile ranges for continuous variables. For each HLA locus, a test for deviation from Hardy–Weinberg equilibrium (HWE) was obtained using a permutation-based approach [14]. Loci with HWE *p*-values less than 0.01 were flagged as being potentially out of equilibrium.

Separate analyses were carried out for each outcome and each HLA locus. Alleles were grouped by HLA type and summaries for the measures of rubella immune response were obtained using medians and inter-quartile ranges. Individuals contributed two observations to these descriptive summaries: one for each of their two alleles. Associations between HLA alleles and immune response measures were then formally evaluated using linear regression models. In these models, regression variables were created for each HLA allele and were coded as 0, 1 or 2, according to the number of copies of the allele that a subject carried. Rare alleles, defined as occurring fewer than five times among all subjects, were pooled into a category labeled “other”. Repeated measures approaches were implemented for the cytokine secretion and CMI variables, simultaneously modeling all six observed measurements. These repeated measures models are similar to paired *t*-tests, in that they compare differences between the two states within each individual among groups of individuals defined by their HLA alleles. In these models, we allowed for within-subject correlations without imposing any constraints on the within-person variance–covariance matrix.

Differences in immune response among all alleles of each HLA locus were first assessed globally. This was achieved by simultaneously including all but one of the ordinal allele variables in a multivariable linear model, together with a variable representing stimulation status. The resulting covariates reflecting the allele-by-stimulation status interaction were then simultaneously tested for statistical significance. Following these global tests, we examined individual allele associations with immune response. Each allele was tested individually by including only the variable corresponding to that allele, as well as stimulation status and the corresponding interaction term, in separate linear models which accounted for repeated measures. These series of tests were performed in the spirit of Fisher’s protected least significant difference test; individual allele associations were not considered statistically significant in the absence of locus-specific global significance.

All global and allelic analyses were adjusted for covariates potentially associated with immune response. These variables were: age at enrollment, race, gender, age at first rubella vaccination, age at second rubella vaccination, and cohort status (cohort 1 versus cohort 2). Data transformations were used to correct for data skewness in all linear regression models. An inverse cumulative normal (probit) transformation was used for all cytokine secretion and ELISPOT outcome variables. All statistical tests were two-sided, and all analyses were carried out using the SAS software system (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Demographics

A total of 738 healthy children were included in our rubella vaccine genetic association analyses (Table 2). The majority of subjects were Caucasians (91%), and the median age at first and second rubella immunization was 15 months and 11 years, respectively. There were 396 (54%) males and 342 (46%) females in the study. We found that cytokine responses to rubella vaccination were not gender or age associated. Cytokine responses to rubella virus antigens were highly variable in the study population. Notably, rubella-specific IFN- γ ELISPOT and IL-10 ELISPOT T cell memory responses as well as ELISA IL-4, IL-5 and IL-12p40 secretion levels were extremely low and hardly detectable in our study subjects.

We found no violations of Hardy–Weinberg equilibrium for HLA class I (A, B, and C) and class II (DQB1) loci. However, a comparison of allele distributions for the DRB1, DQA1, DPB1, and DPA1 loci revealed potential departures from equilibrium ($p < 0.01$ for each). As a result, statistical comparisons involving the DRB1, DQA1, DPB1, and DPA1 loci should be viewed with a certain level of caution.

3.2. HLA polymorphisms and rubella virus-specific T cell memory responses

To determine if associations exist between IFN- γ and IL-10 producing T cells specific for rubella virus and HLA alleles among vaccinated subjects, both IFN- γ ELISPOT and IL-10 ELISPOT assays were performed (Table 3). For these analyses and for the subsequent cytokine secretion analyses, allele-specific results are not presented if the corresponding locus-specific global test was not statistically significant ($p < 0.05$) or suggestive ($p \leq 0.10$). For loci with at least suggestive associations, only those alleles significantly or suggestively associated with cytokine secretion are presented. The global p -value for HLA-A locus and IL-10 ELISPOT secretion was 0.097. IFN- γ responses to rubella by ELISPOT were not detected; however, the role of class I A*0101 ($p = 0.024$), A*0201 ($p = 0.028$), and A*3301 ($p = 0.026$) alleles in IL-10 ELISPOT responses have potential interest in future studies, since the presence or absence of certain HLA alleles may potentially influence T cell memory IL-10 responses.

3.3. Associations between HLA alleles and T-helper type 1 cytokine responses

The associations between HLA class I (A, B and C) and class II (DRB1, DQA1, DQB1, DPA1, and DPB1) alleles and Th1 cytokine (IFN- γ , IL-2, and IL-12p40) secretion levels were examined and statistically significant or suggestive associations are summarized in Table 4. The overall median rubella-specific IFN- γ , IL-2 and IL-12p40 secretion levels were 8.53 (interquartile range (IQR) 2.97, 23.41) pg/ml, 17.59 (IQR 7.73, 30.48) pg/ml, and 0.00 (IQR -7.15, 7.17) pg/ml, respectively. The global tests of significance suggested associations between IFN- γ secretion and HLA-A and DQB1 loci that approached suggestive significance (global p -values 0.082 and 0.081, respectively). Alleles A*2301 (median 12.15 pg/ml, $p = 0.049$) and DQB1*0302 (median 11.29 pg/ml, $p = 0.069$) had marginally significant associations with increased IFN- γ production. On the contrary, alleles HLA-A*3301 (median 3.35 pg/ml, $p = 0.023$), HLA-A*6801 (median 5.32 pg/ml, $p = 0.018$), and DQB1*0603 (median 4.99 pg/ml, $p < 0.001$) had significant associations with decreased IFN- γ production.

Ex vivo rubella virus stimulation induced recall rubella-specific IL-2 cytokine secretion from PBMC of previously vaccinated subjects. The global p -values for HLA-DQA1 and HLA-DQB1 loci and rubella-specific IL-2 production were 0.022 and 0.007, respectively (Table 4). Alleles DQA1*0201 (median 20.53 pg/ml, $p = 0.058$), DQA1*0301 (median 21.53 pg/ml, $p = 0.005$), DQA1*0303 (median 21.80 pg/ml, $p = 0.038$), DQB1*0202 (median 19.85 pg/ml, $p = 0.077$), and DQB1*0302 (median 21.51 pg/ml, $p = 0.005$) demonstrated significant associations with increased IL-2 production. In contrast, alleles DQA1*0102 (median 15.74

pg/ml, $p = 0.099$), DQA1*0103 (median 15.16 pg/ml, $p = 0.047$), and DQB1*0603 (median 14.34 pg/ml, $p = 0.003$) were associated with decreased IL-2 levels.

Lastly, little IL-12p40 release was detected after stimulation of PBMC with rubella virus; however, for the HLA-DPA1 locus and IL-12p40 secretion, the global p -value was significant ($p = 0.012$). Alleles DPA1*0103 (median 0.00 pg/ml, $p = 0.073$) and DPA1*0201 (median 0.00 pg/ml, $p = 0.008$) were associated with variation in IL-12p40 secretion and have potential interest in future studies, because IL-12 has been defined as the functional bridge between innate resistance and the antigen-specific adaptive immune response [15].

3.4. Associations between HLA alleles and T-helper type 2 cytokine responses

The associations between HLA class I and class II alleles and Th2 cytokine (IL-4, IL-5 and IL-10) secretion levels were examined and statistically significant or suggestive associations are summarized in Table 5. The overall median rubella-specific IL-4 and IL-5 secretion levels were 0.30 (IQR, -0.31, 0.96) pg/ml and 0.47 (IQR 0.00, 1.09) pg/ml, respectively; though both IL-4 and IL-5 were hardly detectable in our study subjects. The global p -value for the HLA-DPA1 locus and IL-5 secretion was significant ($p = 0.016$) and may warrant further investigation. Alleles DPA1*0103 (median 0.47 pg/ml, $p = 0.058$) and DPA1*0202 (median 0.81 pg/ml, $p = 0.010$) were marginally associated with higher IL-5 secretion. In addition, a marginally significant increase in the frequency of DPA1*0109 (median 0.21 pg/ml, $p = 0.023$) was found among subjects who failed to produce rubella-specific IL-5. However, these potential associations should be viewed with caution, due to IL-5 secretion detection below the sensitivity of the immunoassay.

The overall median production of IL-10 in our study subjects was 4.20 (IQR 2.29, 6.69) pg/ml. The global tests suggested associations between IL-10 secretion and the HLA-A locus that approached significance (global p -value 0.068). For example, allele HLA-A*0205 (median 4.57 pg/ml, $p = 0.106$) had a suggestive association with increased IL-10 secretion. In contrast, alleles HLA-A*1101 (median 3.95 pg/ml, $p = 0.060$) and HLA-A*6802 (median 2.02 pg/ml, $p = 0.009$) had marginally significant associations with decreased IL-10 production.

3.5. Associations between HLA alleles and innate/proinflammatory cytokine responses

We detected significant release of rubella-specific TNF- α , GM-CSF and IL-6 (Table 6). The median values for secretion of these cytokines were 29.74 pg/ml (IQR -7.00, 89.23), 28.04 pg/ml (IQR 23.56, 32.55), and 3680.99 pg/ml (IQR 3159.97, 4051.96), respectively. For class I loci and rubella-specific TNF- α production, the global p -values were 0.058, 0.035 and 0.023 for HLA-A, -B, and -C, respectively. Alleles A*0301 (median 24.05 pg/ml, $p = 0.064$) and A*1101 (median 23.59 pg/ml, $p = 0.099$) appeared to be marginally associated with lower TNF- α cytokine response, whereas allele A*2902 (median 62.24 pg/ml, $p < 0.001$) was significantly associated with higher TNF- α secretion. As shown in Table 6, our analysis by allele for the HLA-B locus identified associations between higher TNF- α secretion and B*1501 (median 38.35 pg/ml, $p = 0.068$), B*3901 (median 4.19 pg/ml, $p = 0.048$), B*4001 (median 7.11 pg/ml, $p = 0.012$), B*4102 (median 90.05 pg/ml, $p = 0.004$), and B*4403 (median 46.08 pg/ml, $p = 0.022$) alleles. In addition, alleles B*1302 (median 27.47 pg/ml, $p = 0.086$) and B*5301 (median -3.20 pg/ml, $p = 0.056$) had marginally significant associations with increased TNF- α production. For the HLA-C locus, alleles C*0303 (median 37.71 pg/ml, $p = 0.035$), C*1601 (median 57.19 pg/ml, $p = 0.028$), and C*1703 (median 91.45 pg/ml, $p = 0.002$) were associated with increased TNF- α secretion.

The associations between HLA alleles and levels of GM-CSF production by PBMC stimulated with rubella virus were also examined (Table 6). For the HLA-DQB1 locus and rubella-specific GM-CSF secretion, the global p -value for association failed to show a statistically significant

association ($p = 0.150$). The results of our subsequent exploratory analyses by allele for the DQB1 locus suggested a potential association with alleles DQB1*0501 (median 27.17 pg/ml, $p = 0.072$) and DQB1*0609 (median 20.13 pg/ml, $p = 0.004$) and lower GM-CSF production. However, these suggestive associations should be interpreted with caution due to the absence of a significant global test. We found no associations with any of the HLA alleles and rubella-specific IL-6 production.

4. Discussion

Widespread vaccination and development of herd immunity has dramatically reduced the incidence of rubella disease. Studies suggest that polymorphisms of HLA and other genes may account for variability in the development of the immune response to rubella virus vaccine [1,2,16]. It has been suggested that development of detectable antibody responses to rubella vaccine is serologic evidence of rubella immunity [17]. Regardless, details regarding the presence, longevity and level of variability of rubella vaccine-specific cell-mediated immunity remain unclear. Since immunological memory is the foundation of vaccination, it is also important to understand virus-specific memory T cell responses that are required for effective immunity. It is possible that the presence or absence of certain HLA alleles may influence long-term rubella immunity and the dynamics of virus-specific T cell memory outcome in vaccinated subjects.

Cellular immunity to rubella has been reported, as demonstrated by lymphocyte proliferation and by increased levels of IL-10 on day 30 after rubella vaccination [18,19]. In our study the extremely low levels of IL-5, the lack of detectable IL-4, moderate amounts of IFN- γ and GM-CSF and the high levels of IL-6 and TNF- α suggest the predominant development of an innate/Th1-type rubella vaccine-induced cellular immune response in these older children and adolescents. In addition, we detected very low levels of IL-12p40 secretion following stimulation of PBMC with rubella virus. The current data on IL-12 indicate that this cytokine is a required factor for Th1 cell generation and IL-12-induced IFN- γ production and may play a role in the generation of the antiviral and immunoregulatory effects of IL-12 [20,21]. Orange et al. [22] reported that low doses of IL-12 are active *in vivo* against lymphocytic choriomeningitis virus infection and are able to enhance the cytotoxic T lymphocyte (CTL) response against virus-infected cells. The production of a wide spectrum of rubella-specific innate and Th1 cytokines strongly suggests that these cytokines could have important functions in the host reactions to rubella virus vaccine and in the regulation of the immune response.

We demonstrated elsewhere that certain class I A (*0201, *2402, and *6801) alleles are associated with rubella vaccine IFN- γ secretion in 106 healthy children previously immunized with two doses of rubella vaccine [6]. In the current study, the A*6801 allele, which is a part of the A3 supertype [23], was also found to be associated ($p = 0.018$) with lower levels of rubella-induced IFN- γ secretion in this larger cohort of subjects. This is not surprising, since the 106 individuals in the initial study are a subset of the subjects in this current report. The association of the A*6801 allele with a decreased IFN- γ secretion has significance with respect to future rubella vaccine design since the frequency of the A3 supertype in Caucasian, African-American and Hispanic populations is approximately 21%, 16%, 15%, respectively [23].

Our study results suggest that specific class I and class II HLA alleles are associated with variations in cellular (cytokine) immune responses to rubella vaccine. Of special interest is the strong association in our subjects after rubella vaccination between multiple alleles of the HLA-DQA1 (global p -value of 0.022) and HLA-DQB1 (global p -value of 0.007) loci and variations in rubella-specific IL-2 cytokine levels. Although the involvement of these HLA molecules in the IL-2 response to rubella virus remains to be further explored, our findings show that DQA1 and DQB1 gene polymorphisms may influence variation in IL-2 secretion levels after rubella

vaccine. The potential relationships between alleles of the HLA-A locus and IFN- γ , IL-10, and TNF- α secretion levels also have possible interest in future studies, since the balance between IFN- γ , IL-10, and TNF- α production may determine whether effective immunity is established in rubella vaccinated individuals, and/or may influence clinical outcome, such as rubella infection [18,24–26].

Similarly, potential associations between several alleles of the HLA-DQB1 locus and IFN- γ and GM-CSF production point to a major role of DQB1 restriction in rubella antigen presentation to T cells. The notable relationships between alleles of the HLA-B and HLA-C loci and TNF- α secretion levels further confirm the importance of these class I molecules in innate/inflammatory immune response. Tumor necrosis factor (TNF, also known as TNF- α) is a key regulator of the inflammatory response and an important modulator (26 kDa glycoprotein) of the host response to bacterial, viral and parasitic infections [27]. Evidence suggests that physiologically, TNF is important for the normal response to viral and parasitic infections, but excessive levels of production can be damaging [28]. Recent studies have suggested that IL-6 cytokine may induce the differentiation of proinflammatory Th17 cells, which are characterized by CD4+T cells producing IL-17A [29,30]. Even though we found no associations between HLA alleles and rubella-specific IL-6 secretion, our previous study demonstrated associations between single nucleotide polymorphisms (SNPs) in IL-6 gene and variations in antibody responses to measles and rubella disease and/or vaccination [16]. These data together with the results reported in the present paper suggest that impact of the HLA (and other) genes on the immune response to rubella at the level of cytokine production should be further defined. Many statistical tests were performed for this study; therefore, the possibility of false-positive results cannot be excluded. Our analyses examined 11 rubella immune response outcomes, each on 8 HLA loci, resulting in a total of 88 global tests. Assuming independent tests of association, one would expect about 4 or 5 of these tests to be statistically significant at the $p = 0.05$ level and 9 of these tests at the $p \leq 0.10$ level. We found a total of 6 significant tests at the 0.05 level and 12 tests at the 0.10 level, slightly more than estimated. This provides us some assurance that at a minimum some of our results are not false positive. Therefore, future studies involving HLA alleles along with other immunological evaluations of cellular immunity are necessary to validate relationships between HLA gene polymorphisms and rubella vaccine immune responses.

In conclusion, our data enhance our understanding of the mechanisms underlying immune response variations following live viral rubella vaccination. The results of this study suggest that further investigation into the role of HLA molecules in rubella cellular immune responses, particularly the role of cytokines, should be pursued.

Acknowledgments

We thank the Mayo Vaccine Research Group and subjects who participated in our studies. We thank Cheri A. Hart for her editorial assistance. This work was supported by NIH grants AI 48793, AI 33144 and 1 UL1 RR024150-01 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health, and the NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH.

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Table 1

Optimal cell culture conditions for infection of PBMC for detection of rubella-specific cytokine secretion.

Cytokine	Rubella virus MOI	Length in culture
IL-2	5	8 days
IL-4, IL-5	5	24 h
IL-6, IL-10	5	24 h
IL-12p40, GM-CSF	5	18 h
TNF- α	0.05	8 days
IFN- γ	5	2 days

MOI—multiplicity of infection.

Table 2

Characteristics of the study population.

Variable	Number of subjects (percent)
Overall	738
Age at enrollment	
11–13	219(30)
14–15	196(27)
16–17	205(28)
18–19	118(16)
Gender	
Male	396(54)
Female	342(46)
Race	
White, non-Hispanic	672(91)
Other	66(9)
Age at first rubella vaccination	
≤14 months	91(12)
15 months	394(53)
16–17 months	128(17)
≥18 months	125(17)
Age at second rubella vaccination	
≤5 years	209(28)
6–10 years	113(15)
11 years	129(17)
≥12 years	287(39)

Table 3
HLA allelic associations with rubella virus-induced T cell ELISPOT responses.

Locus	Allele	Allele counts	Median response (SFC 1 × 10 ⁶ cells)	First quartile (SFC 1 × 10 ⁶ cells)	Third quartile (SFC 1 × 10 ⁶ cells)	p-Value ^a	Global p-value
IFN-γ ELISPOT	Overall	1438	-20	-60	0		
	HLA-A						0.574
	HLA-B						0.707
	HLA-C						0.860
	HLA-DRB1						0.363
	HLA-DQA1						0.513
	HLA-DQB1						0.406
	HLA-DPA1						0.498
IL-10 ELISPOT	Overall	1450	5	-35	45		0.365
	HLA-A						0.097
	*0101	225	0	-45	50	0.024	
	*0201	412	10	27.5	50	0.028	
	*3301	10	-15	-50	20	0.026	
	HLA-B						0.341
	HLA-C						0.893
	HLA-DRB1						0.285
HLA-DQA1	HLA-DQB1						0.203
	HLA-DPA1						0.440
	HLA-DPB1						0.410
							0.935

^aLinear regression analysis. p-Values for cytokine levels were based on inverse cumulative normal transformation. Analyses adjusted for age at blood draw, gender, race, age at first rubella, age at second rubella vaccine, and cohort status. Only statistically significant or suggestive findings ($p \leq 0.10$) are presented. SFC—spot-forming cells per 1×10^6 T cells.

Table 4
HLA allelic associations with rubella virus-specific Th1 cytokine responses.

Locus	Allele	Allele counts	Median response (pg/ml)	First quartile (pg/ml)	Third quartile (pg/ml)	p-value ^d	Global p-value
IFN- γ HLA-A	Overall	1426	8.53	2.97	23.41		0.082
	*2301	25	12.15	5.25	32.44	0.049	
	*3301	9	3.35	-0.97	12.05	0.023	
HLA-B HLA-C HLA-DRB1 HLA-DQA1 HLA-DQB1	*6801	66	5.32	2.98	13.48	0.018	0.981 0.946 0.493 0.222 0.081
	*0302	161	11.29	4.25	23.51	0.069	
	*0603	94	4.99	1.93	11.46	<0.001	
HLA-DPA1 HLA-DPB1 IL-2 HLA-A HLA-B HLA-C HLA-DRB1 HLA-DQA1	Overall	1426	17.59	7.73	30.48		0.148 0.230 0.611 0.141 0.428 0.123 0.022
	*0102	287	15.74	6.27	29.28	0.099	
	*0103	100	15.16	4.24	25.73	0.047	
HLA-DQB1	*0201	155	20.53	10.01	32.20	0.058	0.005 0.038 0.007
	*0301	147	21.53	11.40	35.09	0.005	
	*0303	102	21.80	9.53	37.82	0.038	
HLA-DPA1	*0202	116	19.85	10.01	34.88	0.077	0.207
	*0302	161	21.51	10.72	35.09	0.005	
	*0603	94	14.34	2.71	25.63	0.003	

Locus	Allele	Allele counts	Median response (pg/ml)	First quartile (pg/ml)	Third quartile (pg/ml)	<i>p</i> -value ^a	Global <i>p</i> -value
HLA-DPB1							0.382
IL12-p40	Overall	1422	0.00	-7.15	7.17		
HLA-A							0.158
HLA-B							0.666
HLA-C							0.724
HLA-DRB1							0.360
HLA-DQA1							0.848
HLA-DQB1							0.894
HLA-DPA1							0.012
	*0103	1105	0.00	-6.89	7.32	0.073	
	*0201	205	0.00	-9.53	4.96	0.008	
HLA-DPB1							0.218

^aLinear regression analysis. *p*-Values for cytokine levels were based on inverse cumulative normal transformation. Analyses adjusted for age at blood draw, gender, race, age at first rubella, age at second rubella vaccine, and cohort status. Only statistically significant or suggestive findings (*p* ≤ 0.10) are presented. Statistically significant global *p*-values are in bold type.

Table 5
HLA allelic associations with rubella virus-specific Th2 cytokine responses.

Locus	Allele	Allele counts	Median response (pg/ml)	First quartile (pg/ml)	Third quartile (pg/ml)	Allele <i>p</i> -value ^d	Global <i>p</i> -value
IL-4	Overall	1382	0.30	-0.31	0.96		
HLA-A							0.779
HLA-B							0.857
HLA-C							0.376
HLA-DRB1							0.473
HLA-DQA1							0.951
HLA-DQB1							0.072
	*0601	11	0.59	0.36	1.77	0.009	
	*0604	56	0.34	-0.13	1.12	0.030	
HLA-DPA1							0.416
HLA-DPB1							0.356
IL-5	Overall	1382	0.47	0.00	1.09		
HLA-A							0.697
HLA-B							0.280
HLA-C							0.403
HLA-DRB1							0.521
HLA-DQA1							0.404
HLA-DQB1							0.389
HLA-DPA1							0.016
	*0103	1076	0.47	0.00	1.07	0.058	
	*0109	13	0.21	0.00	0.46	0.023	
	*0202	59	0.81	0.42	1.31	0.010	
HLA-DPB1							0.536
IL-10	Overall	1426	4.20	2.29	6.69		
HLA-A							0.068
	*0205	9	4.57	3.69	8.70	0.106	
	*1101	69	3.95	1.81	6.51	0.060	
	*6802	6	2.02	0.90	3.38	0.009	
HLA-B							0.661
HLA-C							0.673

Locus	Allele	Allele counts	Median response (pg/ml)	First quartile (pg/ml)	Third quartile (pg/ml)	Allele <i>p</i> -value ^a	Global <i>p</i> -value
HLA-DRB1							0.806
HLA-DQA1							0.783
HLA-DQB1							0.301
HLA-DPA1							0.152
HLA-DPB1							0.417

^aLinear regression analysis. *p*-Values for cytokine levels were based on inverse cumulative normal transformation. Analyses adjusted for age at blood draw, gender, race, age at first rubella, age at second rubella vaccine, and cohort status. Only statistically significant or suggestive findings (*p* ≤ 0.10) are presented. Statistically significant global *p*-values are in bold type.

Table 6
HLA allelic associations with rubella virus-specific innate/proinflammatory cytokine responses.

Locus	Allele	Allele counts	Median response (pg/ml)	First quartile (pg/ml)	Third quartile (pg/ml)	p-Value ^d	Global p-value
TNF- α HLA-A	Overall	1426	29.74	-7.00	89.23		0.058
	*0301	210	24.05	-22.98	67.46	0.064	
	*0302	5	-70.10	-86.95	-1.40	0.023	
	*1101	69	23.59	-0.70	70.87	0.099	
	*2902	47	62.24	7.62	123.66	<0.001	
HLA-B	*1302	33	27.47	-10.08	118.02	0.086	0.035
	*1501	106	38.35	0.41	88.74	0.068	
	*3901	15	4.19	-26.46	47.49	0.048	
	*4001	98	7.11	-18.99	47.70	0.012	
	*4102	6	90.05	43.67	211.19	0.004	
	*4403	65	46.08	7.62	123.66	0.022	
	*5301	5	-3.20	-13.13	8.47	0.056	
HLA-C	*0303	86	37.71	-3.57	79.30	0.035	0.023
	*1601	48	57.19	12.48	118.17	0.028	
	*1703	7	91.45	43.67	211.19	0.002	
HLA-DRB1							0.990
HLA-DQA1							0.758
HLA-DQB1							0.858
HLA-DPA1							0.905
HLA-DPB1							0.221
GM-CSF							
HLA-A	Overall	1422	28.04	23.56	32.55		0.247
HLA-B							0.997
HLA-C							0.708
HLA-DRB1							0.268
HLA-DQA1							0.437
HLA-DQB1							0.150

Locus	Allele	Allele counts	Median response (pg/ml)	First quartile (pg/ml)	Third quartile (pg/ml)	p-Value ^a	Global p-value
	*0501	169	27.17	23.30	31.59	0.072	
	*0609	5	20.13	18.39	21.37	0.004	
HLA-DPA1							0.254
HLA-DPB1							0.687
IL-6	Overall	1426	3680.99	3159.97	4051.96		
HLA-A							0.196
HLA-B							0.235
HLA-C							0.580
HLA-DRB1							0.296
HLA-DQA1							0.905
HLA-DQB1							0.953
HLA-DPA1							0.850
HLA-DPB1							0.457

^aLinear regression analysis. p-Values for cytokine levels were based on inverse cumulative normal transformation. Analyses adjusted for age at blood draw, gender, race, age at first rubella, age at second rubella vaccine, and cohort status. Only statistically significant or suggestive findings ($p \leq 0.10$) are presented. Statistically significant global p-values are in bold type.