Replication of rubella vaccine population genetic studies: Validation of HLA genotype and humoral response associations

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A R T I C L E   I N F O

Article history:
Received 21 June 2009
Received in revised form 27 August 2009
Accepted 31 August 2009
Available online 15 September 2009

Keywords:
Rubella vaccine
HLA antigens
Antibody formation
Adolescent

A B S T R A C T

Purported genetic associations found in population studies require validation for confirmation. We previously reported rubella vaccine-induced immune responses and HLA associations in 346 adolescents, age 12–18 years (1st cohort), following two doses of a rubella-containing vaccine. We sought to replicate the associations discovered in that work by verifying these associations in a new cohort of 396 subjects, age 11–19 years (2nd cohort), all having had two doses of a rubella-containing vaccine. We found that B*2705 (median 1st cohort 20.9 IU/ml, p = 0.028; 2nd cohort 20.5 IU/ml, p = 0.001) and DPA1*0201 (median 1st cohort 32.5 IU/ml, p = 0.048; 2nd cohort 25.8 IU/ml, p = 0.025) alleles were consistently associated with lower rubella-induced antibodies. Further, DPB1*0401 (median 1st cohort 43.3 IU/ml, p = 0.021; 2nd cohort 36.2 IU/ml, p = 0.002) alleles were associated with higher antibody levels in both populations. The association of DRB1*04-DQB1*03-DPB1*03 (mean 1st cohort 25.2 IU/ml, p = 0.011; 2nd cohort 21.4 IU/ml, p = 0.032) and DRB1*15-DQB1*06-DPB1*03 (1st cohort 16.3 IU/ml, p = 0.043; 2nd cohort 19.1 IU/ml, p = 0.023) haplotypes with lower rubella-specific antibodies was observed in both studies. This study provides confirmatory evidence for an association between specific class I and II HLA markers and haplotypes with rubella vaccine-induced humoral responses and lends further weight to their influence on rubella immune responses.

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1. Introduction

Immunity to rubella is an important public health issue worldwide. While it is well appreciated that polymorphic products of immune response genes, including the HLA region, determine variations in host susceptibility to infectious diseases [1], it is less appreciated that such genetic factors also determine variations in host immune responses to vaccines, including rubella [2]. We previously reported rubella vaccine-induced immune responses and HLA associations in 346 healthy adolescents after two doses of rubella vaccine [3–5]. These studies demonstrated that antibody responses to rubella vaccination are influenced by polymorphisms of the class I and II HLA genes. We sought to replicate the associations discovered in that work by verifying these associations in a new cohort of individuals. To confirm these findings, we undertook an independent study with a new cohort of 396 adolescents who received two doses of rubella vaccine and tested the hypothesis that the HLA associations discovered in the previous study would be replicated in a new cohort of similar subjects.

The lack of replication studies of allelic associations, including HLA genes, among different populations continues to cause difficulties in the identification of relationships between immune response gene polymorphisms and variations in adaptive immune responses to vaccines. Indeed, a large proportion of genetic associations fail to replicate in follow-up studies [6]. Although well recognized and accepted, the basis of such inter-population differences is not completely understood [7]. Validation of genetic associations is important not only for understanding mechanisms by which polymorphisms influence immune responses to vaccines, but also for the development of novel vaccine targets [7]. Still, it has not been easy to determine whether specific HLA alleles are consistent or reliable determinants of humoral (and cellular) immune responses to vaccines in different populations [8]. In addition to extreme HLA gene polymorphism, discordances, for instance, have...
come from disparities in ethnicity, sample sizes, laboratory assays for measures of vaccine-induced immunity, statistical methods and other factors [8]. Thus, replication studies in different populations are crucial for better understanding the importance of genotype–phenotype associations and to differentiate true positive from false-positive associations [9].

To address the basic question of whether these same HLA alleles, including HLA supertypes and haplotypes, are associated with antibody responses following two doses of rubella vaccine in an independent population, we took advantage of information obtained from two separate cohorts of adolescents who had participated in two studies of rubella vaccine-induced immunity and HLA gene associations.

2. Materials and methods

2.1. Study patients

As previously described, between December 2001 and August 2002, we enrolled 346 healthy adolescents (age 12–18 years) in Rochester, MN (cohort 1) [3]. Three hundred and forty-two parents agreed to allow their adolescents to take part in the current rubella vaccine study. From December 2006 to August 2007, we enrolled a new cohort of 396 healthy adolescents and young adults (age 11–19 years) in Rochester, MN (cohort 2). All 738 participants (combined cohort) 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 was observed since the earliest year of birth for any subject in our geographic area. The Institutional Review Board of the Mayo Clinic approved the study. Written informed consent was obtained from the adolescents aged 18 and 19. Written parental permission from the parents of adolescents less than 18 years of age was provided, as well as assent from the adolescents themselves.

2.2. Antibody measurement

Original quantitative levels of rubella virus-specific IgG antibodies for cohort 1 were determined by the Enzygnost anti-rubella virus/IgG EIA (Dade Behring, Germany). To ensure consistency in antibody measures across both cohorts, rubella-specific IgG antibodies after two doses of the rubella vaccine in both cohorts were re-assayed by whole virus rubella-specific chemiluminescent immunoassay (Beckman Coulter Access, Fullerton, CA) according to the manufacturer’s instructions. The limit of detection for this assay was 0.5 IU/ml and the coefficient of variation of this assay in our laboratory was 6%.

2.3. Molecular genotyping

Cohort 1 genotyping was performed several years prior to that of cohort 2, 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). Class I HLA-A, -B and -C allele typing was performed using High Resolution SSP (sequence-specific primer) A, B, and C UniTray typing kits, respectively. Any ambiguities were resolved using the Forensic Analytical sequencing kit and AmbioSolv™ when needed (Invitrogen). Class II HLA typing was performed with high resolution DRB1 SSP, DQB1 SSP, DPA1 SSP, and DPB1 SSP UniTray® typing kits with the entire locus on a single tray (Invitrogen). PCR was followed by AmbioSolv™ 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 analysis

The purpose of the efforts reported here was to validate associations of individual HLA alleles, supertype alleles, and haplotypes with rubella antibody levels that were reported previously in our original rubella cohort (cohort 1), using data from an additional new cohort of 396 children (cohort 2). Published results on cohort 1 subjects were based on 346 individuals. However, only 342 of these subjects provided consent for future research and so are included in the current report. Also, previous cohort 1 reports used the Dade Behring Enzygnost Anti-Rubella/IgG enzyme immunoassay kit to determine rubella-specific antibody levels. This assay could not be obtained for cohort 2 subjects, so the Beckman Coulter Access Rubella IgG assay was used in its place. For consistency, cohort 1 subjects were re-assayed using the Beckman assay. A comparison of the two assays found high levels of agreement. We found that the two assays had a high qualitative (95%) and quantitative correlation 0.93 (0.92, 0.95) [Greenwood NP, Ovsyannikova IG, Vierkant RA, O’Byrne MM, and Poland GA, unpublished]. Nevertheless, we wanted to ensure that the associations reported previously remained using the new assay and the smaller set of 342 subjects. Thus, all haplotypes and allelic analyses described below proceeded in the following three-step fashion. We first reanalyzed the 342 cohort 1 subjects using the Beckman assay and compared results to the original 346 subjects based on the Dade Behring assay. If the original and updated results were similar, we then attempted to validate the statistically significant or suggestive cohort 1 results (based on the 342 subjects and the Beckman assay) using cohort 2 subjects. We examined the same associations after combining cohorts 1 and 2 into one overall study group. To minimize the possibility of false negative results and a corresponding missed opportunity for validation, we relaxed criteria for designating cohort 1 associations as statistically interesting. All genetic effects associated with antibody levels with a p-value less than 0.2 in cohort 1 were followed up with association tests in cohort 2 and in the combined study group.

HLA four-digit alleles and supertype alleles were grouped by locus, and summaries of antibody levels were obtained using medians and inter-quartile ranges. Individuals contributed two observations to these descriptive summaries, one for each of their two alleles. Antibody levels were expressed in the original sampling units of IU/ml. Associations between HLA alleles and antibody measures were then formally evaluated using linear regression models. In contrast to the descriptive comparisons, each subject contributed one observation to these analyses, based on an observed genotype. Regression variables were created for each allele of interest and coded as 0, 1, or 2 according to the number of copies of the allele that a subject carried. Each allele variable was included in a separate linear regression analysis, effectively comparing antibody levels for the allele of interest against all other alleles combined. Due to data skewness, original immune response values were replaced with corresponding log-transformed values in the linear regression models.

Haplotype frequencies were created using a maximum likelihood approach. Due to the polymorphic nature of the HLA loci, examination of haplotypes using four-digit alleles was not possible. Thus, all haplotype analyses are based on two-digit HLA allele variables. Two sets of haplotypes were considered: one for the three class I loci (A, C, and B), and one for the three class II loci (DRB, DQB, and DPB). Because each individual’s linkage phase is unknown, there may be multiple pairs of haplotypes which are consistent with the observed HLA alleles. Posterior probabilities of all possible haplotypes for an individual, conditional on the observed geno-
Characteristics of the study cohorts by rubella antibody levels.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Level</th>
<th>Cohort 1 (n = 342)</th>
<th>Cohort 2 (n = 396)</th>
<th>Combined (n = 738)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Median (IU/ml)</td>
<td>Q1 (IU/ml)</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>342</td>
<td>38.8</td>
<td>19.7</td>
</tr>
<tr>
<td>Age at enrollment</td>
<td>11–13</td>
<td>72</td>
<td>40.1</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>14–15</td>
<td>95</td>
<td>39.2</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>16–17</td>
<td>97</td>
<td>40.3</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>18–19</td>
<td>78</td>
<td>34.5</td>
<td>19.2</td>
</tr>
<tr>
<td>Age at first rubella vaccination</td>
<td>≤14 months</td>
<td>32</td>
<td>37.3</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>15 months</td>
<td>171</td>
<td>31.8</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>16–17 months</td>
<td>66</td>
<td>47.0</td>
<td>18.9</td>
</tr>
<tr>
<td></td>
<td>≥18 months</td>
<td>73</td>
<td>39.9</td>
<td>22.8</td>
</tr>
<tr>
<td>Age at second rubella vaccination</td>
<td>≤5 years</td>
<td>31</td>
<td>20.7</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>6–10 years</td>
<td>33</td>
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<td>23.0</td>
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<tr>
<td></td>
<td>11 years</td>
<td>84</td>
<td>38.0</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>≥12 years</td>
<td>194</td>
<td>42.2</td>
<td>22.1</td>
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<tr>
<td>Gender</td>
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<td>161</td>
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<td></td>
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<td>181</td>
<td>31.5</td>
<td>18.9</td>
</tr>
<tr>
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<td>Other</td>
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<td>33.1</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>321</td>
<td>39.5</td>
<td>20.4</td>
</tr>
</tbody>
</table>

Q1 and Q3 represent the first (25th percentile) and third (75th percentile) quartiles, respectively. N represents number of subjects studied.

Table 1

3.2. Associations of HLA genotypes with rubella antibody levels

The associations between HLA alleles and rubella antibody levels were examined in some study cohorts. Only alleles found to be statistically or marginally significant in cohort 1 (p < 0.20) are presented.

Table 2 summarizes the strongest associations between individual class I and class II HLA alleles and rubella vaccine-induced humoral immune responses across each separate and combined cohort.

In cohort 1, individual alleles with the strongest association were B*2705 (median 20.9 IU/ml, p = 0.008), B*4501 (median 74.4 IU/ml, p = 0.039), DPA1*0201 (median 32.5, p = 0.048), DPB1*0401 (median 43.5 IU/ml, p = 0.021), DPB1*1301 (median 24.8 IU/ml, p = 0.046), DPB1*1501 (median 70.8 IU/ml, p = 0.018), and DRB1*1101 (median 56.2 IU/ml, p = 0.033). Specifically, B*2705, DPB1*0201, and DPB1*1301 alleles were associated with lower median levels of rubella vaccine-induced antibodies. Among these statistically significant alleles, the HLA-B*2705 (median 20.5 IU/ml, p = 0.001), DPB1*0201 (median 25.8 IU/ml, p = 0.025) and DPB1*0401 (median 36.2 IU/ml, p = 0.002) alleles were also associated with variations in antibody responses among rubella-vaccinated subjects in cohort 2, and in the same direction as the associations found in cohort 1. Associations were even stronger when combining data across cohorts. We found that HLA-B*2705 and HLA-DRB1*0201 alleles were significantly associated with lower rubella-induced antibody levels in both cohorts, and the DPB1*0401 alleles were significantly associated with higher antibody levels in both populations.
In cohort 1, individual alleles with marginally significant association (p-value between 0.05 and 0.20) were B*4002 (median 65.4 IU/ml, p = 0.09), B*5701 (median 55.8 IU/ml, p = 0.135), C*0704 (median 25.3 IU/ml, p = 0.064), DPA1*0104 (median 64.2 IU/ml, p = 0.098), DPB1*0202 (median 19.5 IU/ml, p = 0.109), DPB1*0301 (median 25.6 IU/ml, p = 0.128), and DQB1*0303 (median 26.8 IU/ml, p = 0.095). Among these, B*5701, DPB1*0202 and DPB1*0301 alleles were also associated with variations in antibody responses among rubella-vaccinated subjects in cohort 2, and in the same direction as the associations found in cohort 1. In the final analysis, associations for the B*5701 and DPB1*0301 alleles were significant at the p < 0.05 level when combining across cohorts. The B*4002, C*0704, DPB1*0202, DPB1*0303 alleles found to be interesting in cohort 1 were not validated in cohort 2. Finally, we were unable to examine A*2901, DPA1*0104, and DPB1*1501 alleles in cohort 2 due to low allele frequencies.

### 3.3. Associations of HLA supertypes with rubella antibody levels

Associations between the most common HLA class I A (A1, A2, A3 and A24) and B (B7, B27, B44, B58 and B62) super-types and rubella-specific IgG antibody levels for all study cohorts were also examined (Table 2). The B27 supertype (median 1st cohort 31.8 IU/ml, p = 0.072; 2nd cohort 25.9 IU/ml, p = 0.051) was marginally associated with lower rubella-specific antibodies in both cohorts 1 and 2. When combining across cohorts, this association reached statistical significance (median 29.6 IU/ml, p = 0.008). Further, the A3 supertype (median 35.0 IU/ml, p = 0.196) found to be marginally associated with lower antibody levels in cohort 1 was not validated in cohort 2.

### 3.4. Associations of HLA haplotypes with rubella antibody levels

We also analyzed potential relationships between HLA haplotypes and mean antibody levels in rubella vaccine study populations (Table 3). Mean rubella-specific IgG antibody levels for cohort 1 (n = 342), cohort 2 (n = 396), and a combined cohort (n = 738) were 39.2 IU/ml, 33.4 IU/ml and 36.0 IU/ml, respectively. Importantly, the association of DRB1*04-DQB1*03-DPB1*03 (mean 1st cohort 25.2 IU/ml, p = 0.011; 2nd cohort 21.4 IU/ml, p = 0.032) and DRB1*15/16-DQB1*06-DPB1*03 (mean 1st cohort 16.3 IU/ml, p = 0.043; 2nd cohort 19.1 IU/ml, p = 0.023) haplotypes with lower rubella-specific antibodies was observed in both studies. In a combined cohort, the DRB1*04-DQB1*03-DPB1*03 (mean 23.6 IU/ml, p = 0.005), and DRB1*15/16-DQB1*06-DPB1*03 (mean 18.3 IU/ml, p = 0.005) haplotypes were associated with lower levels of IgG antibodies. Further, the DRB1*04-DQB1*03-DPB1*04 (mean 42.3 IU/ml, p = 0.065) haplotype found to be interesting in cohort 1 was not validated in cohort 2.

Although our two cohorts have a fairly homogeneous racial composition, and although we adjusted for race in all statistical analyses, we were concerned about the possibility of residual confounding due to race. Thus, we ran a series of sensitivity analyses subset to Caucasian individuals. These results were virtually identical to those reported in Tables 1–3 (data not shown).

### 4. Discussion

Validation of population genetic associations is important for confidence that such associations are not spurious and for determining immunogenetic mechanisms by which polymorphisms influence immune responses to vaccines. A major shortcoming of many population-based studies involving a candidate gene approach, including HLA genes, is the lack of validation of the findings generated [7,11,12]. The present study is one of the few to examine the relationship between individual HLA alleles, HLA supertypes and HLA haplotypes and variations in humoral immune responses in a large group of healthy children after two doses of rubella vaccine and the first to validate the strongest associations found in our previous work in a new cohort of healthy
Table 3

<table>
<thead>
<tr>
<th>HLA Class</th>
<th>Haplotype</th>
<th>Combined (n=738)</th>
<th>Cohort 1 (n=342)</th>
<th>Cohort 2 (n=396)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-Value</td>
<td>Mean (IU/ml)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Overall</td>
<td>39.2</td>
<td>0.012</td>
<td>36.0</td>
<td>(25.6, 46.9)</td>
</tr>
</tbody>
</table>

Only haplotypes statistically or marginally significant (≥0.01) in cohort 1 are presented. Predicted means (95% confidence interval [CIs]) for individuals who carry one copy of the haplotype of interest. Analyses were carried out on log-transformed values; the values were then back-transformed to obtain appropriate means and CIs. *p*-value comparing haplotype of interest to all other haplotypes combined. Statistically significant findings (*p* < 0.05) are bolded.

To examine the independent associations of the HLA alleles presented in our manuscript with rubella antibody levels, we ran a series of multivariate linear regression analyses within our combined cohort of 738 subjects. We first re-assessed the association of B*2705 after adjustment for all other common B alleles (including all observed Bw4 alleles). After adjustment, B*2705 was still significantly associated with lower antibody levels (*p* = 0.025). We then ran one final linear regression model, simultaneously including the nine alleles found to be statistically significant in the combined cohort as reported in Table 2. All post-adjustment associations were in the same direction as reported in Table 2, and *p*-values were as follows: B*2705 (*p* < 0.001), B*4501 (*p* < 0.001), B*5701 (*p* = 0.07), DPA1*0201 (*p* = 0.31), DPA1*0401 (*p* = 0.02), DPA1*1301 (*p* = 0.25), DRB1*1501 (*p* = 0.02), and DRB1*1101 (*p* = 0.03). Each of these alleles, with the possible exception of DPA1*0201 and DPA1*1301, seems to be independently associated with rubella antibody levels after rubella vaccination.

A striking finding was the consistent association of HLA class II haplotypes with rubella-specific antibodies. Two HLA haplotypes were found to be consistently associated with rubella vaccine-induced humoral immunity. The association of DRB1*04-DQB1*03-DPB1*03 and DRB1*15/16-DQB1*06-DPB1*03 haplotypes with lower rubella-specific antibodies was observed in both studies. Our rubella vaccine study suggests that class II polymorphisms dominated the HLA associations observed. Consistent with this interpretation, these results provide evidence of possible multi-genic HLA class II associations with antibody levels after rubella vaccination.

There were other HLA associations that were found in one cohort but not validated in the other—these associations are more likely to be false-positive associations than those that were validated. These false-positive associations are perhaps due to statistical associations arising by chance, underlying systematic biases due to study design and technical artifacts [12]. In general, false-positive associations can be minimized both by using methods designed to control the false discovery rate and by utilization of a validation cohort. However, a sample size of 342 subjects in the first study and subsequent validation cohort of 396 subjects allowed us to define the most important relationships between HLA gene polymorphisms and humoral immune responses following rubella vaccine. Thus, performance of two independent studies of a single rubella vaccine allowed us the unique opportunity to assess and validate the contribution of HLA genes in immune responses to rubella vaccination. Given the importance of understanding the mechanisms behind differential vaccine-induced antibody responses, data from this study will also inform directed and rational development of novel rubella vaccines. Knowing and understanding genetic elements that control (or influence) variations in vaccine-induced immu-
nity allows prediction of individual and population-level immune responses to vaccine, including rubella [2,13].

In one of our earlier studies, we examined 100 twin pairs (45 monozygotic and 55 dizygotic) who had received one to two doses of MMR vaccine. We examined the heritability of immune responses to MMR and found that the heritability for rubella was 46%, whereas, the heritability for measles and mumps was 90% and 39%, respectively [14]. This suggests a strong genetic contribution to variation in rubella vaccine immune response. We were not able to estimate the total variance explained by the HLA system as a whole, because the number of polymorphisms across all the loci would overwhelm the number of enrolled subjects in any regression analysis. However, we were able to fit a series of locus-specific models to calculate the following $r^2$-squared values: HLA-A, 0; HLA-B, 0.0128; HLA-C, 0; DRB1, 0.0075; DQA1, 0.0052; DQB1, 0.0138; DPA1, 0.0152; DPB1, 0.0347. The sum of these $r^2$-squared statistics (0.0892) provides an upper bound for HLA contribution: the HLA system explains no more than (and most likely less than) 8.9% of the total variation in rubella antibody levels. Since the heritability for rubella was approximately 46%, this indicates that variation in the HLA loci only accounted for approximately 19% of the overall genetic variation in rubella antibodies.

Our study has several strengths. Both cohorts had documented MMR vaccination and no known wild type rubella viruses circulated in the community during their lifetimes. Both cohorts have adequate sample sizes to detect small-to-moderate genetic effects, thus lowering the risk of false negative results. Demographic and clinical characteristics were similar in the two cohorts, minimizing the possibility of discrepant results due to confounding effects of these attributes. Both cohorts have a fairly homogeneous racial composition (primarily Caucasians), minimizing the possibility of spurious results due to population stratification. However, this latter attribute also limits the inferential ability of our results to primarily Caucasian populations. Similar studies are needed in non-Caucasian populations to further validate these findings.

In conclusion, we report consistent associations with rubella vaccine-specific IgG antibody levels that validate our previously reported associations of HLA genetic polymorphisms on rubella vaccine antibody responses. A previously recruited sample and subsequent validation cohort allowed us to identify HLA markers associated with humoral immune responses following rubella vaccine. Replication of several associations allows confidence that specific individual HLA alleles and HLA haplotypes can now be considered as determinants of rubella vaccine-induced immunity. Findings from this study could inform and advance the design of improved viral vaccines and help to identify approaches for personalized vaccination [14,15]. Further, this information informs our understanding of genetic restrictions to immunity after vaccination and advances the knowledgebase in the new field of vaccinomics and adversomics [13,16].

Acknowledgments

We thank the Mayo Clinic Vaccine Research Group and subjects who participated in our studies. We thank V. Shane Pankratz, PhD for his assistance with this study. This work was supported by NIH grants AI 48793, AI 33144 and 1 U1L 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.

Conflict of interest statement: Dr. Poland is the chair of a safety evaluation committee for novel non-rubella 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