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The effect of HLA homozygosity on rubella vaccine-induced humoral and cell-mediated immune responses

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Abstract

Human Leukocyte Antigen (HLA) genes play a critical role in host immunity including vaccine responses. HLA molecules present antigenic peptides to T cells and provide inhibitory signals to NK cells, and polymorphisms within HLA genes allows for binding and presentation of a diverse array of self and foreign peptides. Heterozygosity across HLA alleles has been found to play a positive role in host defense for a variety of infections. Homozygosity within one or more HLA loci may restrict this epitope repertoire and limit T cell responses to infection or vaccination. Here we report that homozygosity within the HLA DPB1 locus is associated with increased levels of rubella-specific IgG, an effect driven by a common allele DPB1*0401. We also show that homozygosity within different HLA class I and class II loci is correlated with variations (but not necessarily decreases) in IL-2, IL-5, and IL-10 secretion following rubella virus stimulation.

Keywords

HLA Antigens; Homozygote; Rubella Virus; Measles-Mumps-Rubella Vaccine; Immunity

Introduction

The Human Leukocyte Antigen (HLA) class I and class II molecules present immunogenic epitopes to $CD4^+$ and $CD8^+$ T lymphocytes. The genes encoding the HLA molecules are extremely polymorphic with each allele capable of presenting slightly different peptide repertoires, that are determined by the molecular structure of the peptide binding domains. According to the heterozygote advantage theory, homozygosity at HLA genes should predict poor immune response to vaccines like rubella.

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Disclosures

Dr. Poland is the chair of a Data and Safety Monitoring Board (DSMB) 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. The other authors do not have any disclosures.

Genetic variation within these HLA loci can have profound effects on an individual's susceptibility to infection, autoimmune disorders, or response to vaccines[1–4]. Similarly, homozygosity within HLA could restrict the hosts' ability to present a wider repertoire of immunogenic peptides and result in diminished or ineffective cellular immunity as a result of missing epitope presentation. For example, homozygosity at HLA class I loci is associated with more rapid HIV progression [5–7], impaired $CD4^+$ T cell recovery after antiretroviral therapy [8], increased risk of HIV transmission to the fetus in pregnant women [9], and severe bacterial infections in sickle cell anemia patients [10,11] Likewise, heterozygosity at HLA class II loci is associated with protection against persistent disease after hepatitis B virus (HBV) infection [12], and may confer resistance to infection with hepatitis C virus (HCV)[13].

Rubella infections and congenital rubella syndrome (CRS) are rare in the United States but remain major health concerns in developing countries [14] and worldwide travel combined with poor adherence to vaccine recommendations results in continued exposures across the world[15]. In addition, the current two dose vaccination schedule of Measles, Mumps and Rubella vaccine (MMR) results in seroconversion rates to rubella over 95%, but a significant minority of individuals do not seroconvert or develop suboptimal antibody levels after vaccination[16–22].

Our laboratory has previously studied the role of genetic polymorphisms including HLA haplotypes and HLA homozygosity in immune responses to vaccines containing live viruses such as measles, mumps and rubella. We have previously reported that several HLA class II alleles are associated with variations in rubella-specific antibody levels as well as lymphoproliferative responses to rubella[23]. Additionally, individuals homozygous at either the HLA-A or HLA-DQA1 loci were more likely to be seronegative following measles vaccination[24]. Interestingly, two doses of MMR appear to overcome this "homozygote disadvantage"[25]. We also found that, following two doses of measles vaccine, HLA class II homozygosity was associated with increased IFNγ secretion[26]. Our objective for this study was to examine the effect of HLA homozygosity on immune responses to rubella virus after two doses of the MMR vaccine.

Materials and Methods

Subjects

The subjects for this study were recruited from Olmsted County, Minnesota where vaccination rates are high and there have been no cases of natural rubella infection in the community during the lifetime of the participants. The current study population came from two cohorts (stratified, random samples) of healthy children and young adults (11–19 years old) enrolled in private or public school in Olmsted County. 342 healthy children were enrolled between December 2001 and August 2002, while an additional 396 children were recruited between Dec 2006 – August 2007. The Mayo Clinic Institutional Review Board approved the study, and we obtained parental permission and subject assent (and for those 18 to 19 years of age) informed consent prior to enrollment. We reviewed the medical record of each participant for documentation of receipt of two age-appropriate doses of M-M-R® II (MEASLES, MUMPS, and RUBELLA VIRUS VACCINE LIVE) (Merck and Co., Inc, West Point, PA). We also determined that each participant was in good health at the time of enrollment. Once enrolled, we obtained a single blood sample from each of the 738 study participants.

Rubella IgG enzyme immunoassay

For each subject, serum aliquots were stored at −80° C until use. The samples were thawed and virus-specific rubella IgG levels were determined using an automated paramagnetic particle, chemiluminescent immunoassay (Beckman Coulter, Fullerton, CA) according to the

manufacturer's protocol. The assays were performed using a UniCel DxI 800 Access Immunoassay System (Beckman Coulter, Fullerton, CA). WHO reference standards for anti-rubella serum were used to determine the antibody level for each sample. For this assay the limit of detection was 0.5 IU/mL, and the coefficient of variation in our laboratory was 6.0%.

Preparation of peripheral blood mononuclear cells

The PBMC were isolated from venous blood using BD Vacutainer® CPT™ cell preparation tubes containing sodium citrate. Cells were purified by centrifugation and washed in RPMI medium with glutamine (Gibco, Carlsbad, CA) supplemented with 5% heat inactivated fetal calf serum (Hyclone, Logan, UT), 1 mM sodium pyruvate (Cellgro, Manassas, VA), 100 μg/mL streptomycin and 100 U/mL penicillin (Sigma, St. Louis, MO). The isolated cells were counted and resuspended in freezing media with 10% dimethyl sulfoxide (Protide Pharmaceuticals Inc., Lake Zurich, IL). One mL aliquots containing 10^6 cells were stored in liquid nitrogen until needed.

Cell culture

An aliquot of PBMC for each subject was thawed and resuspended in complete RMPI/5% FCS (supplemented with pyruvate, streptomycin, and penicillin as above). Trypan blue exclusion was performed to determine cellular viability and no difference was found between samples before and after storage in liquid nitrogen. Duration of incubation and multiplicity of infection (MOI) of rubella virus for each assay was optimized as previously described[23]. 2×10^5 cells for each subject in RPMI/5% FCS was added to seven wells of a 96-well plate for each of the cytokines studied. Rubella virus in complete RPMI/5% FCS was added to three of the seven wells for each subject for each cytokine studied to achieve an MOI of 0.05 for TNFα assays, and an MOI of 5 for the IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, GM-CSF, IFNγ assays. For each subject, PHA (Sigma, St. Louis, MO) was added to one well for each cytokine studied to a concentration of 5 μg/mL. Three of the seven wells were left as unstimulated controls for each subject for each cytokine studied. The plates were then incubated at 37 °C and 5% CO_2 for 18 hours for the GM-CSF and IL-12p40 cytokine assays, 24 hours for the IL-4, IL-5, IL-6, and IL-10 cytokine assays, 2 days for the INFγ cytokine assays, and 8 days for the IL-2 and TNFα assays. The supernatants were then transferred to a new 96-well plate, sealed and placed in a −80 °C freezer until the time that ELISA assays were performed.

Cytokine response by ELISA

The 96-well plates were thawed and the supernatants were tested using commercially available ELISA kits specific for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, GM-CSF, INFγ, and TNFα (BD Bioscience, San Jose, CA). All tests were performed according to the manufacturer's instructions.

Cytokine response by ELISPOT

Commercial ELISPOT kits for IL-10 (BD Bioscience, San Diego, CA) and IFN-γ (R&D Systems, Minneapolis, MN) were used as previously described[27,28]. Briefly, PBMCs were seeded in 96-well precoated ELISPOT plates at 2×10^5 cells per well. Unstimulated wells had medium only, stimulated wells were given rubella virus (W-Therien strain – kindly provided by Dr. Teryl Frey, Georgia State University) at an MOI of 2.5. PHA (5 μg/mL) was used as a positive control. The plates were placed in a 37°C and 5% CO_2 incubator for 24 hours and then developed as per manufacturer's instructions.

An ImmunoSpot S4 Pro Analyzer from C.T.L. (Cleveland, OH) was used to count the ELISPOT plates. Counting parameters were created separately for IL-10 and INFγ. The parameters included sensitivity, spot size threshold, background balance, and spot separation

[28]. A random selection of subjects was used to create the initial counting parameters for IL-10 and INFγ. These parameters were then held constant for all ELISPOT plates analyzed in the study. Quality control was performed by a single operator for each plate, removing debris and manually excluding overdeveloped areas.

HLA Typing

Genomic DNA was extracted from fresh blood using the Puregene® extraction kit (Gentra Systems). Classical HLA-A, -B and -C alleles typing was performed using High Resolution SSP (sequence-specific primer) UniTray typing kits, using an internal control primer pair to verify the efficiency of the PCR amplification. Ambiguities were resolved using the Forensic Analytical sequencing kit and AmbiSolv™ as needed.

Class II HLA typing (DRB1, DQA1, DQB1, DPA1, and DPB1) was performed using high resolution SSP Unitray® typing kits specific for the indicated loci. All typing reactions included negative controls and every 50^{th} reaction was repeated for quality control. When necessary, PCR was followed by AmbiSolv™ and analyzed using MatchTools software.

Statistical Analysis

The purpose of the efforts reported here was to assess associations between homozygosity in the HLA loci and rubella immune response. The following outcomes were examined: a measure of circulating rubella antibodies (reported as IU/mL); nine measures of rubella virus-specific *in vitro* cytokine secretion (IFNγ, IL-10, IL-2, IL-6, IL-4, IL-5, IL-12p40, TNFα, and GM-CSF, each reported in units of pg/mL), and two measures of cell-mediated immunity (CMI) via rubella vaccine-induced T cell frequencies (IFNγ and IL-10 ELISPOT results evaluated as count variables). Per manufacturer specifications, quantification of antibody levels resulted in one observation per subject. In contrast, 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 homozygosity status, and medians and inter-quartile ranges for continuous variables.

We compared levels of immune response in homozygous versus heterozygous individuals using linear regression models. Separate analyses were carried out for each immune response outcome. For measured antibody levels, which resulted in a single value per subject, simple linear models were used. For all other outcomes, repeated measures approaches were implemented in order to model all six observed values. To assess the associations of HLA homozygosity with *in vitro* changes in immune response from an unstimulated to stimulated environment within this repeated measures framework, we included in the statistical model the homozygosity variable of interest, an indicator of stimulation status, and the resulting interaction term. The strength of association was then formally tested by examining the statistical significance of the interaction term. 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 homozygosity status. In these models, we allowed for within-subject correlations without imposing any constraints on the nature of the correlations by fitting an unstructured variance-covariance matrix.

Variables representing locus-specific homozygosity status were created for each of the eight available HLA loci. We first fit separate models for each individual locus. We then created a variable indicating whether a subject was homozygous for at least one of the loci and assessed

its relationship with immune response. We then calculated a homozygosity count for each subject. Values of this count could theoretically range from 0 to 8, depending on the number of loci for which the subject was homozygous. However, due to sparseness of data in the higher count values, individuals homozygous for 5 or more loci were grouped with those homozygous for four loci. We used this count variable to assess the possible dose-response relationship between homozygosity and immune response by fitting the count (and, for the cytokine secretion and ELISPOT variables, the corresponding interaction term) as a one degree-of-freedom ordinal variable.

Primary analyses focused on homozygosity within the 4-digit HLA classification. However, secondary analyses were also carried out that examined associations with 2-digit homozygosity. All 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. Data transformations were used to correct for data skewness in all models. We used a log-transformation for antibody level data. This was not possible for the cytokine secretion and ELISPOT data due to the existence of zero or negative values. Thus, for these variables an inverse cumulative normal (probit) transformation was used. All statistical tests were two-sided, and all analyses were carried out using the SAS software system (SAS Institute, Inc., Cary, NC).

Results

Population demographics and rubella-specific immune responses

Our study population consisted of healthy children and young adults between 11 and 19 years of age. The vast majority (91%) was Caucasian and 54% were male. The median age at first and second rubella immunization was 11 months and 15 years, respectively. Both cellular and humoral immune responses to rubella (Table 1) were tested and included rubella-specific IgG levels in serum, and cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p40), GM-CSF, TNFα, IFNγ) secreted by PBMCs in response to rubella virus stimulation. Associations between individual HLA alleles and immune outcomes for this cohort have been analyzed and previously reported[23].

HLA homozygosity and humoral responses

Rubella vaccine induced strong humoral antibody responses in our study population. 644/738 (87.3%) of individuals were seropositive (defined as having a rubella-specific antibody levels ≥ 15 IU/mL) with 63/738 (8.5%) subjects being classified as seroequivocal (between 10 – 15 IU/mL) and only 31 (4.2%) subjects being seronegative (< 10 IU/mL). Thus, the majority of our subjects were seropositive following 2 doses of rubella vaccine. As expected, there was a considerable amount of inter-individual variation in antibody levels. Antibody levels ranged from 2.5 IU/mL to 329.8 IU/mL, with a mean titer of 49.87 IU/mL (IQR 19.2 – 63.7). Homozygosity at the DPB1 locus was significantly associated with variation in rubella-specific IgG levels. Individuals homozygous at the four-digit allele typing resolution had a median antibody level of 46.00 IU/mL (24.2, 75.4), while the lower level of antibody in heterozygous individuals was 32.7 IU/mL (18.3, 60.10) (See Table 2 showing statistically significant associations). The DPB1 association was also significant at the 2 digit allele typing resolution. In addition, homozygosity at the DPA1 locus was also associated with higher antibody levels (36.2 IU/mL [IQR 19.6 – 66.2] for homozygotes versus 32.2 IU/mL [IQR 18.10 – 59.0] for heterozygotes), although the association did not remain significant when analyzed at the 4-digit allele typing level.

HLA homozygosity versus specific HLA allele effects

We have previously reported associations between several HLA class II DPB1 alleles (*0301, *0401, *1301, *1401 and *1501) and variations in rubella specific antibody responses in a subset of the cohort described here[23]. Of particular importance is the DPB1*0401 allele that, together with DPB1*0402, are the most common alleles, present at a frequency of 20–60% in populations world-wide and >60% in North American and European populations. (http://www.allelefrequencies.net) The DPB1*0401 was equally common in our cohort, with an allele frequency of 41.0% (585/1426 allele counts). In fact, 17.8% (127/711) of our cohort was homozygous for DPB1*0401. Given that this allele, associated with higher levels of rubella-specific antibodies, was so prevalent in our cohort it is possible that the homozygosity effects that we observed can be attributed to possession of the DPB1*0401 allele rather than homozygosity at the DPB1 locus. To address this important question we reran any of our analyses demonstrating significant associations between immune outcome and carriage of either DPB1 or DPA1 (given the tight linkage disequilibrium between DPA1 and DPB1) and excluded all DPB1*0401 homozygotes (n=127) during the reanalysis. These revised results for homozygosity and rubella-specific antibody levels are also shown in Table 2. After exclusion of the DPB1*0401 homozygotes, none of the HLA homozygosity associations with rubella antibody level retained significant p values. It should be pointed out that for the DPB1 4-digit association the same trend of increased antibody level among the homozygotes remained, however, our revised analysis consisted of only 32 homozygotes. It is probable that we were underpowered to detect the significance of this trend. This conclusion is supported by the DPB1 2 digit allele analysis, showing the same trend, where a slightly larger pool of homozygotes (n=108) resulted in a smaller p-value. Nevertheless, it is clear that the association between homozygosity at the DPB1 locus and increased humoral immunity can be attributed, in large part, to the DPB1*0401 allele. These results also indicate the DPA1 associations are likely to have been driven by the fairly strong linkage disequilibrium between the DPA1 and DPB1 regions[29]. In fact, 97.6% (124/127) of the DPA1*0401 homozygotes were also homozygous for DPB1. Given these results we performed additional analyses (excluding homozygotes for each specific allele) for each of our significant homozygosity associations where previous reports showed an association between individual alleles for that locus and the immune outcome in question.

HLA homozygosity and cellular responses

While antibody levels to rubella and many other viral vaccines are considered the “gold standard” by which those vaccines are evaluated, cellular immunity also plays a role in protection. We studied the effect that homozygosity at various HLA loci had on markers of cellular immunity. Tables 3–6 list all significant associations between secreted cytokines and HLA loci. No significant associations were detected between homozygosity at HLA loci and the following cytokines: IFNγ, IL-4, IL-6, TNFα and GM-CSF.

Higher levels of IL-2 were associated with homozygosity at the 2-digit resolution for the HLA-DQB1 locus (p = 0.013; 18.24 pg/ml [IQR 8.71 – 33.94] for homozygotes versus 16.65 pg/ml [IQR 7.35 – 29.29] for heterozygotes). While this trend was the same for the higher resolution 4 digit DQB1 typing, it failed to reach significance (p = 0.071) (Table 3). We have previously reported that individual DQB1 alleles are associated with variations in IL-2 secretion [30] and these may introduce a similar confounding effect as DPB1*0401 did on antibody responses. Removal of the DQB*0202 homozygotes indicated that homozygosity at the 2-digit allele resolution for the HLA-DQB1 locus still resulted in higher levels of IL-2 secretion (p = 0.015; 18.88 pg/ml [IQR 8.71 – 33.94] for homozygotes versus 16.65 pg/ml [IQR 7.35 – 29.29] for heterozygotes). Homozygosity at the 2-digit allele resolution for the HLA-DQB1 locus was still significantly associated with lower levels of IL-2 after removal of the DQB*0302 homozygotes (p=0.028; 18.88 pg/ml [IQR 8.71 – 33.67] for homozygotes versus 16.65 pg/ml

[IQR 7.35 – 29.29] for heterozygotes). Although we have previously reported an association between DQB1*0603 and variations in IL-2 secretion after rubella stimulation, our current cohort had no DQB1*0603 homozygotes.

Homozygosity at HLA-A was associated with higher levels of IL-5 (0.62 pg/ml [IQR 0.00 – 1.07] for homozygotes versus 0.47 pg/ml [IQR 0.22 – 1.16] for heterozygotes), while homozygosity at HLA-DPA1 correlated with the opposite effect on IL-5 (0.46 pg/ml [IQR 0.00 – 1.03] for homozygotes versus 0.65 pg/ml [IQR 0.22 – 1.15] for heterozygotes) (Table 4). These associations held true even at the lower resolution 2-digit allele typing. In light of the aforementioned linkage between DPB1 and DPA1 and our previous reports showing an association between DPA1*0202 and IL-5 we reran our analyses excluding either DPB1*0401 or DPA1*0202 homozygotes and in both cases found similar results (Table 4). The levels of IL-5 we detected following rubella stimulation were quite low, and while we did find significant differences associated with homozygosity, the actual differences were minute and may not be clinically or biologically relevant.

For IL-10, homozygosity at the 2-digit level for the DPA1 locus showed a significant correlation with lower IL-10 production (4.08 pg/ml [IQR 2.11 – 6.69] for homozygotes versus 4.57 pg/ml [IQR 2.64 – 6.69] for heterozygotes) (Table 5). While the trend was the same when analyzed using the 4-digit resolution, the effect did not reach the level of significance ($p = 0.107$). We observed that homozygosity at any locus corresponded to a slight but significant decrease in IL-10 secretion. We also found that homozygosity at an increasing number of loci correlated with differences in IL-10 levels. As with the attenuation of the antibody associations, each of the associations between HLA and IL-10 depicted in Table 5 also diminished when the DPB1*0401 homozygotes were removed from the analysis. The DPA1 (2 digit) association p-value increased (from $p = 0.028$ to $p = 0.062$), the homozygosity at any locus p-value likewise increased (from $p = 0.043$ to $p = 0.08$), and the correlation between increasing numbers of homozygous loci and differential IL-10 levels also failed to reach significance (changing from $p = 0.027$ to $p = 0.150$).

HLA homozygosity associations with IL-12p40 levels were also identified; however for the majority of subjects, PBMC stimulation with rubella virus actually resulted in lower levels of IL-12 than those seen in the medium only controls. As shown in Table 6, the median levels for heterozygotes and homozygotes at the DPA1 locus were both 0 pg/mL although the IQRs varied slightly. Interestingly, each of the associations between HLA loci and IL-12p40 reached p values of greater significance after exclusion of the DPB1*0401 homozygotes. The DPA1 4 digit p value decreased from $p = 0.049$ to $p = 0.014$, while the 2 digit association likewise dropped from $p = 0.223$ to 0.074. Finally, the strength of the association between homozygosity at any locus increased (from $p = 0.046$ to $p = 0.012$). Unfortunately, the extremely low levels of IL-12p40 detected in our subjects make it difficult to attribute any clinical relevance to these associations.

In addition to testing cytokine secretion following rubella virus stimulation by ELISA, we performed IFNγ and IL-10 ELISPOT assays to further characterize rubella-specific cellular immunity. Unfortunately, IFNγ responses were undetectable by ELISPOT, and IL-10 responses were marginal at best, making it difficult to assign biologic significance to associations between HLA homozygosity and altered ELISPOT responses.

Discussion

Our laboratory and other research groups have identified associations between genetic factors such as HLA alleles and differential vaccine response outcomes. Homozygosity at HLA loci is thought to restrict the ability of the host to present a diverse array of peptide epitopes to T

cells and negatively impact disease susceptibility, resolution of infection and vaccine response. This phenomenon has perhaps best been studied with HIV [5,7–9], although HLA homozygosity has also been shown to have differential effects on HBV, HCV, measles, mumps and other viral infections[12]. To our knowledge the effect of HLA homozygosity on cellular immune responses to rubella has not been reported. In this study we identified several associations between HLA homozygosity and rubella vaccine response.

Regarding humoral immunity, homozygosity at the HLA class II locus DPB1 was significantly correlated with higher levels of rubella-specific IgG. We have previously examined the effect of homozygosity on a subset of our current cohort, and we reported a significant association between the DPB locus and higher antibody levels[25]. In this manuscript we have retested that smaller cohort using an alternate rubella IgG assay and higher resolution HLA typing procedures. These data were then combined with the results from a previously unstudied cohort and the resulting larger population was analyzed for the current report. With this in mind, it is not surprising that we again found the DPB1 locus associated with higher levels of rubella antibodies. In fact, the majority of the association between homozygosity at the DPB1 locus and increased humoral immunity can be attributed to the DPB1*0401 allele as the homozygosity associations disappear when DPB1*0401 homozygotes are removed from the analysis.

We also found that DPA1 homozygosity was associated with lower levels of both IL-5, and homozygosity at a class I locus, HLA-A was associated with slightly higher levels of IL-5 (0.62 pg/mL in homozygotes versus 0.47 pg/mL in heterozygotes). This association remains despite removal of either DPB1*0401 or DPA1*0202 homozygotes suggesting that it is homozygosity at this locus and not possession of these specific alleles that is driving the variations in IL-5 secretion.

While our initial analysis found significant associations between HLA homozygosity and decreased section of IL-10, once the DPB1*0401 homozygotes were removed from the analysis, the decreases in IL-10 secretion remained but the associations were no longer significant. There are no previous reports showing an association between DPB1*0401 and IL-10 secretion following rubella stimulation, and since the same outcome trend remained it is more likely that removal of 127 subjects decreased our power to the point that we were unable to detect these small effect sizes.

We found that homozygosity at the DQB1 locus was associated with higher levels of IL-2. In the case of DQB1, the 4-digit allele typing showed the same trend but did not reach significance ($p=0.071$). Interestingly this association remained even when DQB1*0202 or DQB1*0302 homozygotes were removed from the analysis.

These results provide further evidence that HLA homozygosity may affect immune responses to vaccines. We have previously reported that HLA homozygosity correlated with a decrease in measles-specific antibody levels after a single dose of MMR[24]. In fact, individuals homozygous for more alleles had progressively lower levels of antibodies. Interestingly, this heterozygote advantage disappeared when we studied individuals receiving the normal 2-dose regimen of measles vaccine, indicating that additional vaccine doses were able to overcome any homozygote disadvantage[25].

We have previously reported associations between individual HLA alleles and rubella vaccine response in part of this same cohort and comparing those results to the data reported here provides several points of interest. It is possible that these associations exhibit an allele-dose effect and that individuals homozygous for these alleles demonstrate an even greater degree of increased/decreased cytokine production. Exclusion of the large number of DPB1*0401 homozygotes caused the DPB1/lower antibody and most of the DPA1/cytokine homozygosity

associations to vanish. Therefore, care should be taken to account for the effect of common alleles when examining the role of homozygosity on vaccine response. On the other hand, correcting for potentially confounding alleles for the DQB1/IL-2, and DPA1/IL-5 homozygosity associations did not alter our findings indicating that homozygosity suggesting that HLA homozygosity can affect cytokine production by vaccine-elicited cellular immune responses.

There are a number of potential mechanisms for the effect of HLA homozygosity on cytokine production. T cell responses to pathogens are frequently directed against a number of immunodominant epitopes. Genetic variation within the HLA region results in a large number of HLA alleles each capable of binding to subtly different peptide repertoires. Homozygosity at any locus reduces the breadth of that peptide repertoire able to be presented by the host. This could be a two-edged sword, as an individual may not have the appropriate HLA allele capable of recognizing a critical epitope or may, in fact, have two copies of the “right” HLA allele. Further complicating the issue are the viruses that exhibit a high degree of sequence diversity such as influenza or HIV where HLA homozygosity leads to a reduced epitope repertoire and increases the chances of viral escape variants. Along similar lines, HLA alleles can bind the same epitope with vastly different binding affinities which can translate into high or low affinity T cells which are at a competitive advantage or disadvantage during the evolution of an immune response. Yet another mechanism of immune variation is the ability of HLA genes on infected cells to interact with NK cells and control their function[31]. One can add viral immune evasion factors to the list as well. A large number of viruses possess molecules capable of suppressing HLA antigen presentation and the molecular interactions and effectiveness of the viral protein may differ between HLA alleles[32,33].

Given the number of independent tests of association we have performed, it is possible that some of the associations we found are false positives. We found 13 associations (only 1 more than would be expected by chance alone). Of the associations between HLA homozygosity and differences in immune outcome, all showed the same trend regardless of the typing used for the tests of association (2-digit versus 4-digit allele specificities); given that the tests of association are structurally correlated, it is expected that the two-digit and four-digit associations would be similar.

Contrary to what one would expect from the premise of ‘heterozygote advantage’, many of our associations identified homozygotes with higher immune responses. This is similar to what we have previously reported for cellular immune responses to measles following two doses of MMR[26]. In that report homozygosity within HLA class II loci correlated with increased secretion of IFNγ in response to viral infection.

In conclusion, our results show that HLA homozygosity at class I and class II loci can be associated with variations in antibody and cytokine production both to the advantage and disadvantage of the host. Importantly, the strongest homozygosity associations were found with antibody responses and could be directly attributed to a single allele (DPB1*0401), already known to be associated with increased rubella antibody titers. These data support other findings which implicate HLA polymorphisms and homozygosity in altered susceptibility to disease and heterogeneity of vaccine response. It should be noted that several of the differences seen, while significant, were small and it remains to be determined whether or not these associations contribute to different clinical outcomes. Further insights into the genetic control of the immune response to rubella vaccination will help to identify individuals who remain unprotected after the current vaccination schedule, develop improved vaccination strategies for these susceptible individuals, and to better understand immunologic markers of protection from rubella infection.

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Table 1

Humoral and Cellular Immune Responses to Rubella in Vaccinated Subjects.

Immune outcome	Number of Subjects	Median Response (IQR)[a]
Rubella-specific IgG	738	34.4 IU/mL (19.2, 63.7)
IL-2	713	17.6 pg/mL (7.7, 30.5)
IL-4	691	0.3 pg/mL (−0.3, 1.0)
IL-5	691	0.5 pg/mL (0.00, 1.1)
IL-6	713	3,681.0 pg/mL (3160.0, 4052.0)
IL-10	713	4.2 pg/mL (2.3, 6.7)
IL-12p40	711	0.0 pg/mL (−7.2, 7.2)
GM-CSF	711	28.0 pg/mL (23.56, 32.6)
IFNγ	713	8.5 pg/mL (3.0, 23.4)
TNFα	713	29.7 pg/mL (−7.0, 89.2)

[a] IQR indicates the interquartile range.

Table 2

HLA Associations with Rubella Antibody Levels

Parameter		N	Median secretion in IU/mL (IQR)[a]		
Overall Rubella Antibody Level		738	34.4 (19.2, 63.7)		
HLA Locus	**Zygosity**	**All subjects**		**Excluding DPB1*0401[b]**	
		N	Antibody Level[a]	N	Antibody Level[a]
DPA1 (4 digit)	Heterozygous	250	33.20 (18.10, 59.90)	247	33.20 (18.10, 59.90)
	Homozygous	488	34.90 (19.55, 65.20)	364	32.15 (19.05, 61.80)
			p = 0.131[c]		p = 0.611[c]
DPB1 (4 digit)	Heterozygous	579	32.70 (18.30, 60.10)	579	32.70 (18.30, 60.10)
	Homozygous	159	46.00 (24.20, 75.40)	32	38.60 (21.35, 69.75)
			p <.001[c]		p = 0.635[c]
DPA1 (2 digit)	Heterozygous	219	32.20 (18.10, 59.00)	219	32.20 (18.10, 59.00)
	Homozygous	519	36.20 (19.60, 66.20)	392	33.20 (19.05, 62.20)
			p = 0.042[c]		p = 0.301[c]
DPB1 (2 digit)	Heterozygous	503	32.60 (18.10, 59.00)	503	32.60 (18.80, 59.00)
	Homozygous	235	43.30 (23.00, 75.90)	108	36.85 (20.95, 74.60)
			p <.001[c]		p = 0.080[c]

Rubella-specific Ab response for all loci with significant p-values at either the 2 or 4 digit typing resolution are shown. Bold indicate loci with p values < 0.05.

[a] Antibody level is measured in IU/ml, IQR indicates the interquartile range.

[b] Analyses repeated excluding all individuals homozygous for the indicated HLA allele.

[c] Repeated measures regression analysis adjusted for age, gender, race, age at first immunization, age at second immunization, and cohort status.

Table 3

HLA Associations with IL-2 Levels

Parameter		N	Median secretion in pg/mL (IQR)[a]				
Overall IL-2		713	17.59 (7.73, 30.48)				
HLA Locus	**Zygosity**		**All subjects**		**Excluding DQB1*0202**[b]		**Excluding DQB1*0302**[b]
		N	IL-5 secretion[a]	N	IL-5 secretion[a]	N	IL-5 secretion[a]
DQB1 (2 digit)	Heterozygous	504	16.65 (7.35, 29.29)	504	16.65 (7.35, 29.29)	504	16.65 (7.35, 29.29)
	Homozygous	209	18.24 (8.71, 33.94)	201	18.88 (8.71, 33.94)	201	18.88 (8.71, 33.67)
			p = 0.013[c]		**p = 0.015**[c]		**p = 0.028**[c]
DQB1 (4 digit)	Heterozygous	623	17.59 (7.71, 29.90)	623	17.59 (7.71, 29.90)	623	17.59 (7.71, 29.90)
	Homozygous	90	17.76 (8.02, 37.47)	82	18.08 (7.60, 39.26)	82	17.36 (8.02, 37.11)
			p = 0.071[c]		p = 0.08[c]		p = 0.182[c]

IL-2 response for all loci with significant p-values at either the 2 or 4 digit typing resolution are shown. Bold indicate loci with p values < 0.05.

[a] Cytokine secretion is measured in pg/ml, IQR indicates the interquartile range.

[b] Analyses repeated excluding all individuals homozygous for the indicated HLA allele.

[c] Repeated measures regression analysis adjusted for age, gender, race, age at first immunization, age at second immunization, and cohort status.

Table 4

HLA Associations with IL-5 Levels

Parameter		N	Median secretion in pg/mL (IQR)[a]				
Overall IL-5		691	0.47 (1.11, 1.09)				
HLA Locus	**Zygosity**		**All subjects**	**Excluding DPB1*0401[b]**		**Excluding DPA*0202 [b]**	
		N	IL-5 secretion[a]	N	IL-5 secretion[a]	N	IL-5 secretion[a]
A (4 digit)	Heterozygous	572	0.47 (0.00, 1.07)				
	Homozygous	119	0.62 (0.22, 1.16)				
			p = 0.026[c]				
DPA1 (4 digit)	Heterozygous	229	0.65 (0.22, 1.15)	227	0.66 (0.22, 1.15)	229	0.65 (0.22, 1.15)
	Homozygous	462	0.46 (0.00, 1.03)	344	0.46 (0.00, 1.08)	456	0.45 (0.00, 1.01)
			p = 0.046[c]		**p = 0.043[c]**		**p = 0.024[c]**
A (2 digit)	Heterozygous	566	0.47 (0.00, 1.07)				
	Homozygous	125	0.63 (0.22, 1.16)				
			p = 0.016				
DPA1 (2 digit)	Heterozygous	204	0.67 (0.23, 1.16)	204	0.67 (0.23, 1.16)	204	0.67 (0.23, 1.16)
	Homozygous	487	0.45 (0.00, 1.01)	367	0.46 (0.00, 1.08)	481	0.45 (0.00, 1.00)
			p = 0.010[c]		**p = 0.010[c]**		**p = 0.004[c]**

IL-5 response for all loci with significant p-values at either the 2 or 4 digit typing resolution are shown. Bold indicate loci with p values < 0.05.

[a] Cytokine secretion is measured in pg/ml, IQR indicates the interquartile range.

[b] Analyses repeated excluding all individuals homozygous for the indicated HLA allele.

[c] Repeated measures regression analysis adjusted for age, gender, race, age at first immunization, age at second immunization, and cohort status.

Table 5

HLA Associations with IL-10 Levels

Parameter		N	Median secretion in pg/mL (IQR)[a]		
Overall IL-10		713	4.2 (2.29, 6.69)		
HLA Locus	**Zygosity**		**All subjects**		**Excluding DPB1*0401[b]**
		N	IL-5 secretion[a]	N	IL-5 secretion[a]
DPA1 (4 digit)	Heterozygous	239	4.49 (2.55, 6.64)	236	4.49 (2.62, 6.64)
	Homozygous	474	4.08 (2.13, 6.74)	353	4.15 (2.36, 6.65)
			p = 0.0107[c]		p = 0.149[c]
DPA1 (2 digit)	Heterozygous	209	4.57 (2.64, 6.69)	209	4.57 (2.64, 6.69)
	Homozygous	504	4.08 (2.11, 6.69)	380	4.15 (2.34, 6.64)
			p = 0.028[c]		p = 0.062[c]
Any Locus (4 digit)	Heterozygous	151	4.27 (2.64, 6.49)	151	4.27 (2.64, 6.49)
	Homozygous	562	4.17 (2.18, 6.81)	438	4.29 (2.39, 6.74)
			p = 0.043[c]		p = 0.080[c]
Number of Homozygous Loci (4 digit)	0	151	4.27 (2.64, 6.49)	151	4.27 (2.64, 6.49)
	1	252	4.16 (2.22, 7.22)	249	4.18 (2.38, 7.18)
	2	197	4.21 (2.36, 6.98)	122	4.27 (2.64, 6.38)
	3	61	4.47 (2.09, 6.61)	38	4.56 (1.78, 6.61)
	4+	52	3.29 (1.57, 6.09)	29	5.21 (1.69, 6.24)
			p = 0.027[c]		p = 0.150[c]

IL-10 response for all loci with significant p-values at either the 2 or 4 digit typing resolution are shown. Bold indicate loci with p values < 0.05.

[a] Cytokine secretion is measured in pg/ml, IQR indicates the interquartile range.

[b] Analyses repeated excluding all individuals homozygous for the indicated HLA allele.

[c] Repeated measures regression analysis adjusted for age, gender, race, age at first immunization, age at second immunization, and cohort status.

Table 6

HLA Associations with IL-12(p40) Levels

Parameter		N	Median secretion in pg/mL (IQR)[a]		
Overall IL-12(p40)		711	0.0 (−7.15, 7.17)		
HLA Locus	**Zygosity**		**All subjects**		**Excluding DPB1*0401[b]**
		N	IL-5 secretion[a]	N	IL-5 secretion[a]
DPA1 (4 digit)	Heterozygous	239	0.00 (−8.43, 5.26)	236	0.00 (−8.28, 5.26)
	Homozygous	472	0.00 (−6.58, 7.53)	352	0.00 (−5.88, 7.9)
			p = 0.049[c]		**p = 0.014[c]**
DPA1 (2 digit)	Heterozygous	209	0.00 (−7.75, 5.42)	209	0.00 (−7.75, 5.42)
	Homozygous	502	0.00 (−6.89, 7.40)	379	0.00 (−6.56, 7.71)
			p = 0.223[c]		**p = 0.074[c]**
Any Locus (4 digit)	Heterozygous	151	0.00 (−9.53, 5.42)	151	0.00 (−9.53, 5.42)
	Homozygous	560	0.00 (−6.81, 7.32)	437	0.00 (−6.46, 7.52)
			p = 0.046[c]		**p = 0.012[c]**

IL-12(p40) response for all loci with significant p-values at either the 2 or 4 digit typing resolution are shown. Bold indicate loci with p values < 0.05.

[a] Cytokine secretion is measured in pg/ml, IQR indicates the interquartile range.

[b] Analyses repeated excluding all individuals homozygous for the indicated HLA allele.

[c] Repeated measures regression analysis adjusted for age, gender, race, age at first immunization, age at second immunization, and cohort status.