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Independence of Measles-Specific Humoral and Cellular Immune Responses to Vaccination

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Abstract

With a larger, independent cohort and more sophisticated measures, we sought to confirm our work that indicated independence of humoral and cellular immunity following measles vaccination. We recruited an age-stratified random cohort of 764 healthy subjects from all socio-economic strata, all with medical-record documentation of two age-appropriate doses of measles-containing vaccine. We quantified measles-specific neutralizing antibody levels and assayed the IFN- γ ELISPOT response to measles virus. We also measured secreted cytokines from the PBMCs in response to measles virus by performing enzyme-linked immunosorbent assays as secondary measures of cellular immune status. The median antibody level and median IFN- γ ELISPOT response were 844 mIU/mL (IQR: 418 to 1,752) and 36 (IQR: 13.00 to 69.00) spot-forming cells (per 2×10^5 PBMCs), respectively. We found only a very weak and negative correlation [Spearman's r_s or rho of -0.090 (95 percent confidence interval -0.162 to -0.018)]. We found a similar lack of quantitatively important correlations between the neutralizing antibody level and any of the secondary measures. Our data confirm the independence of humoral and cellular immune responses after the second dose of measles vaccination. As researchers pursue novel measles vaccine and measles vaccine delivery systems, they must not infer that humoral responses predict cellular responses.

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DISCLOSURES

Dr. Poland is the chair of a data monitoring committee for novel non-measles vaccines undergoing clinical study by Merck & Co, Inc. Dr. Jacobson serves on a safety review committee for a post-licensure study of non-measles vaccine undergoing clinical study as well as a data monitoring committee for a novel non-measles vaccine undergoing clinical study, both by Merck & Co, Inc.

Keywords

Measles Vaccine; Immunity, Humoral; Immunity, Cellular; Antibody Formation; Cytokines

INTRODUCTION

In 2005 we published a study demonstrating an apparent independence between humoral and cellular measles-specific immune response following two doses of measles vaccine [1]. At that time, global measles mortality was falling—from 773,000 in 2000 to 164,000 in 2008 [2]. However, the drop in mortality leveled off in 2007, and experts raised concerns about the achievability and sustainability of a 90 percent reduction in worldwide measles mortality [2]. More recent mortality statistics are not available [3]. Efforts to eliminate measles in developing nations are hampered by many issues. First, the current live viral vaccine requires the maintenance of a cold chain for shipping and storage [4–7]. Second, administration issues exist because the intramuscular injections require healthcare workers [4–7]. Third, issues arise with using a live viral vaccine in countries with significant rates of HIV infection with not just safety [4–7], but with the potential for reduced efficacy and even long-term carriage [4–7]. Fourth, passive transfer of maternal immunity prevents earlier administration in infancy despite earlier occurrence of measles in young infants [4–7]. Furthermore, parental and community acceptance of the current measles vaccine remains challenging [8]. Since 2008, measles outbreaks in highly industrialized nations across four continents have re-emerged [9,10].

Approaches to eradicating measles require consideration of novel vaccines and vaccine delivery methods [5–7]. Specifically, Moss and Strebel wrote in 2011 that among the four major research needs in pursuing the global eradication of measles, we must make “progress towards developing the ideal measles vaccine, which would be inexpensive, safe, heat-stable, immunogenic in neonates or very young infants, and administered as a single dose without needle or syringe [7].” While vaccine development and licensure depend primarily upon measurements of humoral immunity, researchers recognize the importance of cellular immunity as well as humoral immunity with regard to severity and complications of infection as well as durability of vaccine immunity [11–15]. The importance of cellular immunity cannot be ignored; some researchers speculate that the killed measles vaccine of the 1970s actually caused atypical measles by the vaccine’s failure to generate cellular immunity [16].

We now have an opportunity to revisit our 2005 work examining the impact of a second measles vaccine dose upon cellular immunity and its apparent independence from humoral immunity. Through an unrelated study, we recruited a larger, independent cohort of children who received two doses of vaccine. In this recent study, we used what we believe is a better measure of cellular immunity. In place of the lymphocyte proliferation assay (LPA) as the primary measure of cellular immunity, we used an ELISPOT assay for interferon-gamma. We selected the ELISPOT assay as it gives a better readout with more accuracy and precision [17]. In addition, we have available to us the results of cytokine ELISAs as secondary measures of cellular immunity. Furthermore, we improved upon our original study because we measured humoral immunity with a plaque reduction microneutralization assay rather than IgG ELISA. Thus, with this larger cohort and more sophisticated measures, we sought to confirm the independence of humoral and cellular immunity following a second dose of measles-containing vaccine.

1. MATERIALS AND METHODS

1.1 Study subjects

We have previously described these study subjects [18,19]. The study cohort comprised a combined sample of 764 subjects from two independent age-stratified random cohorts of healthy schoolchildren and young adults from all socioeconomic strata in Rochester, MN. "For each cohort, using a process approved by the Mayo Clinic Institutional Review Board and the local school district, we recruited the subjects through a random selection of individuals eligible by age and documented vaccine status on the school registry rolls. We contacted those selected first by a letter to their parents at their home addresses followed by a telephone call if after two weeks we did not hear a response. Subjects who refused, whose parents refused, or who were not reached were replaced again by random selection." Between December 2006 and August 2007, we enrolled and sampled 388 healthy children (age 11 to 19 years) (Cohort 1). Between November 2008 and September 2009, we enrolled and sampled an additional 376 healthy children and young adults (age 11 to 22 years). All subjects provided medical records showing they received two doses of measles-containing vaccine, the first at 12 months of age or later, and the second dose occurring at least one month after the first dose. No known circulating wild measles virus occurred in the greater Rochester community since the earliest year of birth for any subject. The Mayo Clinic Institutional Review Board approved the study, and we obtained written informed consent from the parents of all children who participated in the study as well as written assent from children old enough to assent.

1.2 Laboratory Methods

We have previously published the following laboratory methods [18,19].

1.3 Plaque Reduction Microneutralization Assay (PRMN)

We quantified measles-specific neutralizing antibody levels using a high throughput fluorescence-based PRMN, as previously described [20] with small modifications. We diluted heat-inactivated sera four-fold from 1:4 to 1:4,096 (six replicates for each dilution) in Opti-MEM® I Reduced Serum Medium (Gibco, Invitrogen), mixed with an equal volume of low passage challenge virus Measles Virus-expressing Enhanced Green Fluorescent Protein (final dilutions 1:8 to 1:8,192) and incubated for one hour at 37°C. We used a standard inoculum of challenge virus in Opti-MEM® I Reduced Serum Medium at a dilution adjusted to yield 20 to 60 plaque-forming units (PFU) per well in the control wells with virus only. We transferred serum/virus mixtures (50 µl) to a new 96-well plate and mixed those with an equal volume of Vero cell suspension in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen), containing 10 percent fetal bovine serum (Hyclone, Thermo Fischer Scientific, Logan, UT). We incubated plates for 43 hours at 37°C under 5 percent CO₂. We scanned and counted the brightly fluorescent green plaques (syncytia) on an automated Olympus IX71 fluorescent microscope using the Image-Pro Plus Software Version 6.3 (MediaCybernetics). We calculated the 50 percent endpoint titer (Neutralizing Dose, ND50) using Karber's formula.[21] Our use of the WHO 3rd International Standard for Anti-Measles (3,000 mIU/mL, NIBSC Code No. 97/648) enabled us to transform quantitative ND50 values into mIU/mL as previously described [20].

1.4 Isolation of peripheral blood mononuclear cells (PBMCs)

We collected 100 mL of whole blood from each participant in BD Vacutainer® Cell Preparation Tubes (CPT™) with Sodium Citrate (Becton, Dickinson and Company, Franklin Lakes, NJ) and isolated the PBMCs according to a standard protocol [17]. Isolated PBMCs were resuspended at a concentration of 1×10^7 cells/mL in RPMI 1640 media containing 1-

Glutamine (Invitrogen, Carlsbad, CA), supplemented with 10 percent dimethyl sulfoxide (Protide Pharmaceuticals, St. Paul, MN) and 20 percent FBS (Hyclone, Thermo Fischer Scientific, Logan, UT), frozen overnight at -80°C in a controlled-rate freezing container, and transferred to liquid nitrogen for storage until use.

1.5 Interferon-Gamma (IFN γ) ELISPOT

We assessed the IFN- γ cytokine response using commercially available, total PBMC IFN- γ ELISPOT assays from R&D Systems (Minneapolis, MN) as previously described [17]. Briefly, we blocked an ELISPOT plate pre-coated with anti-human IFN- γ antibody (Ab) for 20 minutes with RPMI culture medium supplemented with 5 percent FBS. We thawed and counted one aliquot of 5×10^6 cryo-preserved PBMCs and then resuspended it in RPMI culture medium supplemented with 5 percent FBS. We plated seven wells with 2×10^5 human PBMCs per subject. We supplemented three wells with the Edmonston B vaccine strain of live attenuated measles virus using a multiplicity of infection (MOI) = 0.5, three wells with culture medium (our negative controls), and one well with 5 $\mu\text{g/mL}$ of phytohemagglutinin or PHA (our positive control). A single individual manually reviewed each plate and eliminated any spurious spots resulting from debris or overdeveloped regions. We read plates with an ImmunoSpot S4 Pro Analyzer from C.T.L (Cleveland, OH). We optimized counting parameters to precisely and accurately count all ELISPOT plates. Briefly, we assessed several plates to optimize the counting parameters, which included sensitivity, spot size thresholds, background hue, and spot separation. Once we established the counting parameters, we used these parameters to count all ELISPOT plates ensuring that we counted the spots consistently. The intra-class correlation coefficients (ICCs) comparing the multiple observations per subject were 0.94 for the stimulated values, and 0.85 for the unstimulated values, indicating high levels of measurement reliability.

1.6 Measurement of Secreted Cytokines

We performed enzyme-linked immunosorbent assays (ELISA) to measure the level of twelve (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFN- α , IFN- γ , IFN- λ 1, and TNF- α) cytokines secreted by PBMCs (five replicates for each cytokine) following *in vitro* stimulation with measles virus as previously described [22]. However, we could not detect by ELISA measles-specific IL-4, IL-5, IL-12, IL-13, and IL-17 cytokines secreted by PBMCs, and thus we did not include them in our study. Briefly, we thawed and counted 1.5×10^7 cryopreserved PBMCs and resuspended them in RPMI culture medium supplemented with 5 percent FBS. We plated eleven wells on 96-well round bottom plates with 2×10^5 cells/well. We supplemented five wells with the Edmonston B vaccine strain of live attenuated measles virus (the MOI is dependent on the cytokine, as described below), five wells with RPMI culture medium with 5 percent FBS (our negative controls), and one well with PHA (our positive control). A response surface methodology approach was applied to predict optimal combinations of length in culture and measles virus MOIs for maximum virus-specific cytokine production for each specific cytokine of interest [23]. See Table 1 for cytokine-specific MOIs and incubation times.

After incubation, we harvested cell-free supernatants from each plate, transferred them to a 96-well flat-bottom plate, and froze them at -80°C until analysis. We measured cytokine levels with commercial kits according to the manufacturer's instructions. We measured IL-2, IL-6, IL-10, IFN- γ , and TNF- α using commercial kits from BD Biosciences (San Jose, CA). We measured IFN- α using commercial kits from Mabtech (Cincinnati, OH). We measured IFN- λ 1 using commercial kits from R&D systems (Minneapolis, MN). We determined cytokine concentrations by measuring absorbance at 450 nm and correlated those with a standard curve created by performing serial dilutions of the manufacturers' reference standards. The levels of sensitivity for the IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN- λ 1, and

TNF- α assays were 7.8 pg/ml, 4.7 pg/ml, 7.8 pg/ml, 12.5 pg/ml, 4.7 pg/ml, 7.8 pg/ml, and 7.8 pg/ml, respectively. Cytokine-specific ICCs ranged from 0.65 (IL-2, unstimulated values) to 0.94 (IFN- α and IL-6, stimulated values).

1.7 Statistical Analysis

We extracted a summary measurement of each of the ELISPOT and secreted cytokine assessments for each study participant by subtracting the median value of their unstimulated wells from the median value of their stimulated measurements. We summarized the subjects' demographic characteristics, as well as these single per-individual measures of measles immune response descriptively across individuals using frequencies and percentages for categorical variables, and medians and inter-quartile ranges for continuous variables. We assessed differences between the demographic variables (age, gender, race and ethnicity, and timing of immunization relative to recruitment) and study cohort using chi-square tests for categorical variables and Mann-Whitney tests for quantitative variables.

We assessed pair-wise correlations between measles humoral and cellular immune response measures. For our primary objective we tested the presence of a correlation between the humoral measure—the levels of measles-specific neutralizing antibody (measured in units of mIU/mL)—and our principle measure of cellular immunity using measles virus-induced IFN- γ cell frequencies (evaluated as a count using an ELISPOT kit). For our secondary objective, we also analyzed our data from seven measures of measles virus-specific *in vitro* cytokine secretion (IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN- λ 1, and TNF- α), each reported in units of pg/mL).

The assessment of antibody levels resulted in a single recorded value per individual. The IFN- γ ELISPOT resulted in three recorded values prior to stimulation (negative controls) and three post-stimulation. A single response measurement per individual was obtained for each outcome by subtracting the median of the multiple unstimulated values from the median of the multiple stimulated values. Assessments of cytokine secretion resulted in five recorded values per outcome prior to stimulation with measles virus (negative controls) and five values post-stimulation.

We formally assessed correlations between immune response measures using Spearman correlation coefficients and their 95 percent confidence intervals. Primary analyses focused on simple, unadjusted correlation coefficients. Secondarily, we calculated partial correlation coefficients to evaluate associations after adjusting for the potential confounding effects of age at enrollment, gender, race, age at first measles vaccination, age at second measles vaccination, and cohort status (Cohort 1 vs. Cohort 2). We used the Cohen et al.'s thresholds for the strength of correlation with $r_s = \pm 0.1$ small, $r_s = \pm 0.3$ medium, and $r_s = \pm 0.5$ large [24]. Additionally, we employed linear regression analyses to determine the degree to which the various measures of cellular immunity were able to predict our observed measures of humoral immunity. These analyses used transformations to satisfy the assumptions required by linear regression. We performed all analyses using the SAS version 9 (SAS Institute, Inc., Cary, NC) and S-Plus version 8 (Insightful Corporation, Seattle, WA) software systems.

2.0 RESULTS

The combined cohort included 764 individuals with a median age of 15 years who received their first dose of measles-containing vaccine at a median age of 15 months and their second dose at a median age of five years of age. Table 2 provides further demographic details regarding the members of each cohort as well as a statistical test for differences in the distributions of gender, age, race, and ethnicity between the two cohorts that were combined

in subsequent analyses. Cohort 2 subjects tended to be younger at the second immunization as a result of the harmonization of the American Academy of Pediatrics and the Advisory Committee on Immunization Practices in their recommendations for the age at the second dose. This resulted in Cohort 2 subjects having a longer period of time elapse from the second dose to study enrollment. Also, Cohort 2 reflects the changing diversity of Rochester school children and young adults with a higher proportion of non-Caucasian individuals recruited.

The median PRMN antibody level was 844 (IQR: 418 to 1,752) mIU/mL, well above the threshold for sero-protection of 210 mIU/mL (corresponding to a PRMN titer of 120 in our study) [25]. In fact, 68 of the subjects, or 8.9 percent, had PRMN values less than 120. Table 3 provides a distribution for each of the nine measures of measles vaccine immunity. No thresholds have been determined for these measures other than the PRMN assay with respect to sero-protection. The median IFN- γ ELISPOT response was 36 (IQR: 13 to 69) spot-forming cells per 200,000 PBMCs. We detected high secretion levels of IL-6, IFN- α , and IFN- γ ; moderate secretion levels of IL-2 and IFN- λ 1; and low secretion levels of IL-10 and TNF- α .

We sought to determine correlations across the array of measures. Table 4 reports an assessment of the associations between all pairs of the measures, providing the Spearman's rank correlation coefficients (Spearman's rho or r_s) and their corresponding 95 percent confidence intervals. We found only a very weak and negative correlation between the PRMN antibody level and the IFN- γ ELISPOT. Similarly, we found no quantitatively important correlations between PRMN antibody level and our secondary measures of cellular immunity. Specifically, we found small correlations of PRMN antibody level with measles-specific secreted IL-2 (positive), IL-6 (negative), and IFN- α , very weak correlations with measles-specific secreted IFN- α (negative), IFN- γ , and IFN- λ 1 (negative), and no statistically significant correlations for measles-specific secreted IL-10 or TNF- α .

As shown in Table 4, we found more substantive correlations for secreted IL-2 with secreted IL-10 ($r_s = 0.354$; 95% confidence interval or CI of 0.290 to 0.415; moderately strong [24], positive, and statistically significant) and with secreted IFN- γ ($r_s = 0.545$; 95% CI: 0.493 to 0.593; strong, positive, and statistically significant). We also found substantive correlations for secreted IL-10 with both secreted IFN- α and secreted IFN- γ ($r_s = 0.327$ and 0.324 respectively; 95% CI: 0.252 to 0.380 and 0.258 to 0.386; both moderately strong, positive, and statistically significant). Furthermore, we found a substantive correlation—the strongest correlation found—between secreted IFN- α and secreted IFN- λ 1 ($r_s = 0.719$; 95% CI: 0.683 to 0.752; strong, positive, and statistically significant).

In addition to these simple estimates of correlation, we also estimated partial correlations while controlling for demographic and clinical variables. This controlled for the length of time between immunization and sample acquisition. Despite doing so, the partial correlations were essentially equal to the simple correlations and thus are not shown.

Finally, we performed a multiple linear regression to determine if, and to what extent, the cellular measures of measles vaccine-induced immune response predict the PRMN results. The partial correlations for antibody levels with each of the various cellular immune markers adjusted for all other cellular markers are shown in Table 5 and are similar to the simple correlation coefficients provided in the first row of Table 4. While these markers were significantly associated with PRMN antibody levels ($p < 0.001$) as a group, together they explained only 4.7 percent of the variability of the PRMN antibody levels.

3. DISCUSSION

Our results confirmed the independence of the humoral and cellular immune status following two doses of measles vaccine as we previously reported. [26] Replication of these findings in a new, non-overlapping, and larger cohort is a substantial strength of this work. Furthermore, by using PRMN instead of ELISA and IFN- γ ELISPOT instead of LPA, we used more sophisticated, sensitive, and accurate measures for both the humoral and cellular immune status [17,20]. The original study's lack of correlation arguably might have resulted from using a less specific metric of cellular immune response (e.g., LPA); replicating the independence with a better measure indeed strengthens the confidence in our conclusion of the independence of measles humoral and cellular immune status resulting from two doses of measles vaccine. Additional strengths of our approach include the use of a cohort whose subjects resided in an area where for their entire lifetime no cases of measles disease were known to have occurred. Furthermore, we relied on medical record documentation of two appropriately timed doses of M-M-R®-II (Merck & Co., Inc., Whitehouse Station, NJ) vaccine.

We recognize some limitations. The humoral and cellular immune measures most likely represent exposure to two documented doses of the attenuated measles vaccine virus rather than infection or exposure to the wild virus. However, we could not absolutely prove this was the case. Many of those recruited lived elsewhere for some part of their lives and nearly all traveled outside of the county. The primarily non-Hispanic, Caucasian population limits the inferences we can make with regard to race and ethnicity. Finally, we did not measure the so-called primary immune response after the second dose (e.g. 28 days after); we have only measurements months to years after that dose. For this reason, our results may reflect some waning of measurable immunity. Still, this may be a strength in regard to understanding long-term immunity.

Measles immunization activates memory T cells with CD4 and CD8 positive subsets. Cytokine production from these T cell subsets are critical in the development and regulation of the entire immune response [27,28]. Cell signaling of the Th1 type favors cytokine production that governs cellular immunity, such as IFN- γ , IL-2 and TNF- α . Cell signaling of the Th2 type cell signaling produces interleukins 4, 5 and 13 driving the humoral response. Together, these mechanisms determine the overall heterogeneity and composition of the immune response [29,30]. Our results demonstrated minimal correlation between neutralizing antibodies and our primary (IFN- γ ELISPOT) measure of cellular immunity and a lack of correlation between neutralizing antibodies and secondary (secreted cytokines) measures for cellular immunity, suggesting the independence of humoral and cellular immune responses after the second dose of measles vaccine. We recognize studies have established a role for cytokines in regulating humoral immune response. Beyond its role in triggering the Th1 immune response, IFN- γ , for example, is also known to promote isotype switching to IgG2a [31].

We do, however, wish to speculate on the meaning of the interplay among cytokines. First, we found the strongest correlation between secreted IFN- α and IFN- λ 1 ($r_s = 0.719$). Both IFN- α and IFN- λ 1 (also known as IL-29) have potent anti-viral activities and play a role in anti-viral protection [32]. IFN- α can also modulate T cell responses by influencing the development of a Th1 immune response. Second, we expected no correlation between measles-specific IFN- γ ELISPOT and secreted IFN- γ , and we found only a weak positive correlation. IFN- γ ELISPOT correlated with all but IL-2 among the seven cytokines; all were positive and statistically significant, but all were weak. IFN- γ is produced by Th1 cells through the Jak-STAT1 signaling pathway and it plays an important role in immunity. IFN- γ functions are influenced by cellular responses to other cross-regulated cytokines and

inflammatory factors, such as IL-4, IL-6, IL-10, and others [33]. Third, we found a correlation between Th2 cytokine, IL-10, and IFN- γ as well as IL-10 and IFN- α secretion. In this regard, IFN- γ and IL-10 are cytokines with opposite signaling and functional immunoregulatory activities, including different effects on class I and class II HLA antigen presentation [34]. Of note, the correlation with secreted IFN- γ was weaker than three of the other five cytokines. IFN- γ ELISPOT and secreted IFN- γ measure different biologic phenomenon and thus the discrepancies found should be expected. These T cell assay correlations should be validated in future studies as we were not testing these relationships as study hypotheses and believe these results to be only hypothesis generating.

Previous studies have demonstrated the importance of cellular immunity to vaccine efficacy. [34] This work, using a larger cohort with more sensitive measures, confirms the apparent independence of cellular and humoral immunity with regard to measles vaccine. While we certainly acknowledge that the cellular immune response can and does directly and indirectly affect humoral immunity through the interplay of cytokine interactions as well as through modification of antigen presentation in the course of infection, it does appear at least with the current vaccine, investigations of measles vaccination cannot presume cellular immunity from the humoral response. Furthermore, we should not presume that alternative vaccine strategies will achieve the same effects just because they achieve the same humoral immune response. Future studies of measles vaccination, with the current vaccine or with alternative vaccines, should measure both humoral and cellular immune responses in the efforts to fully characterize measles vaccine immune response rather than select only the more traditional measures of immunogenicity—the antibody responses. In this way, investigators can more convincingly demonstrate non-inferiority with the current vaccine, avoid the specter of concerns raised with the previously licensed killed measles vaccine, [16] and identify potential areas for improvement in the next generation of measles vaccines.

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ABBREVIATIONS

°C	degrees Celsius
Ab	Antibody
CO ₂	Carbon Dioxide
CPT™	Cell Preparation Tube
ELISA	Enzyme-Linked Immuno-Sorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent SPOT
FBS	Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
ICC	Intra-Class Correlation coefficient
IFN- α	Interferon-alpha
IFN- γ	Interferon-gamma

IFN-λ1	Interferon-lambda 1
IgG	Immunoglobulin G
IL-#	Interleukin-#
IQR	Inter-Quartile Range
LPA	Lymphocyte Proliferation Assay
mIU	milli-International Units
mL	milli-Liter
MOI	Multiplicity Of Infection
ND50	Neutralizing Dose giving 50 percent neutralization of maximal activity
NIBSC	National Institute for Biological Standards and Control
nm	Nano-Meter
PBMC	Peripheral Blood Mononuclear Cell
PFU	Plaque Forming Units
pg	picograms
PHA	Phyto-Hem-Agglutinin
PRMN	Plaque Reduction Micro-Neutralization assay
RPMI	Roswell Park Memorial Institute medium
r_s	spearman's rank-order correlation or spearman's rho
TNF-α	Tumor-Necrosis-Factor-alpha
WHO	World Health Organization

References

1. Dhiman N, Ovsyannikova IG, Ryan JE, Jacobson RM, Vierkant RA, Pankratz VS, Jacobsen SJ, Poland GA. Correlations among measles virus-specific antibody, lymphoproliferation and Th1/Th2 cytokine responses following measles-mumps-rubella-II (MMR-II) vaccination. *Clin Exp Immunol*. 2005; 142:498–504. [PubMed: 16297162]
2. Global measles mortality, 2000–2008. *MMWR Morb Mortal Wkly Rep*. 2009; 58:1321–1326.
3. World Health Organization. Immunization surveillance, assessment and monitoring Measles. 2011 Aug 29. 2011
4. Davey, S. Bulletin of the World Health Organization; 2001. Measles eradication still a long way off; p. 584-585.
5. Moss WJ. Measles control and the prospect of eradication. *Curr Top Microbiol Immunol*. 2009; 330:173–189. [PubMed: 19203110]
6. Griffin DE, Moss WJ. Can we eradicate measles? *Microbes*. 2007; 1:409–413.
7. Moss WJ, Strebel P. Biological feasibility of measles eradication. *J Infect Dis*. 2011; 204(Suppl 1):S47–S53. [PubMed: 21666201]
8. Meissner HC, Strebel PM, Orenstein WA. Measles vaccines and the potential for worldwide eradication of measles. *Pediatrics*. 2004; 114:1065–1069. [PubMed: 15466106]
9. Weekly Epidemiological Record; 2011. Measles outbreaks in Europe; p. 173p. 174
10. Blostein J. Global measles outbreaks threaten US. *Michigan Medicine*. 2008; 104:20–21. [PubMed: 18669065]

11. Burnet FM. Measles as an index of immunological function. *Lancet*. 1968; 2:610–613. [PubMed: 4175162]
12. Kaplan LJ, Daum RS, Smaron M, McCarthy CA. Severe measles in immunocompromised patients. *JAMA*. 1992; 267:1237–1241. [PubMed: 1538561]
13. Markowitz LE, Chandler FW, Roldan EO, Saldana MJ, Roach KC, Hutchins SS, Preblud SR, Mitchell CD, Scott GB. Fatal measles pneumonia without rash in a child with AIDS. *J Infect Dis*. 1988; 158:480–483. [PubMed: 3403997]
14. Mitus A, Holloway A, Enders JF, Enders JF. Attenuated measles vaccine in children with acute leukemia. *Am J Dis Child*. 1965; 103:413–418. [PubMed: 14474744]
15. Ward BJ, Boulianne N, Ratnam S, Guiot MC, Couillard M, De Serres G. Cellular immunity in measles vaccine failure: demonstration of measles antigen-specific lymphoproliferative responses despite limited serum antibody production after revaccination. *J Infect Dis*. 1995; 172(6):1591–1595. [PubMed: 7594723]
16. Polack FP, Auwaerter PG, Lee SH, Nousari HC, Valsamakis A, Leiferman KM, Diwan A, Adams RJ, Griffin DE. Production of atypical measles in rhesus macaques: Evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody. *Nat Med*. 1999; 5:629–634. [PubMed: 10371500]
17. Ryan JE, Ovsyannikova IG, Poland GA. Detection of measles virus-specific interferon-gamma-secreting T-cells by ELISPOT. *Methods Mol Biol*. 2005; 302:207–218. [PubMed: 15937354]
18. Haralambieva IH, Ovsyannikova IG, Kennedy RB, Vierkant RA, Pankratz SV, Jacobson RM, Poland GA. Associations between single nucleotide polymorphisms and haplotypes in cytokine and cytokine receptor genes and immunity to measles vaccination. *Vaccine*. 2011; 29:7883–7895. [PubMed: 21875636]
19. Ovsyannikova IG, Haralambieva IH, Vierkant RA, Pankratz VS, Poland GA. The role of polymorphisms in Toll-like receptors and their associated intracellular signaling genes in measles vaccine immunity. *Hum Genet*. 2011; 130:547–561. [PubMed: 21424379]
20. Haralambieva IH, Ovsyannikova IG, Vierkant RA, Poland GA. Development of a Novel Efficient Fluorescence-based Plaque Reduction Microneutralization Assay for Measles Immunity. *Clin Vaccine Immunol*. 2008; 15:1054–1059. [PubMed: 18463223]
21. Ballew, HC. Neutralization. In: Specter, S., editor. *Clinical Virology Manual*. New York, NY: Elsevier; 1992.
22. Ovsyannikova IG, Dhiman N, Jacobson RM, Vierkant RA, Pankratz VS, Poland GA. HLA homozygosity does not adversely effect measles vaccine-induced cytokine responses. *Virology*. 2007; 364:87–94. [PubMed: 17397893]
23. Ryan JE, Dhiman N, Ovsyannikova IG, Vierkant RA, Pankratz VS, Poland GA. Response surface methodology to determine optimal cytokine responses in human peripheral blood mononuclear cells after smallpox vaccination. *J Immunol Methods*. 2009; 341:97–105. [PubMed: 19038260]
24. Cohen, J. *Statistical power analysis for the behavioural sciences*. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.
25. Haralambieva IH, Ovsyannikova IG, O'Byrne M, Pankratz VS, Jacobson RM, Poland GA. A large observational study to concurrently assess persistence of measles specific B-cell and T-cell immunity in individuals following two doses of MMR vaccine. *Vaccine*. 2011; 29:4485–4491. [PubMed: 21539880]
26. Yewdell JW, Norbury CC, Bennink JR. Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. *Adv Immunol*. 1999; 73:1–77. [PubMed: 10399005]
27. Biron CA. Cytokines in the generation of immune responses to resolution of, virus infection. *Curr Opin Immunol*. 1994; 6:530–538. [PubMed: 7946039]
28. Mosmann TR. Cytokine secretion patterns and cross-regulation of T cell subsets. *Immunol Res*. 1991; 10:183–188. [PubMed: 1659603]
29. Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol*. 1989; 46:111–147. [PubMed: 2528896]

30. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol.* 1989; 7:145–173. [PubMed: 2523712]
31. Schroder K, Hertzog P, Ravasi T, Hume D. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol.* 2004; 75:163–189. [PubMed: 14525967]
32. Khan NU, Pulford KA, Farquharson MA, Howatson A, Stewart C, Jackson R, McNicol AM, Foulis AK. The distribution of immunoreactive interferon-alpha in normal human tissues. *Immunology.* 1989; 66:201–206. [PubMed: 2647627]
33. Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity.* 2009; 31:539–550. [PubMed: 19833085]
34. Amanna IJ, Slifka MK. Contributions of humoral and cellular immunity to vaccine-induced protection in humans. *Virology.* 2011; 411:206–215. [PubMed: 21216425]

Table 1

Optimized Multiplicity of Infections (MOIs) and Incubation Times for Cytokines Specific to Measles Vaccine Virus

Cytokine	MOI	Incubation time (hours)
IL-2	0.5	48
IL-6	1.0	72
IL-10	0.5	48
IFN- α	1.0	24
IFN- γ	1.0	72
IFN- λ 1	1.0	72
TNF- α	1.0	24

ELISA assays were performed to measure the level of secreted cytokines (IL-2, IL-6, IL-10, IFN- α , IFN- γ , IFN- λ 1, and TNF- α) after *in vitro* stimulation with measles virus using pre-optimized conditions for culture time and virus MOI, as previously described [23].

Table 2

Demographic Description and Comparison of the Two Cohorts (N=764)

	Cohort 1	Cohort 2	Combined	P Value*
Total N Count (percentage)	388 (49.9)	376 (50.1)	764 (100)	NA
Median age at enrollment, years (IQR)	15.0 (13.0 to 17.0)	15.0 (13.0 to 17.0)	15.0 (13.0 to 17.0)	<0.001
Median age at first measles vaccination, months (IQR)	15.0 (15.0 to 16.0)	15.0 (15.0 to 16.0)	15.0 (15.0 to 16.0)	0.96
Median age at second measles vaccination, years (IQR)	8.0 (5.0 to 11.0)	5.0 (4.0 to 10.0)	5.0 (4.0 to 11.0)	<0.001
Median time from 2nd measles vaccination to enrollment, years (IQR)	6.7 (4.9 to 8.2)	8.5 (6.4 to 10.4)	7.4 (5.6 to 9.2)	<0.001
Females Count (percentage)	176 (45.4)	161 (42.3)	337 (44.1)	0.48
Race Count (percentage)				<0.001
Caucasian	345 (88.9)	271 (72.1)	616 (80.6)	
African-American	4 (1.0)	85 (22.6)	91 (11.9)	
All Other Races	39 (10.1)	20 (5.3)	59 (7.7)	
Hispanic Count (percentage)	6 (1.6)	9 (2.4)	15 (2.0)	0.02

* P-values are calculated using chi-square tests for categorical variables and Mann-Whitney tests for continuous variables.

Table 3

Distribution of Measures of Measles Vaccine Immune Responses

	Mean	Median	Inter Quartile Range
PRMN* (mIU/mL)	1,263.9	844.0	418.0 to 1,752
IFN- γ ELISPOT (SFC** per 2×10^5 PBMCs)	46.19	36.00	13.00 to 69.00
Secreted IL-2 (pg/mL)	46.00	37.51	20.46 to 64.39
Secreted IL-6 (pg/mL)	362.47	354.65	248.5 to 461.4
Secreted IL-10 (pg/mL)	23.50	18.26	11.26 to 28.51
Secreted IFN- α (pg/mL)	742.4	551.0	272.6 to 1,031
Secreted IFN- γ (pg/mL)	95.64	67.44	35.15 to 120.5
Secreted IFN- λ 1 (pg/mL)	53.36	34.05	14.14 to 73.22
Secreted TNF- α (pg/mL)	15.63	13.67	9.20 to 18.82

* PRMN - plaque reduction microneutralization assay

** SFC – spot-forming cells

Table 4

Two-Way Measures of Correlations Between Humoral and Cellular Immunity Measures*
(Spearman's r_s or rho and 95 percent confidence interval)

	PRMN	IFN- γ ELISPOT	Secreted IL-2	Secreted IL-6	Secreted IL-10	Secreted IFN- α	Secreted IFN- γ	Secreted IFN- $\lambda 1$	Secreted TNF- α
PRMN		-0.090 (-0.162 to -0.018)	0.100 (0.029 to 0.170)	-0.147 (-0.216 to -0.076)	-0.041 (-0.111 to 0.031)	-0.107 (-0.177 to -0.035)	0.093 (0.022 to 0.164)	-0.082 (-0.153 to -0.011)	-0.015 (-0.087 to 0.056)
IFN- γ ELISPOT			-0.019 (-0.092 to 0.054)	0.196 (0.125 to 0.265)	0.099 (0.027 to 0.171)	0.131 (0.058 to 0.202)	0.106 (0.033 to 0.177)	0.091 (0.019 to 0.163)	0.175 (0.103 to 0.245)
Secreted IL-2				0.010 (-0.061 to 0.081)	0.354 (0.290 to 0.415)	0.108 (0.037 to 0.178)	0.545 (0.493 to 0.593)	0.010 (-0.061 to 0.081)	0.072 (0.001 to 0.143)
Secreted IL-6					0.032 (-0.039 to 0.103)	0.001 (-0.070 to 0.073)	0.001 (-0.070 to 0.073)	0.035 (-0.037 to 0.106)	0.224 (0.155 to 0.291)
Secreted IL-10						0.317 (0.252 to 0.380)	0.324 (0.258 to 0.386)	0.264 (0.196 to 0.329)	0.207 (0.138 to 0.275)
Secreted IFN- α							0.056 (-0.015 to 0.127)	0.719 (0.683 to 0.752)	0.146 (0.075 to 0.215)
Secreted IFN- γ								0.097 (0.026 to 0.167)	0.227 (0.158 to 0.294)
Secreted IFN- $\lambda 1$									0.130 (0.059 to 0.200)

* We also ran partial correlation adjusting for age at enrollment, gender, race, age at first MMR, age at second MMR, cohort status (first versus second), and found no substantial differences from the data reported in this table.

PRMN - plaque reduction microneutralization assay

Bold text reflects moderately strong (0.3 to 0.5) and strong correlations (>0.5)

Table 5

Partial Correlation Estimates Obtained When Simultaneously using All Measures of Cellular Immunity to Predict Plaque Reduction Microneutralization Assay Results

Cellular Immune Measure	Partial Correlation	95 percent Confidence Interval
IFN- γ ELISPOT	-0.055	-0.128 to 0.018
Secreted IL-2	0.073	0.000 to 0.146
Secreted IL-6	-0.152	-0.223 to -0.080
Secreted IL-10	-0.044	-0.117 to 0.030
Secreted IFN- α	-0.049	-0.122 to 0.024
Secreted IFN- γ	0.086	0.013 to 0.159
Secreted IFN- λ 1	-0.006	-0.080 to 0.067
Secreted TNF- α	0.032	-0.041 to 0.106

P-value tests whether correlation of interest differs from zero.