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Predominant Inflammatory Cytokine Secretion Pattern in Response to Two Doses of Live Rubella Vaccine in Healthy Vaccinees

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Abstract

We conducted a population-based study on 738 schoolchildren who received two doses of rubella vaccine in order to determine cytokine secretion patterns and their associations with demographic and clinical variables. The results showed a robust rubella-specific inflammatory cytokine response characterized by high median [inter-quartile range (IQR)] secretion levels (in pg/mL) of IL-6 [3681.0 (3160.0, 4052.0)], GMCSF [28.0 (23.6, 32.6)] and TNF-α [29.7 (−7.0, 89.2)]. We also detected modest levels of rubella-specific secretion of Th1 cytokines IL-2 and IFN-γ, while IL-12p40 was undetectable. In contrast, rubella-specific Th2 responses were hardly detectable. Age at vaccination, enrollment, and time elapsed between last vaccination and enrollment was significantly associated with the outcome of IL-2, IL-6 and IFN-γ secretion. These results suggest an immune-deviation or "skewing" from Th1/Th2 cytokine patterns towards a predominant inflammatory response upon in vitro rubella virus stimulation.

Keywords

rubella; cytokines; vaccine; inflammatory

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1. Introduction

Rubella virus (RV), an important pathogen in humans from the *Togaviridae* family, is a small, enveloped, nonsegmented, plus-strand RNA virus, which causes German measles (rubella). Natural infection in children and young adults is often characterized by a low-grade fever, sore throat, lymphadenopathy and a maculopapular rush. However, the virus is teratogenic, and maternal infection during the first trimester of pregnancy leads to a pattern of birth defects, collectively referred to as congenital rubella syndrome [1]. The vaccine currently used in the United States and in most of the developed world is the live attenuated Wistar RA 27/3 strain of rubella virus. The incidence of adverse reactions is lower to vaccination than that to natural rubella infection with age, gender, and genetics playing a role [1–3].

Antibody measures have been widely accepted as the "gold standard" surrogate for protection for many vaccines, including the measles-mumps-rubella (MMR) vaccine [4]. A threshold of 10–15mIU/ml of rubella-specific antibodies has been established as a marker of protective immunity [5,6]. Most immunogenicity and seroprevalence rubella vaccine studies have shown high rates of seroconversion and seropositivity, ranging from 92% to 100% in different populations [7–11].

Some studies have also demonstrated gender-based differences in rubella-specific humoral and cell-mediated immune responses [12,13], with females having significantly higher antibody titers than males [14,15], while others found no difference in rubella antibody status with respect to gender [9,10]. Increase in age has also been found to be significantly associated with poorer serological status [9,10].

Cell-mediated immune functions are critical in protection against intracellular pathogens, particularly in viral clearance, and CD4+ T lymphocytes are necessary to help B cell development [4]. Our research group and others have demonstrated the induction of rubella-specific lymphoproliferative responses, as well as IFN γ and IL-10 cytokine production as surrogate measures of cell-mediated immunity (CMI), following vaccination or natural infection [16–18]. Rubella-specific cytotoxic and helper T-lymphocyte responses have been mapped to the viral E1 and E2 glycoproteins, as well as the C (capsid) protein; minimal T cell epitopes have been defined for the design of new vaccines [19–23]. It is well established that the pattern and nature of cytokine production by CD4+T cells significantly impacts the immune response, as the T-cell response progresses along the Th1, Th2 or Th17 pathway [24–27].

There are no comprehensive studies on rubella-specific cytokine secretion patterns related to the cellular and humoral immune responses after rubella infection or immunization. Earlier studies concentrated on rubella virus-specific induction of interferons, including IFN-α and IFN-γ in cell lines and human cultures [28–31]. Recent microarray gene expression studies, when stimulated with rubella virus, revealed modulation of key cytokine and cytokine receptor genes in human cells [32,33]. Impaired cytokine production and moderate immunosuppression, including a defective lymphocyte response to mitogens, was reported in schoolgirls who received a rubella vaccination [34]. In patients with rubella infection, serum levels of IL-10 were markedly elevated early in the disease with a significant negative correlation between IL-10 and IgM/IgG antibody concentrations, suggesting that this cytokine may play an important immunosuppressive role in the pathogenesis and immune response to rubella [35].

To characterize cytokine patterns and rubella-specific memory T cell responses after immunization, we undertook an extensive host profiling of Th1 (IL-2, IFN-γ, IL-12p40), Th2 (IL-4, IL-5, IL-10), and inflammatory (IL-6, TNF-α, GM-CSF) cytokines in a combined cohort of 738 healthy subjects. Subjects were between 11–19 years of age and had received two doses of rubella vaccine. We demonstrated a robust and predominant inflammatory response, along with a modest Th1 rubella-specific immune response. We did not detect any significant Th2

response. Age and race were associated with the outcome of specific cytokine measures, however, no gender-based differences were observed in cytokine secretion levels.

2. Materials and Methods

2.1. Study subjects

Our study cohort comprised a combined sample of 738 subjects from two independent age-stratified random cohorts of healthy children and young adults (ages 11–19 years) from all socioeconomic strata, identified by Minnesota Independent School District 535, in Rochester. We recruited 346 healthy children for Cohort 1 (age 12 to 18 years) between December 2001 and August 2002, of which 342 consented to participate [14]. For cohort 2, we recruited an additional 396 healthy children and young adults (age 11 to 19 years) between December 2006 and August 2007. All 738 participants had a written medical record of having received two doses of measles-mumps-rubella (MMR) vaccine containing the attenuated RA27/3 Wistar strain ($TCID_{50} \geq 1000$) of rubella virus (Merck Whitehouse Station, NJ). No known circulating rubella virus in the community was observed since the earliest year of birth for any subject. A single venipuncture to obtain sera and peripheral blood mononuclear cells (PBMC) was approved by the Mayo Clinic Institutional Review Board. Written, informed consent was obtained from subjects' parents/guardians as well as written assent from age-appropriate children at the time of recruitment in the study.

In addition, we enrolled three MMR-II vaccinees (ages 20–29 years) as optimization subjects in order to determine the optimal rubella virus multiplicity of infection (MOI) for PBMC and supernatant harvest times specific to each cytokine of interest.

2.2 Rubella-specific secreted cytokine detection by ELISA

W-Therien strain of rubella virus used in the study (a kind gift from Dr. Teryl Frey, Georgia State University, Atlanta, GA) was propagated on Vero cells and titrated on RK-13 or Vero cells by a standard plaque assay. [36,37] The virus was free of lipopolysaccharide (LPS) contamination, as verified by the Limulus Amebocyte Lysate PYROGEN test kit (Cambrex, Walkersville, MD) per the manufacturer's instructions.

For all cellular assays, cryopreserved PBMC were thawed, resuspended at a concentration of 2×10^6 cells/mL in RPMI 1640 (Invitrogen), 5% FCS (HyClone); culture media with antibiotics and cell viability was tested using the trypan-blue exclusion test [38,39].

Rubella-specific cytokines were examined in the PBMC cultures using pre-optimized conditions for culture time and virus MOI for each cytokine of interest using an approach described previously [40] (Outlined in Supplementary Table 1,Table 2 and Table 3). PBMC were cultured in triplicate (2×10^5 cells/well) in 96-well round bottom culture plates with live rubella virus or media alone (negative unstimulated control). PHA (5 μg/mL) was used as a positive control. For the detection of rubella-specific IL-4 secretion, PBMC were cultured as above with the addition of 2 μg/mL of monoclonal anti-human IL-4R antibody (R&D Systems, Minneapolis, MN) [41]. Cell-free culture supernatants, from both stimulated and control wells, were harvested for each cytokine at a specified pre-optimized time point for maximal secretion at 18h (IL-12p40, GM-CSF), 24h (IL-4, IL-5, IL-6, IL-10), 2 days (IFN-γ) and 8 days (TNF-α, IL-2), and frozen at −80°C until the ELISA cytokine detection was performed. We performed ELISA assays for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, IL-12p40, TNF-α and GM-CSF, using kit-specific manufacturers' recommendations (BD Pharmingen), and we read plates for absorbance at 450 nm on a microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The limits of detection for cytokines, based on the lowest standard, were 4.7 pg/mL (IL-6, GM-CSF, IFN-γ and TNF-α), 7.8 pg/mL (IL-2, IL-4, IL-5 and IL-10) and 31.3 pg/mL

(IL-12p40). Optimal time and virus MOI parameters for each cytokine are shown in Supplementary Table 3.

2.3. Statistical analysis

The purpose of our efforts was to characterize rubella-specific cytokine secretion in healthy schoolchildren and assess associations of demographic and clinical variables with these outcomes of interest. Three Th1 (IL-2, IFN-γ, IL-12p40), three Th2 (IL-4, IL-5, IL-10), and three inflammatory (IL-6, TNF-α, GM-CSF) cytokines were examined as the outcomes of interest. Six values for each of these outcomes were recorded per individual: three values for rubella virus-stimulated cytokine response, and three for unstimulated (negative, background) response. For descriptive purposes, we obtained a single response measurement per individual for each outcome by subtracting the median of the three unstimulated values from the median of the three stimulated values. Data were descriptively summarized across individuals using frequencies and percentages for categorical variables, and medians and inter-quartile ranges (IQRs) for continuous variables.

Associations of cytokine secretion with demographic and clinical variables were formally evaluated using linear regression models with results from all six observed values within an individual as the outcome measurements, while accounting for multiple measures obtained for each subject. Demographic or clinical variables, the stimulation status, and the interaction between the two, were included as predictor variables. Interaction between the cytokines and stimulation status assesses the degree of change in the outcome of interest from the unstimulated to stimulated state, associated with differences in the demographic or clinical variable. Therefore, the p-value for the interaction between the cytokines and the stimulation status was used to assess statistical significance of the association between the demographic or clinical variable and these outcomes of interest. Correlations among the 6 within-subject repeated measures were accounted for using a repeated measures approach with n constraints on the structure of the correlations; data transformations were used to correct for data skewness in all linear regression models. For each immune response measure, we used an inverse normal (probit) transformation to ensure that distributional assumptions were met. Separate analyses were carried out for each cytokine and each demographic and clinical variable, with each model using the corresponding measure of secreted cytokine as the outcome of interest. Secondary analyses examined the independent (adjusted) effects of each variable on a given cytokine by simultaneously including all demographic and clinical variables in a multivariate linear regression model. 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 Demographic variables of the study population

Demographic variables of our cohort of 738 healthy children are shown in Table 1. Subjects were primarily Caucasian (91%), and the median age at enrollment was 15 years. The median age of the study cohort at first and second rubella immunization was 15 months and 11 years, respectively. The median time elapsed between the second rubella immunization and enrollment was 5.8 years. We had a slight over-representation of males (54%) in our study cohort.

3.2 Characterization of rubella-specific cytokine responses

Cytokine secretion patterns in PBMCs in response to in vitro rubella virus stimulation are shown in Table 2. We detected modest levels of rubella-specific secretion of the Th1 cytokines IL-2 and IFN-γ, while IL-12p40 was undetected. In contrast, the rubella-specific Th2 response was hardly detectable with no secretion of IL-4 and IL-5 and very low levels of IL-10 secretion.

We observed a robust rubella-specific inflammatory cytokine response. IL-6 and GM-CSF secretion were detected in 99 % and 100% of the subjects respectively, with median (IQR) secretion levels of 3681.0 (3160.0, 4052.0) pg/mL for IL-6 and 28.0 (23.6, 32.6) pg/mL for GM-CSF. TNF-α was also detected in 69% of the subjects with median (IQR) secretion levels of 29.7 (−7.0, 89.2) pg/mL.

3.3 Associations between rubella-specific Th1 cytokines and study cohort variables

Table 3 shows the associations between Th1 (IL-2 and IFN-γ) cytokine secretion patterns and demographic and clinical variables of rubella vaccinees. We observed a significant ($p<0.001$) decreasing trend in IL-2 secretion, with an increase in age at enrollment. Age at second rubella vaccination was associated with IL-2 ($p<0.001$) and IFN-γ ($p=0.002$) secretion levels. IFN-γ secretion was also significantly ($p<0.001$) influenced by the time elapsed between second dose and the time of enrollment, with a decreasing trend in IFN-γ secretion with an increase in time interval. Because we did not detect the secretion of IL-12p40, and because the levels of Th2 cytokines (IL-4, IL-5 and IL-10) were below levels of detection, these data are not shown in Table 3.

3.4 Associations between rubella-specific inflammatory cytokines and study cohort variables

Table 4 shows the associations between inflammatory (IL-6, TNF-α, GM-CSF) cytokine secretion patterns and demographic and clinical variables of rubella vaccinees. Age at enrollment was significantly ($p=0.018$) associated with IL-6 and GM-CSF production. We observed a strikingly higher level of TNF-α secretion ($p=0.009$) in Caucasians as compared to the rest of the cohort. Subjects who received a second dose of rubella vaccine at 11 years of age or more had significantly higher IL-6 levels ($p<0.001$) than those that received the vaccine at 10 years or younger. There was also a trend towards decreased IL-6 production ($p<0.001$) with an increase in time from the second rubella vaccination to blood draw.

4. Discussion

Rubella is well controlled by an efficacious vaccine in the developed world [1]. Therefore, the currently licensed rubella vaccine provides an excellent model to study immune mechanisms that are critical for a successful outcome, specifically after rubella vaccine, and possibly viral vaccines in general.

We conducted this study to profile rubella-specific cytokine response patterns after immunization and demonstrated an immune deviation from balanced Th1/Th2 responses towards a predominant inflammatory response post second dose of rubella vaccination. We observed a robust inflammatory response, characterized by high levels of IL-6 production in almost all vaccinees in response to in vitro stimulation with rubella virus. IL-6 is a key inflammatory cytokine, essential for the regulation of immune processes that induces IL-2 dependent antigen-specific helper and cytotoxic T cell proliferation and differentiation [42]. IL-6 has been shown to act as the critical “immunological switch” that resolves innate responses effectively and directs the immune system to transition towards an adaptive response [43]. More recently, a novel role of IL-6 in conjunction with other proinflammatory cytokines (TGF-β and IL-23) in the development and differentiation of the Th17 cell subset has been established [27,44–48]. Th17 cell expansion is negatively regulated in the presence of Th1/Th2 signature cytokines, IFN-γ and IL-4, via inhibiting the IL-23 driven expansion of these cells [49,50]. In addition to IL-6, we observed modest levels of TNF-α and GM-CSF production in response to rubella stimulation. TNF-α secretion has previously been reported in response to rubella vaccination [34]; however, the role of GM-CSF has not been studied.

Overall, both Th1 and Th2 responses were suppressed in rubella vaccinees after the second dose of vaccine, and the immune system was directed towards a predominant inflammatory response. We did detect a modest Th1 response to rubella vaccination with low levels of IL-2 and IFN-γ secretion and extremely low or undetectable Th2 cytokines. In light of our present findings, we are aware that rubella-induced secreted cytokine values cannot be truly negative and that negative values are an indication of assay measurement variability.

It is well known that the Th1 and Th2 responses are mutually regulated and that the predominance of one type redirects the other. Thus, the presence of modest levels of IL-2 and IFN-γ in rubella vaccinees is expected not only to favor Th1, but also inhibit Th2 specific IL-4, IL-5 and IL-10 production [25,51,52]. Our subjects demonstrated a lack of IL-12 secretion, which is a critical factor required for Th1 cell generation and IFN-γ production [53,54]. Further, the presence of TNF-α in our subjects is also expected to down-regulate the anti-inflammatory cytokine IL-10, as these two cytokines are known to play opposite roles in immune response [55–57].

Ours is the first study that attempts to elucidate the cellular adaptive mechanisms that govern vaccine immunity by characterizing cytokine patterns several years after rubella vaccination. Previous studies have focused primarily on determination of immediate cytokine secretion patterns post vaccination or in cell lines and human cultures [28–31,34,35]. While the early response to rubella vaccination/infection may involve Th1/Th2 and innate cytokines such as IFN-α, IFN-γ and IL-10 [29,31,34,35], this study demonstrates that the cytokine milieu several years post rubella vaccination is redirected towards an inflammatory response after live virus challenge. We speculate that the predominance of IL-6 as well as TNF-α secretion in the absence of IL-10, IL-4, IL-12 and IFN-γ may lead to deviation of the immune system towards a Th17 phenotype in response to viral encounter, as reported recently [24]. Induction of antigen-specific Th17 cells was previously reported in hepatitis C virus infected patients, and the role of IL-17 in the pathogenesis and outcome of human immunodeficiency virus, herpes simplex virus, cytomegalovirus, rotavirus and vaccinia virus infections has been suggested [58–63]. Recent microarray gene expression studies in rubella virus stimulated human fetal/adult fibroblasts and umbilical endothelial cells have shown modulation of a number of cytokine and cytokine receptor genes such as IL-6, IL-15, IL-24 and IFNα, several IFN-stimulated genes, IL-13RA2 and IL-7R [32,33]. Taken together, the predominance of inflammatory cytokines in our population-based study and recent microarray studies (21 fold up-regulation of IL-6) [32,33] suggest that the inflammatory cytokines may play an important role in rubella-specific immunity.

We noticed the age at vaccination demonstrated an influence on rubella virus-specific responses. Overall, we observed a trend towards a decrease in cytokine production with an increase in age at enrollment and an increase in time elapsed between last immunization and blood draw, which suggests that virus-specific cytokine responses may wane over time. A few studies have determined associations between age and serological status [9,10], however, to our knowledge, there have been no reports of the association of age with cellular status after rubella vaccination and the underlying mechanisms. We studied a relatively small window of age, from 11–19 years, and believe a study design with a greater spread of age may be necessary to characterize better age-dependent differences in vaccine response. We did not observe any gender-based differences in cytokine secretion patterns in response to in vitro stimulation with rubella virus in age-appropriate vaccinees.

The large age-stratified random sample, which was ethnically homogenous and age-appropriately vaccinated, is the major strength of our study, as it decreases the influence of confounding variables such as ethnicity and previous viral exposure history, and allows us to measure immunity variables that were true responses to vaccination, rather than wild virus

exposure. Based on our results, we speculate that a complex network of cytokines regulate rubella vaccine responses. Further, immune-deviation from Th1/Th2 cytokine patterns towards a predominant inflammatory response is seen in rubella vaccinees. Whether and how these long-term viral responses may be regulated by inflammatory cytokines that are also the mediators of the Th17 pathway is a cutting edge question and an area for further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1
Demographic variables of the study population

Variable		Result
Median age at enrollment, years (IQR[a])		15.0 (13.0,17.0)
Median age at first rubella immunization, months (IQR[a])		15.0 (15.0,16.0)
Median age at second rubella immunization, years (IQR[a])		11.0 (5.0,12.0)
Median time from second rubella immunization to enrollment, years (IQR)		5.8 (3.8,7.4)
Gender, N (%)		
	Male	396 (53.7)
	Female	342 (46.3)
Race, N (%)		
	Others	66 (8.9)
	White	672 (91.1)

[a]IQR=inter-quartile range (1st quartile – 3rd quartile)

Table 2
Rubella-specific cytokine responses in study cohort

Rubella-specific cytokine responses in study subjects were determined by ELISA. Cytokine response in pg/mL was defined as the subject-specific median rubella-stimulated response (measured in triplicate) minus the median unstimulated response (also in triplicate). Subjects were considered to have a positive cytokine response if the median of the stimulated cells was larger than the median of the unstimulated cells.

Response Type	Cytokine	No. of Subjects	Median response (IQR[a])	No. subjects positive (%)[b]
Th1	IL-2	713	17.6 (7.7,30.5)	652 (91.4)
	IFN-γ	713	8.5 (3.0,23.4)	644 (90.3)
	IL-12p40	711	0.0 (−7.1,7.2)	326 (45.9)
Th2	IL-4	691	0.3 (−0.3,1.0)	392 (56.7)
	IL-5	691	0.5 (0.0,1.1)	482 (69.8)
	IL-10	713	4.2 (2.3,6.7)	641 (89.9)
Inflammatory	IL-6	713	3681.0 (3160.0,4052.0)	707 (99.2)
	TNF-α	713	29.7 (−7.0,89.2)	490 (68.7)
	GM-CSF	711	28.0 (23.6,32.6)	711 (100.0)

[a] IQR=inter-quartile range (1^{st} quartile – 3^{rd} quartile)

Table 3

Th1 cytokine associations with demographic and clinical variables

Associations between rubella-specific Th1 secreted cytokines and demographic and clinical variables of the study cohort were evaluated using linear regression models. Response in pg/mL was defined as the subject-specific median rubella-stimulated response (measured in triplicate) minus the median unstimulated response (also in triplicate). Reported p-values were based on repeated measures analysis, unadjusted for any covariates. The calculated adjusted p-values did not influence the outcome of the results.

Parameter	Level	Median IL-2 in pg/mL (IQR[a])	p-value	Median IFN-γ in pg/mL (IQR[a])	p-value
Age at enrollment	11–13	20.6 (9.0,35.1)	<0.001	8.1 (2.1,23.4)	0.254
	14–15	18.2 (7.7,34.1)		7.7 (3.0,22.1)	
	16–17	15.0 (7.5,31.1)		11.1 (3.6,28.0)	
	18–19	14.3 (5.9,23.5)		7.7 (3.1,22.0)	
Gender	Male	18.2 (8.3,31.1)	0.623	7.6 (2.9,25.9)	0.153
	Female	15.8 (6.6,30.4)		9.9 (3.1,22.1)	
Race	Others	18.8 (9.4,30.8)	0.117	8.2 (3.1,23.6)	0.660
	White	17.6 (7.6,30.5)		8.7 (3.0,23.4)	
Age at first rubella vaccination, months	<= 14	16.2 (4.4,31.4)	0.117	11.1 (3.0,21.8)	0.961
	15	18.1 (8.2,31.4)		8.3 (2.8,26.7)	
	16–17	17.3 (8.5,26.6)		8.2 (2.9,23.4)	
	>= 18	14.8 (4.5,29.3)		8.3 (3.6,19.2)	
Age at second rubella vaccination, years	<= 5	21.5 (9.8,34.7)	<0.001	6.5 (1.6,19.2)	0.002
	6–10	19.1 (8.0,31.2)		8.8 (2.9,20.3)	
	11	20.0 (9.3,32.4)		10.6 (3.6,23.3)	
	>= 12	14.1 (5.4,25.4)		8.7 (3.7,29.0)	
Time from second rubella vaccination to enrollment, years	< 4	15.1 (6.6,33.9)	0.386	8.1 (3.0,23.3)	<0.001
	4–5.9	16.9 (6.1,29.0)		12.2 (4.3,32.5)	
	6–7.9	18.1 (8.9,31.1)		9.4 (3.1,22.4)	
	>= 8	19.7 (7.9,29.4)		5.9 (1.6,17.0)	

[a]IQR=inter-quartile range (1st quartile – 3rd quartile)

Table 4

Inflammatory cytokine associations with demographic and clinical variables

Associations between rubella-specific inflammatory secreted cytokines and demographic and clinical variables of the study cohort were evaluated using linear regression models. Response in pg/mL was defined as the subject-specific median rubella-stimulated response minus the median unstimulated response. Reported p-values were based on repeated measures analysis, unadjusted for any covariates. The calculated adjusted p-values did not influence the outcome.

Parameter	Level	Median IL-6 in pg/mL (IQR[a])	p-value	Median TNF-α in pg/mL (IQR[a])	p-value	Median GM-CSF in pg/mL (IQR[a])	p-value
Age at enrollment	11–13	3630.5 (3028.6,3988.8)	0.017	35.4 (−2.0,96.0)	0.120	28.5 (23.7,32.8)	0.018
	14–15	3636.3 (3128.8,4032.9)		12.1 (−13.8,82.1)		28.0 (24.0,32.8)	
	16–17	3691.9 (3282.8,4075.1)		31.2 (−8.9,92.7)		28.3 (23.1,32.5)	
	18–19	3878.7 (3302.4,4092.1)		38.7 (−3.0,72.5)		26.9 (23.5,31.3)	
Gender	Male	3700.0 (3090.5,4045.4)	0.916	27.3 (−6.7,88.2)	0.653	27.7 (23.1,32.2)	0.621
	Female	3672.5 (3182.7,4062.4)		34.9 (−9.2,91.4)		28.4 (24.6,32.7)	
Race	Others	3675.3 (2986.4,4264.0)	0.574	10.9 (−10.8,51.3)	0.009	29.6 (24.2,34.4)	0.610
	White	3681.0 (3172.9,4039.0)		34.6 (−7.0,92.4)		27.9 (23.5,32.2)	
Age at first rubella vaccination, months	<= 14	3556.8 (3056.4,3963.4)	0.191	25.4 (−4.2,85.9)	0.796	28.5 (24.8,32.9)	0.446
	15	3670.7 (3122.2,4083.9)		29.8 (−10.8,87.8)		28.0 (23.2,32.6)	
	16–17	3770.7 (3186.9,3988.6)		30.3 (−3.2,99.4)		28.7 (24.5,31.8)	
	>= 18	3733.3 (3223.9,4128.3)		36.0 (−4.2,89.2)		26.9 (23.8,32.8)	
Age at second rubella vaccination, years	<= 5	3501.7 (2974.1,3864.9)	<0.001	23.4 (−5.5,89.3)	0.316	27.7 (22.9,32.2)	0.566
	6–10	3501.9 (2908.4,3967.1)		33.2 (−5.5,121.0)		27.4 (22.5,31.8)	
	11	3814.5 (3290.2,4110.4)		35.2 (−10.8,87.6)		28.7 (24.6,32.2)	
	>= 12	3825.0 (3381.0,4122.7)		35.9 (−9.5,79.7)		28.7 (23.8,32.9)	
Time from second rubella vaccination to enrollment, years	< 4	3822.7 (3397.2,4195.6)	<0.001	36.4 (−6.9,97.4)	0.375	29.3 (24.9,33.7)	0.097
	4–5.9	3681.0 (3266.6,4061.2)		27.1 (−18.4,82.1)		28.7 (23.4,32.2)	
	6–7.9	3644.8 (2998.1,4006.3)		35.0 (−5.5,85.9)		27.2 (22.8,31.7)	
	>= 8	3590.4 (3043.4,3988.8)		21.5 (−1.8,103.6)		27.8 (23.3,32.2)	

[a] IQR=inter-quartile range (1st quartile – 3rd quartile)