Decreased humoral immunity to mumps in young adults immunized with MMR vaccine in childhood

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In the past decade, multiple mumps outbreaks have occurred in the United States, primarily in close-contact, high-density settings such as colleges, with a high attack rate among young adults, many of whom had the recommended 2 doses of mumps-measles-rubella (MMR) vaccine. Waning humoral immunity and the circulation of divergent wild-type mumps strains have been proposed as contributing factors to mumps resurgence. Blood samples from 71 healthy 18- to 23-year-old college students living in a non-outbreak area were assayed for antibodies and memory B cells (MBCs) to mumps, measles, and rubella. Seroprevalence rates of mumps, measles, and rubella determined by IgG enzyme-linked immunosorbent assay (ELISA) were 93, 93, and 100%, respectively. The index standard ratio indicated that the concentration of IgG was significantly lower for mumps than rubella. High IgG avidity to mumps Enders strain was detected in sera of 59/71 participants who had sufficient IgG levels. The frequency of circulating mumps-specific MBCs was 5 to 10 times lower than measles and rubella, and 10% of the participants had no detectable MBCs to mumps. Geometric mean neutralizing antibody titers (GMTs) by plaque reduction neutralization to the predominant circulating wild-type mumps strain (genotype G) were 6-fold lower than the GMTs against the Jeryl Lynn vaccine strain (genotype A). The majority of the participants (80%) received their second MMR vaccine ≥10 years prior to study participation. Additional efforts are needed to fully characterize B and T cell immune responses to mumps vaccine and to develop strategies to improve the quality and durability of vaccine-induced immunity.

mumps, measles, rubella | MMR vaccine | memory B cells (MBCs) | plaque reduction neutralization titers | IgG ELISA

Mumps is an acute viral infection characterized by fever and salivary gland inflammation (parotitis) in humans (1). The spectrum of illness may also include orchitis, meningitis, encephalitis, and, rarely, deafness (1). In the United States, a live-attenuated mumps vaccine was first licensed in 1967 and had been administered as a single dose, initially as a monovalent vaccine and then as a trivalent measles-mumps-rubella (MMR) vaccine, which was licensed in 1971 (1). Subsequently, a revised MMR II vaccine was licensed in 1978, which continues to be in use. In 1986, there was a mumps resurgence in the United States and, in 1989, to improve measles control, the Advisory Committee on Immunization Practices (ACIP) recommended 2 doses of MMR vaccine at ages 12 to 15 mo, with the second dose at 4 to 6 y (1). The American Academy of Pediatrics (AAP) recommended the second dose at 11 to 12 y (2). In 1994, the AAP and ACIP harmonized the schedule at 12 to 15 mo and 4 to 6 y (2). The highly successful MMR vaccine and the implementation of the 2-dose vaccine schedule since 1991 led to a reduction in mumps cases from >185,000 cases per y in the prevaccine era to <400 cases per y by 2005 (1).

Unexpectedly, in the past decade, multiple mumps outbreaks have been reported in the United States, primarily in close-contact, high-density settings such as colleges, with a high attack rate among vaccinated young adults (2, 3). Starting in 2006, a multistate outbreak of mumps occurred among vaccinated persons on college campuses in the Midwest followed by outbreaks in the northeastern United States and Guam in 2009 to 2010 (4–8). From 2012 to the present time, there has been an increase in the number of reported cases, from 229 cases in 2012 to over 6,000 cases in both 2016 and 2017 (4). Outbreaks have occurred mostly in university settings and close-knit communities, largely among vaccinated persons (5–9). Outbreaks have also been reported in the Netherlands (2009 to 2012), Canada (2009 to 2010), France (2013), Spain, Israel, Korea, Australia, and others (10–13). Previously, mumps outbreaks were common in nonimmunized populations who declined vaccination (14). As a result of continued outbreaks among groups with high 2-dose vaccination coverage, the ACIP recently recommended the use of a third dose of MMR for persons with 2 doses of MMR who are identified by public health authorities as being part of a group or population at increased risk for acquiring mumps because of an outbreak (15).

Multiple factors have been proposed for resurgence of mumps, including secondary vaccine failure due to waning of

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Significance

The live-attenuated mumps-measles-rubella (MMR) vaccine has been highly successful in the United States since its introduction 47 years ago. However, for the past decade, mumps outbreaks have been occurring among young adults who were vaccinated as children. Waning immunity has been proposed as a key contributing factor to mumps resurgence. In our sample ($n = 71$) of 18- to 23-year-old college students, the majority had detectable mumps IgG antibodies by enzyme-linked immunosorbent assay (ELISA) but the magnitude was lower than rubella. Neutralizing antibody titers were 6-fold lower to a circulating genotype G mumps strain versus the vaccine strain. Ten percent of our participants had no detectable memory B cells to mumps. Strategies are needed to improve immunity to the mumps vaccine.

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vaccine-induced immunity, increased susceptibility due to lack of natural boosting from circulating wild-type mumps virus, lower mumps vaccine effectiveness (85 to 90%) compared with measles and rubella vaccine, high-density settings that favor intense exposure, and the evolution of wild-type virus strains (11, 16). The predominant circulating wild-type strain responsible for outbreaks in the United States is genotype G, which is phylogenetically distinct from the Jeryl Lynn (JL) vaccine strain (genotype A) (5, 6, 17–25). Mumps SH (small hydrophobic) sequences available in GenBank (https://www.ncbi.nlm.nih.gov/genbank) show that since 2015, 98.5% of sequences from the United States were identified as genotype G and the remaining 1.5% of sequences were identified as genotypes C, H, K, or J. Only genotype G was detected in recent outbreaks in the United States (5, 6, 17–25). Serum samples obtained 10 y after mumps vaccination were able to neutralize genotype G viruses although with up to 4-fold reduced efficiency relative to the homologous vaccine strain (17, 26). The reduction in cross-neutralizing capacity could be of significance if there is waning immunity at or below the threshold to achieve herd immunity (13, 27). In addition, cases of reinfection have been reported among persons who have had naturally acquired mumps, suggesting that disease-induced immune memory may not be protective in some individuals (28).

While there is no established correlate of protection for mumps, binding IgG antibodies and neutralizing antibodies by plaque reduction neutralization (PRN) are considered markers of protective immunity (29). Mumps PRN titers and IgG antibodies appear to wane with time even though there is a high rate of seroconversion after the first and second doses of MMR vaccine (30). Waning of antibody levels may not be disadvantageous if immunologic memory is preserved by memory B cells (MBCs), as in the case of hepatitis B (31). But the role of MBCs in maintaining long-term immunity has yet to be fully evaluated for mumps. A moderate to weak correlation exists between MBCs and antibody levels (32, 33). In this study, we describe humoral immunity to mumps in college-aged students who were previously immunized with 2 doses of MMR vaccine as children. We measured mumps antibody levels by IgG ELISA, neutralizing antibody titers to JL and a genotype G strain by PRN, and IgG avidity using an end-point dilution assay. We also evaluated circulating mumps-specific memory B cells among the total IgG-secreting memory B cells after stimulation with B cell mitogens and enumeration with ELISpot assay. Measles- and rubella-specific binding antibodies measured by IgG ELISA and MBC frequencies were compared with the results obtained for mumps.

Results

Of the 71 evaluable participants, 84.5% were born in the United States, with a balanced representation of gender and race/ethnicity (Table 1). Almost all of the participants (69/71) received 2 doses of MMR vaccine in childhood. Among them, 86% received the first MMR between 1 and 2 y of age (Table 1). For the second MMR vaccine, 52% were vaccinated between 4 and 6 y of age, and 23% were vaccinated between 10 and 12 y of age (Table 1). More than one-third of the participants (37%) received the second dose of MMR 15 to 17 y prior to enrollment in the study; 43% of participants received MMR 10 to 14 y prior to enrollment. None of the participants reported exposure to a mumps outbreak or had mumps disease before study participation.

Mumps IgG-Binding Antibodies Are Low. Antibodies to mumps, measles, and rubella were detected in 93, 93, and 100% of participants, respectively. No significant differences in seropositivity rates were observed based on gender, time from receipt of second MMR, or age at receipt of second MMR. The mean mumps index standard ratio (ISR) was significantly lower than the mean ISR for rubella (Fig. 1). A high proportion of participants (34/71 or 47.8%) had a rubella ISR that was higher than the ISRs for measles or mumps. A low proportion of participants (11/71 or 15.5%) had a mumps ISR that was higher than the ISRs for measles or rubella. Accordingly, 3 times as many participants had higher ISR values to rubella than to mumps. As each of the antigens in MMR is unique, seronegativity to one component of the MMR vaccine was not predictive of seronegativity to the other 2 components. Four participants were seronegative and one was equivocal for mumps. For measles, 3 other participants were seronegative and 2 were equivocal. However, one participant was seronegative for both mumps and measles even though the participant was vaccinated with MMR at ages 1 and 4 as a child in Colombia and at age 11 in the United States (9 y prior to study enrollment).

Table 1. Demographics of participants

Variable	Total (%) (n = 71)
Gender	
Male	30 (42.3)
Female	41 (57.7)
Race/ethnicity	
Non-Hispanic	63 (88.7)
White	38 (53.5)
Black	14 (19.7)
Asian	18 (25.4)
Others	1 (1.4)
Born in the United States	60 (84.5)
Age, y	
18 to 19	12 (17)
20 to 22	57 (80)
23	2 (3)
Age at immunization with first MMR vaccine, y	
1 to 2*	61 (85.9)
>3	8 (11)
Age at immunization with second MMR vaccine, y[†]	
4 to 6	36 (52)
6 to 10	13 (18.8)
10 to 12	16 (23)
12 to 19	4 (5.8)
Time from last MMR vaccine to study enrollment, mo	
10 to 108	8 (11)
109 to 216	58 (82)
217 to 252	5 (7)

*Two participants received their first MMR dose before 1 y of age.
[†]Two participants did not receive a second MMR dose.

Mumps Neutralizing Antibody Titers to Jeryl Lynn vs. Genotype G Strain. PRN titers to the JL mumps vaccine strain and a wild-type genotype G strain are shown in Fig. 2. Geometric mean neutralizing antibody titers (GMTs) determined by PRN to the genotype G strain were 6 times lower than those against the JL strain (GMT 35 vs. 217, $P < 0.0001$). The lowest JL and genotype G titers were 23 and 3, respectively. In Fig. 3, the distribution of PRN titers to JL and genotype G showed that there were 5/69 subjects with genotype G PRN titers <8 and 12/69 with titers ≥8 to <16 compared with 0/69 subjects with titers <16 for the JL vaccine strain. A moderate positive correlation was noted between JL and genotype G titers (Spearman $r = 0.6517$, $P = 0.0001$). A weaker correlation was noted between IgG and PRN titers to JL and genotype G strains (Spearman $r = 0.4207$, $P = 0.0003$ and Spearman $r = 0.463$, $P = 0.0001$, respectively). Interestingly, the 3 participants who were seronegative by IgG ELISA to mumps had JL PRN titers of 23, 54, and 103, highlighting the previously reported discordance between the ELISA and PRN assays (34, 35). The discrepancy is likely due to differences in the predominant

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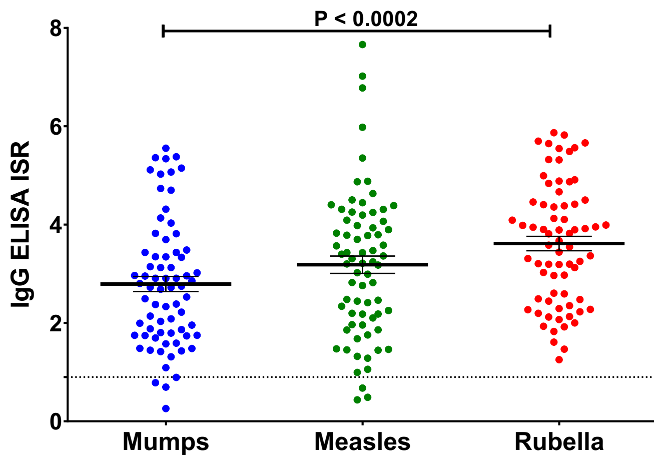

Fig. 1. Index Standard Ratio (ISR) of IgG antibodies to mumps, measles and rubella are shown in 71 participants. Mean, median, and range are as follows: 2.72, 2.79, and 0.25 to 5.55 for mumps; 3.24, 3.18, and 0.43 to 7.66 for measles; and 3.67, 3.61, and 1.25 to 5.87 for rubella. Horizontal lines represent mean with SEM. *P* values were determined comparing 3 groups using unpaired *t* test. The dotted line represents the limit of detection.

antibody species detected by each method. The whole-virus antigen used in this ELISA detects both neutralizing and nonneutralizing antibodies (predominantly nucleoprotein or NP), while the PRN assay detects antibodies to hemagglutinin and fusion proteins. In a previous study, the majority of individuals seropositive for mumps IgG by ELISA had relatively high levels of NP-specific antibodies and a wide variation in PRN titers and hemagglutinin-neuraminidase (HN)–specific antibodies (35).

Mumps-Specific Memory B Cells Are Low Compared with Measles and Rubella. The mean frequency of mumps-specific MBCs was significantly lower than measles and rubella ($P < 0.0001$) (Fig. 4). Conversely, measles- and rubella-specific MBCs (mean) were 5 and 10 times greater than mumps, respectively. Likewise, the medians of measles and rubella MBCs were 8 and 11 times greater than mumps, respectively. Percent antigen-specific MBCs were as follows: mumps mean 0.041%, median 0.022%, and range 0.000 to 0.244%; measles mean 0.23%, median 0.18%, and range 0.000 to 1.33%; rubella mean 0.39%, median 0.24%, and range 0.02 to 3.81%. In 7 out of 71 participants (10%), mumps-specific MBCs were below the level of detection. Three individuals had undetectable measles-specific MBCs, but all participants had rubella-specific MBCs. As a comparator, baseline influenza-specific MBCs in the same group of participants were 52 times higher than mumps (Fig. 4).

Comparison of mumps-, measles-, and rubella-specific MBCs with IgG ISRs revealed weak to moderate positive correlations, which is not unexpected, since these 2 parameters likely represent different compartments of humoral immunity (*SI Appendix*, Fig. S1). Similarly, a weak to moderate positive correlation was observed for mumps-specific MBCs and JL PRN titers (Spearman $r = 0.4354$, $P = 0.0002$; *SI Appendix*, Fig. S2). Of the 7 participants who had no detectable mumps memory B cells, 2 were seronegative by ELISA IgG and 1 of the 2 was seronegative in the PRN assay.

Mumps Avidity. The mean IgG avidity index to the mumps Enders strain as determined in 59 specimens was 75% (95% confidence interval [CI], 71 to 79%), with an index >30% considered high-avidity. The majority (37/59) of participants had an end-point titer avidity index between 60 and 80%. Avidity could not be determined in 10 specimens, due to undetectable levels of IgG.

Discussion

Humoral immunity to mumps vaccine is incompletely understood. Here we have shown that up to 10% of vaccinated young adults have no detectable mumps MBCs as well as a significantly lower frequency of MBCs compared with measles and rubella. Lower mumps-specific MBC frequencies were reported in 2 previous studies, but the sample sizes were small and did not focus on young adults (36, 37). Factors associated with reduced MBCs to mumps remain unknown, but a weaker primary B cell memory response or a faster decay is plausible. In a longitudinal study, measles- and rubella-specific MBCs had a 2- to 3-fold decline 5 to 10 y after the first dose of MMR but mumps-specific MBCs were not determined (33). Longitudinal studies of mumps neutralizing antibody titers showed a decay rate of 9.9% (compared with 7.1% for measles and 8.2% for rubella) and 9.2% per y, 8 and 12 y after receipt of the second dose of MMR, respectively (38, 39).

Furthermore, previous studies have shown that the frequency of antibody-secreting cells (ASCs) measured at 1 wk after a third dose of MMR vaccine was lower for mumps compared with measles and rubella (36). The hierarchy of responses to MMR antigens appears to be similar for MBCs and ASCs (i.e., rubella > measles > mumps). This is consistent with the observed results given that ASCs are thought to be derived from the memory B cell pool. For instance, among influenza-specific MBCs, a new subset of B cells known as activated B cells committed to the MBC lineage has been reported (40). MBCs provide long-term humoral immunity by recall responses upon reexposure to antigen (41, 42). Although significant progress has been made in the general understanding of MBC generation, affinity maturation, and kinetics, very little is known about vaccine-induced mumps-specific MBCs and their role in protection against disease.

A key factor in the mumps resurgence appears to be waning of immunity over time (11, 13, 16, 30, 38, 39). In our study, all of the participants with undetectable MBCs (except for one) were 2-dose recipients who received their recommended second MMR a mean of 16 y prior to participation in the study. Participants ($n = 6$) with the highest level of MBCs to mumps were on average 12 y from receipt of their last vaccination. But the overall correlation between time from the receipt of last MMR vaccine and frequency

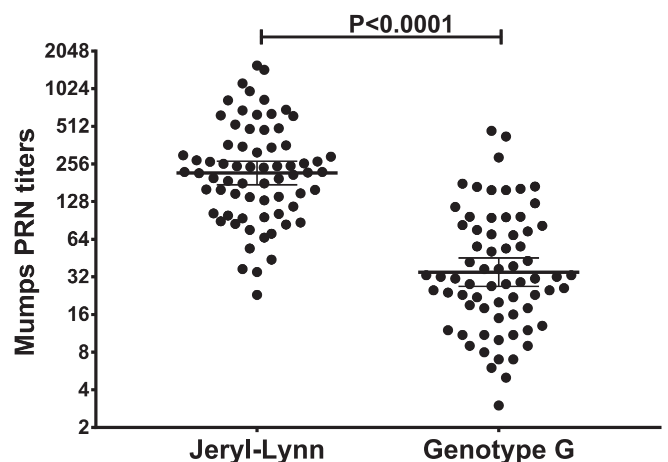

Fig. 2. Neutralizing antibody titers to mumps Jeryl Lynn versus genotype G strain. Antibody titers were determined by plaque reduction neutralization. The PRN end titer was determined to be the highest dilution of serum that gave 50% or higher plaque reduction compared with the average number of plaques formed in the absence of serum by using the Kärber formula. The geometric mean titer of 69 participants for JL was 217 compared with 35 for genotype G (Error bars are 95% CI, 174 to 270 for JL and 27 to 45 for genotype G). *P* values were determined comparing 2 groups using unpaired *t* test.

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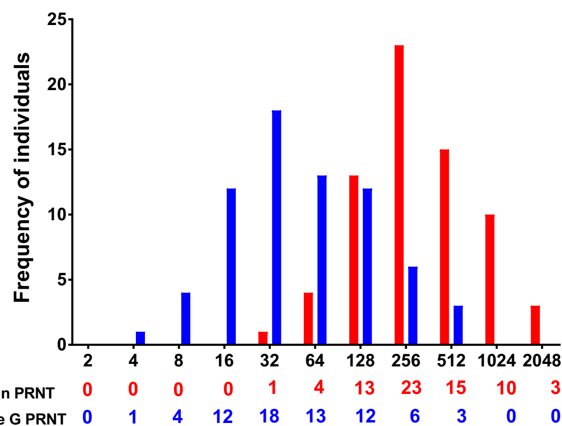

	2	4	8	16	32	64	128	256	512	1024	2048
Jeryl-Lynn PRNT	0	0	0	0	1	4	13	23	15	10	3
Genotype G PRNT	0	1	4	12	18	13	12	6	3	0	0

Fig. 3. Frequency distribution of neutralizing antibody titers to mumps JL or genotype G. PRN titers (PRNT) to the JL vaccine strain are shown in red and PRN titers to genotype G are shown in blue. The PRN end titer was determined for each strain based on the highest dilution of serum that resulted in 50% or higher plaque reduction compared with plaques formed in the absence of serum. The number of participants with a given titer to each strain is represented in the table (*Lower*).

of mumps specific MBCs was weak. Our data are limited by the cross-sectional nature of the study with one-time sampling.

Waning of immunity over time is also notable for mumps PRN titers and IgG levels in our study; participants with the lowest levels of mumps IgG and PRN titers were generally more than 15 y from the last MMR vaccination. In a case–control study of mumps vaccine performance in an outbreak on a college campus, students with mumps were more likely to have received a second dose of MMR ≥10 y earlier (43). During the 2013 mumps outbreaks in France, the odds of mumps increased by 10% for every year that passed after the second dose of MMR immunization (13). In a Finnish longitudinal cohort study, mumps antibody titers decreased by 75% over 20 y, compared with a 58 and 65% decline for measles and rubella, respectively (44).

The quality (avidity) of mumps-specific IgG antibodies may also affect susceptibility to mumps virus infection. Mumps antibody avidity maturation from low to high occurs around 6 mo following acute illness (45, 46). Vaccine-induced IgG avidity was lower than IgG avidity induced by wild-type mumps infection (46). A correlation between mumps-specific IgG concentration and avidity was reported by some (47, 48) and not observed by others (44). In this study, an end-point dilution avidity assay that is independent of IgG concentration was used. As observed previously (45), IgG antibody avidity was high (index > 30%) among vaccinees with sufficient IgG for testing, and the majority (37/59) of participants had an end-point titer avidity index between 60 and 80%, which is lower than the average avidity observed for measles using a similar end-point dilution avidity assay (49).

Epidemiological studies suggest that low titers of IgG antibodies may result in increased susceptibility to mumps. In a mumps outbreak among college students, proportionately more case patients than exposed nonpatients had a preoutbreak IgG ISR <1.40 (64 vs. 9%, P = 0.002) and <1.71 (73% vs. 14%, P = 0.001) (50). But there were overlapping values among cases and noncases and no cutoff point was absolute (50). The rate of mumps seroprevalence was high (93%) in our study, similar to previous reports (51). In contrast, a study in the US military reported a lower mumps seroprevalence of 80.5% (determined by IgG multiplex flow immunoassay) among recruits with a high level of MMR uptake (52). Seroprevalence needed for mumps herd immunity is estimated to be between 88 and 92% (53). In our study, 6 participants may have been potentially susceptible to mumps; 4 participants were seronegative, and 2 had ISRs below 1.4.

Functional antibodies such as PRN titers are a key element in immunity to mumps (43). Studies designed to determine a protective level of PRN titers to JL and genotype G strains had limited success (50, 54). In a 2006 US mumps outbreak, more cases (than exposed noncases) had preoutbreak JL titer <31 and genotype G titer <8 (50). In other studies, JL titers <8 have been considered negative and titers between 8 and <16 were designated low-positive (27, 55). If a hypothetical cutoff value of ≤32 is used for a protective level for JL and <8 for genotype G titers, 19/69 (28%) individuals in our study would be susceptible to mumps. In addition, strong positive correlations between the magnitudes of IgG antibody by ELISA and PRN titers have not been found (34, 35). The PRN assay is not routinely performed in commercial laboratories for determining immune status since it is more time-consuming and labor-intensive than the ELISA (34). Reliance on ELISA, which measures both nonneutralizing and neutralizing antibodies to mumps, may lead to overestimation of seroprotection since subjects with moderate levels of IgG antibodies may have low PRN titers (34, 35).

It has been suggested that individuals vaccinated with the attenuated mumps Jeryl Lynn vaccine strain may not have protective titers to wild-type strains due to the differences in antigenic epitopes (26). Using immunoinformatics and in silico modeling, Homan and Bremel (56) noted that the B and T cell epitopes of the HN protein of the Jeryl Lynn strain were outliers when compared with wild-type strains. Similar to our finding of a 6-fold-lower antibody titer to the genotype G strain, Rubin et al. (26) reported that titers to the genotype G strain in a 2006 mumps outbreak were half those of the Jeryl Lynn strain. Administration of a third MMR dose temporarily boosted antibody titers, but they returned to near-baseline after 1 y (55, 57). Epidemiological evidence suggests that a third dose of MMR may have a protective role in outbreak settings (23, 54, 57). Adding a routine third dose to the current vaccine schedule is feasible to implement (when combined with meningococcal vaccine at age 16), but it does not lead to a sustained increase in antibody responses (57). It is possible that a third-dose booster with a different genotype (i.e., genotype G) may lead to sustained cross-genotype immunity, but this would require considerable research to demonstrate safety and short- and long-term effectiveness.

In conclusion, the lower frequency of MBCs in our college-aged young adults supports weak long-term humoral immunity to mumps vaccine. Further efforts are needed to understand the

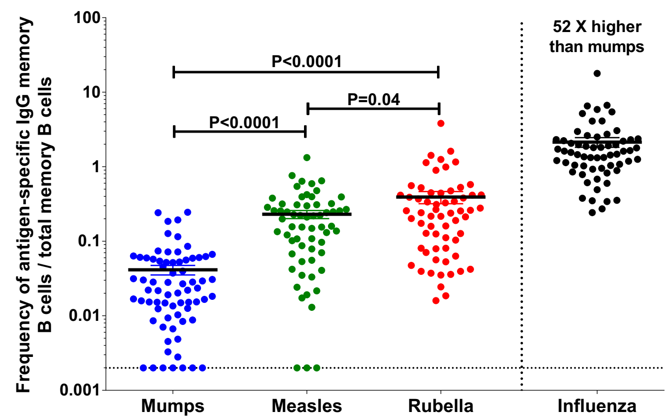

Fig. 4. Mumps-, measles-, and rubella-specific memory B cells. The frequency of antigen-specific IgG-secreting MBCs was measured in PBMCs of healthy participants after short-term culture and reported as percent of total IgG-secreting cells. Dots represent individual participants. Horizontal lines represent mean with SEM and the horizontal dotted line represents the limit of detection. Baseline influenza-specific MBCs in the same individuals were determined using the seasonal influenza vaccine as the antigen.

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mumps MBC pools, Fc receptor-mediated antibody functions such as antibody-dependent cellular cytotoxicity, the durability of protective levels of antibodies, and the contribution of CD4 and CD8 T cells to mumps immunity. However, one must place this in the broader context in that the MMR vaccine has led to a 99% reduction in disease as evidenced by average annual cases of 162,344 reported in the prevaccine era of the 20th century compared with 2,251 cases in 2018 (4). Also, outbreaks have been limited to areas with crowded living conditions, where the force of infection can overwhelm lower herd-immunity levels. Strategies to improve the longevity and quality of the humoral immune responses to the current mumps vaccine need consideration. With advances in adjuvant platforms, reverse immunology, and structure-stabilized recombinant immunogens, a mumps vaccine or a booster containing antigens of circulating strains that could induce broad, sustained immunity to all mumps genotypes could be attainable.

Materials and Methods

Study Design and Human Subjects. Healthy college students 18 to 23 y of age were recruited by placing advertisements on a college campus between February and August 2010. The study initially aimed to enroll participants who received the recommended 2-dose series of age-appropriate MMR vaccine but was later broadened to include receipt of any number of MMR vaccines in the past. Written informed consent was obtained from all study participants. Receipt of vaccinations was verified by review of physician immunization records. Participants were excluded if they were immunocompromised, were taking immunosuppressive medicines, received recent blood transfusions, or received any immunizations 30 d prior to enrollment. The study was conducted at the Hope Clinic of the Emory Vaccine Center in collaboration with the Centers for Disease Control and Prevention (CDC). The study was reviewed and approved by the Emory University Institutional Review Board and CDC Institutional Review Board and was registered at ClinicalTrials.gov (NCT00962819). A one-time blood sample was collected from 71 out of 74 recruited participants; we were unable to collect blood samples from the remaining 3. Specimens were assigned unique codes and analyzed at the Emory Vaccine Center and the CDC. Participants were asked to complete a survey regarding mumps natural infection or potential exposure to mumps, measles, and rubella 3 y prior to enrollment in the study.

ELISA for Mumps, Measles, and Rubella. Three commercially available indirect ELISAs using vaccine strain antigens (measles IgG ELISA II, mumps IgG ELISA II, and rubella IgG ELISA II) from Wampole Laboratories/Zeus Scientific were used for qualitative determination of serum IgG antibodies. Test sensitivity/specificity stated by the manufacturer was 96.6/90.4% for mumps, 93.3/97.4% for measles, and 100/100% for rubella. Positive, equivocal, and negative categorization of sera were determined using the cutoff values specified by the manufacturer based on an index standard ratio (ISR). ISR values for all 3 viruses were defined as follows: $\leq$0.90, seronegative; 0.91 to 1.09, equivocal; and $\geq$1.10, seropositive.

Plaque Reduction Neutralization Assay for Mumps. Neutralizing antibody titers to the JL mumps vaccine strain and the wild-type strain, genotype G, were determined by the PRN assay as described previously (17, 34). PRN end-point titers were determined to be the highest dilution of serum that gave 50% or higher plaque reduction compared with the average number of plaques formed in the absence of serum by using the Kärber formula. Serum was heat-inactivated for 30 min and serially diluted 4-fold starting from 1:2 to 1:2,048 and mixed with an equal volume of either JL or genotype G virus that yielded 40 to 60 plaque-forming units, containing a final dilution ranging from 1:4 to 1:4,096. We did not assess measles and rubella PRN titers.

Mumps Avidity Assay. IgG avidity testing was performed using a commercial (Zeus Scientific) mumps-specific IgG enzyme immunoassay modified to include 3 protein-denaturing washes and designed to measure avidity in serum serially diluted to the end point (45, 49). Receiver operating characteristic (ROC) analysis of unpaired serum specimens from 15-mo-old infants collected 1 mo and 2 y after first-dose MMR was used. Results were calculated using a threshold based on an optical density ratio of 1.5 and expressed as end-titer avidity index percentages (etAI%s) as previously described (49, 58). The results were classified as low-avidity if etAI% $\leq$30% and high-avidity if etAI% >30%. Samples at 1:10.5 dilution with undetectable IgG after denaturant agent diethylamine treatment were classified as low-avidity. Per the ROC curve, the assay is accurate (area under the curve is 0.994 [95% CI, 0.956 to 1.000]), 96.5% sensitive (95% CI, 87.9 to 99.6%), and 92.2% specific (95% CI, 81.1 to 97.8%) at an avidity index of 30%.

Memory B Cell and ELISpot Assays. The assay to measure the frequency of MBCs was performed as previously described (32). Briefly, 5×10^5 peripheral blood mononuclear cells (PBMCs) were seeded in each well of a 24-well plate in 1 mL RPMI medium 1640 in the presence of 6.0 μg/mL CpG oligodeoxynucleotide ODN-2006 (Operon Technologies), 1:1,000 dilution pokeweed mitogen (made at Emory University), and *Staphylococcus aureus* Cowan, fixed (Sigma). Control wells had cells with complete media alone. Cells were incubated for 6 d at 37 °C and 5% CO_2. To harvest the culture, cells from each well were transferred into 1.2-mL-capacity 96-well plates (Thermo Scientific) and centrifuged at 1,200 rpm for 8 min. After decanting the supernatant, cell pellets were resuspended in 200 μL complete medium and transferred to 96-well round-bottom plates. Cells were washed twice and IgG-secreting cells were enumerated using ELISpot (*SI Appendix*, *Methods*). Appropriate negative controls were used during both in vitro stimulation (media alone without any stimulation mix) and ELISpot analysis (uninfected Vero cell lysate, matched to the mumps-infected lysate). As a comparator, the influenza MBC assay was done using the trivalent influenza virus vaccine as the antigen (Fluarix 2008 to 2009: A/Brisbane/59/2007 [H1N1]-like virus; A/Brisbane/10/2007 [H3N2]-like virus; B/Florida/4/2006-like viruses).

The frequency of antigen-specific MBCs among the circulating total IgG-secreting MBCs was determined using a previously described assay based on in vitro polyclonal stimulation of PBMCs followed by an ELISpot assay (32, 59). For the ELISpot assay, the following viruses were used as capture antigens: mumps whole virus, Jeryl Lynn strain grown in Vero cells provided by Merck, and measles (Edmonston strain) and rubella (HPV77 strain) viruses grown in Vero cells obtained from Meridian Life Sciences. Since the Enders strain used in commercial IgG ELISA is different from the Jeryl Lynn strain we used for the MBC ELISpot, we performed an in-house IgG ELISA using the Jeryl Lynn strain whole-virus lysate (60). A strong correlation was observed between the commercial IgG ELISA and the in-house ELISA (Spearman $r = 0.7566$, $P = 0.0001$; *SI Appendix*, Fig. S3), affirming our decision to utilize the Jeryl Lynn strain as a capture antigen for the MBC ELISpot assay. Based on our preliminary observations and to be certain that we captured potentially low levels of MBCs, we performed 6 replicates for determining total IgG and 8 to 12 replicates for determining mumps-, measles-, and rubella-specific IgG-secreting MBCs.

Any participant who was either seronegative or lacked detectable MBCs to mumps or measles was offered a booster dose of MMR vaccine. Postboosting immune responses were not assessed in this study.

Statistics. For univariate analysis, frequency tables were used to summarize the variables and strip plots were used to visualize continuous variables. The association between variables was analyzed using Spearman's rank correlation coefficient. A permutation test was used to assess the statistical significance of the correlation. GraphPad Prism software (version 5.04) was used for statistical analysis and graphing of the data. Statistical significance was assigned to P values <0.05 for all analyses. A 2-tailed paired t test was used to determine the significance of JL vs. genotype G PRN titers.

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