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Identification of Antigen-Specific B Cell Receptor Sequences Using Public Repertoire Analysis

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High-throughput sequencing allows detailed study of the BCR repertoire postimmunization, but it remains unclear to what extent the de novo identification of Ag-specific sequences from the total BCR repertoire is possible. A conjugate vaccine containing *Haemophilus influenzae* type b (Hib) and group C meningococcal polysaccharides, as well as tetanus toxoid (TT), was used to investigate the BCR repertoire of adult humans following immunization and to test the hypothesis that public or convergent repertoire analysis could identify Ag-specific sequences. A number of Ag-specific BCR sequences have been reported for Hib and TT, which made a vaccine containing these two Ags an ideal immunological stimulus. Analysis of identical CDR3 amino acid sequences that were shared by individuals in the postvaccine repertoire identified a number of known Hib-specific sequences but only one previously described TT sequence. The extension of this analysis to nonidentical, but highly similar, CDR3 amino acid sequences revealed a number of other TT-related sequences. The anti-Hib avidity index postvaccination strongly correlated with the relative frequency of Hib-specific sequences, indicating that the postvaccination public BCR repertoire may be related to more conventional measures of immunogenicity correlating with disease protection. Analysis of public BCR repertoire provided evidence of convergent BCR evolution in individuals exposed to the same Ags. If this finding is confirmed, the public repertoire could be used for rapid and direct identification of protective Ag-specific BCR sequences from peripheral blood. *The Journal of Immunology*, 2015, 194: 000–000.

The human humoral response is anticipatory, with diverse Ab specificities present even prior to Ag stimulation, to account for the extensive range of potential Ags likely to be encountered. The basis for this diverse repertoire is the multiple variable (V), diversity (D; H chain only), and junctional (J) B cell gene segments encoding the V region of the Ab H and L chain proteins (1). Further variation is created by combinatorial association, junctional diversity, and somatic hypermutation, leading to the creation of up to 10^{11} unique Ab molecules (2). Within the variable domains of each H and L chain are the three CDRs that

encode the amino acid loops of the Ag binding site, which are particularly susceptible to somatic hypermutation (3). Of these, the variable heavy (V_H) CDR3 plays a dominant role in Ag binding and specificity (4, 5).

Next-generation sequencing (NGS) technologies perform large-scale DNA sequencing (6), allowing in-depth analysis of the BCR repertoire of the circulating B cell pool (7, 8). The Roche 454 platform generates reads of sufficient length to interrogate the entire recombined H chain VDJ region. 454 sequencing of Ab variable regions has been used to obtain estimates of BCR repertoire diversity (2, 9), to detect and track clonal expansions in lymphoid malignancy (10), and to investigate the characteristics of different B cell lineages (11–13). However, understanding the diversity of the BCR repertoire in relation to Ag specificity remains challenging. This is an important area to advance understanding in autoimmunity, immunity against infectious diseases, and immunization. Studies of the BCR repertoire generated in response to specific Ags, such as bacterial polysaccharides (14–16), viral glycoproteins (17–19), and autoimmune Ags (20), used small numbers of immortalized B cell lines and suggested that genetically diverse individuals used similar combinations of H chain VDJ segments in response to a given Ag. However, there is some evidence that VDJ gene segment usage may be relatively independent of Ag specificity; this is supported by the fact that BCR sequences that differ markedly in the CDR3 sequence can have the same V(D)J usage (J.T., unpublished observations). NGS approaches have the potential to advance understanding of this area through access to vastly increased numbers of BCR sequences across a larger number of individuals. Although isolation of Ag-specific B cells is possible, this requires the development of Ag-specific staining and sorting protocols to detect low-frequency B cell populations. Several studies used the relative enrichment for Ag-specific B cells that occurs at day 7 following immunization. Although these demonstrated changes in the large-scale structural features of the repertoire, they did not investigate which features of BCR sequences indicate Ag specificity. Two

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Abbreviations used in this article: Hib, *Haemophilus influenzae* type b; MenC, serogroup C meningococcal; MID, multiplex identifier; NGS, next-generation sequencing; PRP, polyribosyl ribitol phosphate; TT, tetanus toxoid; V_H , variable heavy.

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recent studies found that conserved CDR3 sequences were produced in patients recovering from acute dengue infection (21) and during the immune response following pandemic influenza H1N1 vaccination (22). Similar CDR3 sequences that dominate the immune response in different individuals following Ag stimulation are often referred to as a convergent or public repertoire. We used a model Ag in the form of a vaccine in which *Haemophilus influenzae* type B (Hib) and serogroup C meningococcal (MenC) polysaccharides are conjugated to tetanus toxoid (TT) to stimulate human B cell responses. A significant amount of BCR sequence data is available for the Hib polysaccharide, showing that clonotypes are similar between individuals and revealing usage of a single V_H (V3-23) and only two J_H gene segments combined with two variable and joining L chain gene segments (23–26). The canonical Hib-specific Ab has a conserved CDR3 amino acid motif of Gly-Tyr-Gly-Phe/Met-Asp (GYGMD or GYGFD) (27). Previous investigations used low-resolution methods and, therefore, were only able to describe a small number of Hib-specific sequences, whereas information about the relative abundance of different sequences between time points and individuals, their mutational rate, and the isotype subclasses is lacking. For TT, the limited data indicate that there is a much more diverse repertoire with very few sequences that are shared (28, 29). The use of Ags with CDR3 sequences known to specifically bind Ag, together with deep sequencing of B cell H chain variable domains following Ag stimulation, provides an opportunity to investigate several aspects of the repertoire. The main objective of the current study was to identify Ag-specific BCR sequences following vaccination by searching for sequences shared between participants and by comparing sequences with previously described Ag-specific sequences. Sequence analysis focused on CDR3 amino acid sequences, although other V gene regions, such as CDR1 and CDR2, may be important for Ag binding (3). Ag-specific repertoires were then used to characterize CDR3 length, V(D)J usage, mutational rate, and isotype (subclasses). Finally, the Hib-specific repertoires were compared with anti-Hib Ab concentration and avidity index to investigate the use of repertoire sequencing as an alternative measure of immunogenicity.

Materials and Methods

Participants and vaccine

Ab responses specific for Hib were studied as part of a single-center, open-label clinical study in healthy adults aged 18–65 y, undertaken to generate a Hib reference serum for the U.K.’s National Institute for Biological Standards and Control. Written informed consent was obtained from the participants before enrollment. Ethical approval was obtained from the Oxfordshire Research Ethics Committee (Reference 08/H0605/74). Volunteers received a Hib-MenC polysaccharide-protein conjugate vaccine (Menitorix; GlaxoSmithKline), containing Hib polyribosyl ribitol phosphate (PRP; 5 µg) polysaccharide and MenC polysaccharide (5 µg) individually conjugated to TT carrier protein (total 17.5 µg). Blood was taken from participants immediately prior to vaccination and at 7 and 28 d after vaccination.

Anti-PRP Ab and avidity index 1 mo postvaccination

Serum anti-PRP IgG concentrations were measured by ELISA. Ab avidity can be used as a surrogate measure for Ab quality, and it tends to be increased in memory responses (30). Ab avidity was determined by elution using a 0.15-M solution of the chaotrope sodium thiocyanate as a separate step after the initial binding of the serially diluted sera. Avidity index was expressed as the percentage reduction in IgG concentration compared with the concentration in the absence of the chaotrope (31).

B cell isolation and cell sorting

PBMCs were obtained by density gradient centrifugation over Lymphoprep (Axis-Shield). CD19⁺ cells were separated at baseline from all participant samples and, at day 7, either plasma or CD19⁺ cells were obtained. Enrichment of cell populations was performed by magnetic cell separation using CD19 MicroBeads or a Plasma Cell Isolation Kit II and AutoMACS (Miltenyi

Biotec). The purity of sorted populations was checked using flow cytometry and was 95–99% for plasma cells and 82–97% for the CD19⁺ B cells.

cDNA synthesis and PCR

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized using random hexamers and Superscript III reverse transcriptase (Invitrogen), with reverse transcription at 42°C (60 min) and inactivation at 95°C (10 min). Ab H chain sequences were amplified using previously published V_H family consensus forward primers and reverse primers enabling identification of IgM, IgA, and IgG (11). Two rounds of PCR amplification were performed using Taq DNA polymerase (QIAGEN), with initial denaturation at 94°C (3 min), followed by 30 (first round) or 15 (second round) cycles of denaturation at 94°C (30 s), annealing at 58°C (30 s), extension at 72°C (1 min) and final extension at 72°C (10 min).

Library preparation and 454 sequencing

The PCR amplicons were prepared for sequencing and blunt-end ligated to multiplex identifier (MID) tags using the Roche GS FLX Titanium Rapid Library Preparation Protocol. Emulsion PCR and pyrosequencing were performed on each library using the GS FLX Titanium XL+ sequencing platform.

Sequence analysis

Output files were converted into FASTA files, and sequences were assigned a sample and isotype based on the MID sequence. For two individuals at both time points, isotype-specific amplicons were sequenced without separate MID tags and, therefore, sequences were assigned an isotype by C region sequence motifs; sequences that could not be matched for isotype were discarded. Remaining sequences were analyzed using IMGT/HighV-QUEST, allowing for insertions and deletions (32), and only productive sequences were considered further. Analysis was performed using RStudio (version 0.98.490) and R (33) and graphically displayed using ggplot2 (34). Sequence clonality was calculated by dividing the total number of sequences for each sample by the number of unique VDJ combinations. Of note, an increase in clonality at the sequence level could reflect either an increase in overall clonality of the B cell population or a change in Ig mRNA expression levels in a subset of B cells within a population of fixed clonality.

Sequences with identical CDR3 amino acid sequences, which were shared between at least two individuals, were used to define the public repertoire. Hib-specific sequences were identified as those that contained either the amino acid string GYGMD or GYGFD (27) and were exactly 10-aa long. For each of these sequences, VJ usage and the relative abundances were calculated by dividing the number of Hib-specific sequences by the total number of sequences for each sample. Further, for each of the Hib-specific CDR3 amino acid sequences, the number of study participants using identical VJ combinations to create the same Hib-specific CDR3 amino acid sequence was calculated.

CDR3 amino acid sequences also were compared with previously described sequences specific for Hib (24, 25, 27, 35), TT (36–39), H1N1 influenza (40, 41), and MenC (42, 43) by searching for identical and closely related sequences in the dataset. Approximate matches to known CDR3 amino acid string patterns were searched within the data by the “agrep” function in R (33), using the default parameters but excluding insertions and deletions (maximum Levenshtein edit distance between pattern substring and search strings equals 10% of the pattern substring length [e.g., for a 10-aa string, the maximal number of substitutions allowed to call it “related” is 1 aa]).

Proportions of Hib-specific sequences/total number of sequences were calculated for each sample, and their log-transformed values correlated with the log-transformed Ab concentrations and avidity indices. The same analysis was performed to calculate the correlation between the frequencies of unique Hib-specific CDR3 amino acid sequences and Ab.

Isotype subclass information for IgA and IgG was determined by mapping C region nucleotide sequences to all possible isotypes (subclasses) using Stampy (44) and Vertebrate and Genome Annotation C region sequences as reference (45). The number of V gene mutations was taken from IMGT output files and compared between sequences of different Ag specificities and isotype subclasses.

Results

General overview

At baseline, CD19⁺ B cells were isolated from all five participants. Seven days following vaccination, CD19⁺ B cells were isolated from two individuals, and plasma cells were isolated from three individuals. IgG, IgA, and IgM BCR libraries were prepared from

each sample, with one library preparation failing (IgG from plasma cells). In total, 460,077 sequences were obtained (average 15,860/sample), of which 184,844 (average 6,374/sample) were considered productive by IMGT (Table I).

Approximately 12% of C region sequences from IgA sequence libraries (7,094/61,023 sequences) were too short for confirmation of an IgA subclass, and the isotype of 110 sequences (0.2%) differed from the original PCR primer isotype. For IgG sequence libraries, 2,781/25,280 (11%) sequences were too short for identification, and the isotype of 19 sequences (0.08%) differed from the original PCR primer isotype. Before vaccination, IgA sequences were dominated by IgA₁, and IgG₁ was the most common among the IgG sequences. After vaccination, there was a relative increase in IgA₁ from 65 to 70% and in IgG₂ from 33 to 48% of assigned sequences (Supplemental Fig. 1A).

Sequence clonality and CDR3 length distributions

At baseline, clonality was similar for each of the isotypes. The day-7 plasma cell samples were significantly more oligoclonal than their paired baseline CD19⁺ B cell samples, whereas for paired pre- and postvaccination CD19⁺ B cell samples, an increase in clonality was seen for IgG, but not IgA or IgM, sequences (Supplemental Fig. 1B). Graphical representation of the VDJ repertoire also showed increased clonality after vaccination (Supplemental Fig. 2). CDR3 length distributions of all isotypes were similar between isotypes and before and after vaccination (Supplemental Fig. 1C).

The postvaccination public repertoire is dominated by Hib-specific sequences

The postvaccination public repertoire was defined as the collection of CDR3 amino acid sequences that were shared by at least two of

the five individuals; a total of 47 such CDR3 amino acid sequences were found. More than 50% of these CDR3 amino acid sequences were exactly 10 aa long, which was considerably shorter than the average CDR3 amino acid sequence lengths from all sequence data at day 7 (Fig. 1). We further looked for the presence of these 47 shared CDR3 amino acid sequences in all samples, and the result is represented as an unsupervised heat map with dendograms (Fig. 2). Thirty percent (14/47) of the day-7 public CDR3 amino acid sequences showed distinctive characteristics of previously identified Hib-specific motifs (27). The frequency of sequences determined as Hib specific was significantly enriched postvaccination in all of the isotypes tested, although the frequency of those sequences differed considerably between individuals (Table I). The three most abundant CDR3 amino acid sequences, which were shared by all five individuals and were present in 10–14 of the 15 postvaccination samples, contained Hib-specific motifs (Fig. 2). Postvaccination, another nine of the public CDR3 amino acid sequences also were 10 aa long but differed by only 1 aa from previously described Hib-specific CDR3 amino acid motifs (Supplemental Fig. 3A). Hence, almost half (23/47) of the CDR3 amino acid sequences in the postvaccination public repertoire showed characteristics that were similar or identical to those previously described as binding the Hib polysaccharide Ag.

VJ usage of Hib-specific sequences

Sequences containing known Hib-specific CDR3 amino acid motifs were analyzed further. These sequences were dominated by gene rearrangements consisting of the gene segments V3-23 and J6 in all isotypes. The Hib-specific CDR3 amino acid sequences shared by all participants (which were also the most abundant sequences) showed the broadest range of VJ usage (Supplemental Fig. 3B). In

Table I. Characteristics of sequence data

Participant	Day Postvaccination	Isotype	B Cell Subset	No. Total Sequences	No. of Productive Sequences	No. of Hib CDR3 Amino Acid Sequences ^a	No. of Unique Hib CDR3 Amino Acid Sequences ^b	Anti-PRP Ab (μg/ml)/Avidity Index
1	0	IgA	CD19 ⁺	3,313	2,527	0	0	49.0/59.83
		IgG		2,277	1,689	0	0	
		IgM		7,843	5,998	0	0	
	7	IgA	Plasma cells	3,750	3,026	277	4	45.89/343.29
		IgG		2,165	1,710	78	3	
		IgM		8,694	6,932	601	6	
2	0	IgA	CD19 ⁺	5,071	3,997	0	0	7.3/30.15
		IgM		12,802	10,256	1	1	
		IgA	Plasma cells	3,899	3,289	620	6	
	7	IgG		1,612	1,342	446	10	29.1/66.51
		IgM		7,131	5,819	1,221	13	
		IgA		23,857	9,522	1	1	
3	0	IgG		19,412	3,763	0	0	123.7/3.52
		IgM		20,773	10,879	1	1	
		IgA	Plasma cells	24,768	12,073	143	4	
	7	IgG		15,334	5,350	97	8	16.379
		IgM		27,272	10,156	71	5	
		IgA		21,952	7,858	1	1	
4	0	IgG		32,545	2,391	0	0	16.379
		IgM		24,633	11,237	0	0	
		IgA	CD19 ⁺	22,630	9,080	624	10	
	7	IgG		20,871	3,723	614	10	16.379
		IgM		23,315	9,279	399	8	
		IgA		26,016	9,051	0	0	
5	0	IgG		18,655	3,227	0	0	16.379
		IgM		23,552	11,774	0	0	
		IgA	CD19 ⁺	19,134	7,804	50	3	
	7	IgG		16,379	4,885	5	2	16.379
		IgM		20,422	6,207	1	1	

^aNumber of CDR3 amino acid sequences of length 10 aa containing the string “GYGMD” or “GYGFD” (27).

^bNumber of different CDR3 amino acid sequences showing Hib-specific characteristics as in footnote ^a.

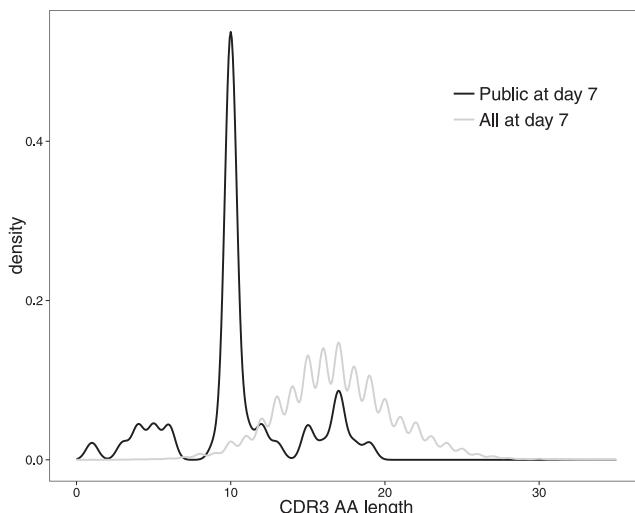


FIGURE 1. CDR3 amino acid length distributions of unique sequences 7 d postvaccination shared by at least two study participants (“public repertoire”) compared with all sequences at day 7 (“total repertoire”). The proportion of sequences of a particular length (x-axis) without counting duplicate sequences in each group is shown on the y-axis as a density plot.

general, less diversity of VJ usage was observed in the IgG repertoire compared with the IgM and IgA repertoires (Supplemental Fig. 3B). Although there were variations in the breadth of VJ usage among individuals, within a given participant a similar usage of VJ segments was seen across the different isotypes (Fig. 3). We also assessed how many participants used the same VJ combination to create similar Hib-specific CDR3 amino acid sequences. VJ usage of some of the Hib-specific CDR3 amino acid sequences was very diverse, whereas only a single VJ rearrangement was found for other CDR3 amino acid sequences (Supplemental Fig. 4A).

TT-specific BCR repertoire

In addition to the Hib-specific sequences that dominated the shared repertoire at day 7 postvaccination, one shared CDR3 amino acid sequence that we identified was described previously as being TT specific (CASGSTLDYW) (36), with another sequence of similar length closely resembling this sequence (CTSGSTFDYW) (Fig. 2). By allowing some mismatch between the CDR3 amino acid sequences in the dataset and previously described sequences specific for TT, we identified several other sequences related to known TT-specific sequences. The majority of CDR3 amino acid sequences identified in this manner had the same length and were highly similar (Levenshtein edit distance between pattern substring and search strings ≤ 2) to previously described sequences (data not shown). These TT-related sequences found in the dataset were enriched and shared between individuals 7 d postvaccination (Fig. 4). In a similar manner, the dataset was investigated for sequences previously described as being specific for H1N1 influenza and MenC. Sequences related to these Ags were not enriched postvaccination (Fig. 5).

The Hib and TT repertoires consist of limited isotype subclasses and show increased mutation postvaccination

Postvaccination Hib-specific sequences were dominated by the subclasses IgA₂ and IgG₂ (Supplemental Fig. 4B). A relative increase in subclasses IgA₁ and IgG₁ was seen for sequences related to known TT sequences (Supplemental Fig. 4C).

Frequencies of nucleotide mutations in V genes (V_{MUT}) differed among isotypes, with IgM sequences having fewer mutations both before and after vaccination than other isotypes. V_{MUT} increased

significantly for IgM and IgG, but not for IgA, sequences after vaccination (data not shown). At baseline, sequences identified as Hib- or TT-specific had similar V_{MUT} compared with sequences of unknown specificity, although the numbers of Ag-specific sequences were low. Seven days postvaccination, V_{MUT} differed significantly among sequences of Hib, TT, and unknown specificity ($TT > >$ unknown $> >$ Hib, $p < 10^{-16}$ for all comparisons, t test; Fig. 6).

Hib-specific CDR3 amino acid frequency and diversity are correlated with functional anti-Hib Ab

The proportion of Hib-specific CDR3 amino acid sequences/sample by isotype at day 7 correlated strongly with anti-Hib avidity indices 1 mo following vaccination (Fig. 7A, Pearson’s $r = +0.8\text{--}0.94$, significant for IgG and IgM) but not with anti-PRP Ab concentrations measured at 1 mo (Supplemental Fig. 4D). Similarly, the number of nonidentical Hib-specific CDR3 amino acid sequences/sample correlated with avidity indices (Fig. 7B, $r = +0.68\text{--}0.93$, significant for IgM) but not with Ab concentrations (Supplemental Fig. 4E).

Discussion

We demonstrated that high-throughput sequencing of BCR H chain transcripts, before and after vaccination, can be used to identify Ag-specific sequences by studying response to a vaccine containing capsular polysaccharides from Hib and MenC, individually conjugated to TT carrier protein. Known and presumptive novel Ag-specific sequences were found by searching for H chain CDR3 amino acid sequences that were shared between participants and by comparing sequences with previously described Ag-specific sequences within the pool of postvaccination sequences. Postvaccination sequences shared between study participants were rare as a proportion of total sequence diversity (47/32,186 [0.15%] unique CDR3 amino acid sequences) but constituted a much greater proportion of the total (8,099/90,675 [8.9%] CDR3 amino acid sequences). Although sequences (especially short sequences) may be shared by chance, the approach used in this study identified sequences that were highly enriched only in postvaccine samples. These sequences also showed isotype subclass distribution similar to previous studies using serum and were similar to those with previously described Ag specificity. The data from IgG sequences suggest that it may be possible to identify public repertoire sequences, following vaccination, from total CD19⁺ B cells without the need for isolation of plasma cells. Public repertoire sequences had an unusual CDR3 amino acid length distribution and were dominated by CDR3 sequences with a length of 10 aa (Fig. 1). This feature of shorter postvaccination CDR3 sizes was shown previously using spectratype analysis following combined influenza and 23-valent pneumococcal vaccination (46). It is unknown whether this is a property of newly generated Ag-specific CDR3 amino acid sequences (versus resting memory or naive B cells) or is characteristic of sequences stimulated by polysaccharide-containing Hib and pneumococcal vaccines. A large proportion of shared (unique) CDR3 motifs (14/47, corresponding to 5206/8099 [64%] sequences) showed an amino acid motif that was characterized previously as specific for the capsular polysaccharide of Hib (27). Almost all of the sequences that were 10 aa long and contained previously identified Hib motifs (5206/5248 [99.2%] sequences) were shared among participants. Unsupervised hierarchical clustering of samples containing day-7 shared CDR3 amino acid sequences distinguished baseline and day-7 samples, further indicating that these CDR3 amino acid sequences were produced in response to vaccination (Fig. 2). We identified another 9/47 (14%) CDR3 amino acid motifs within the postvaccination public repertoire closely

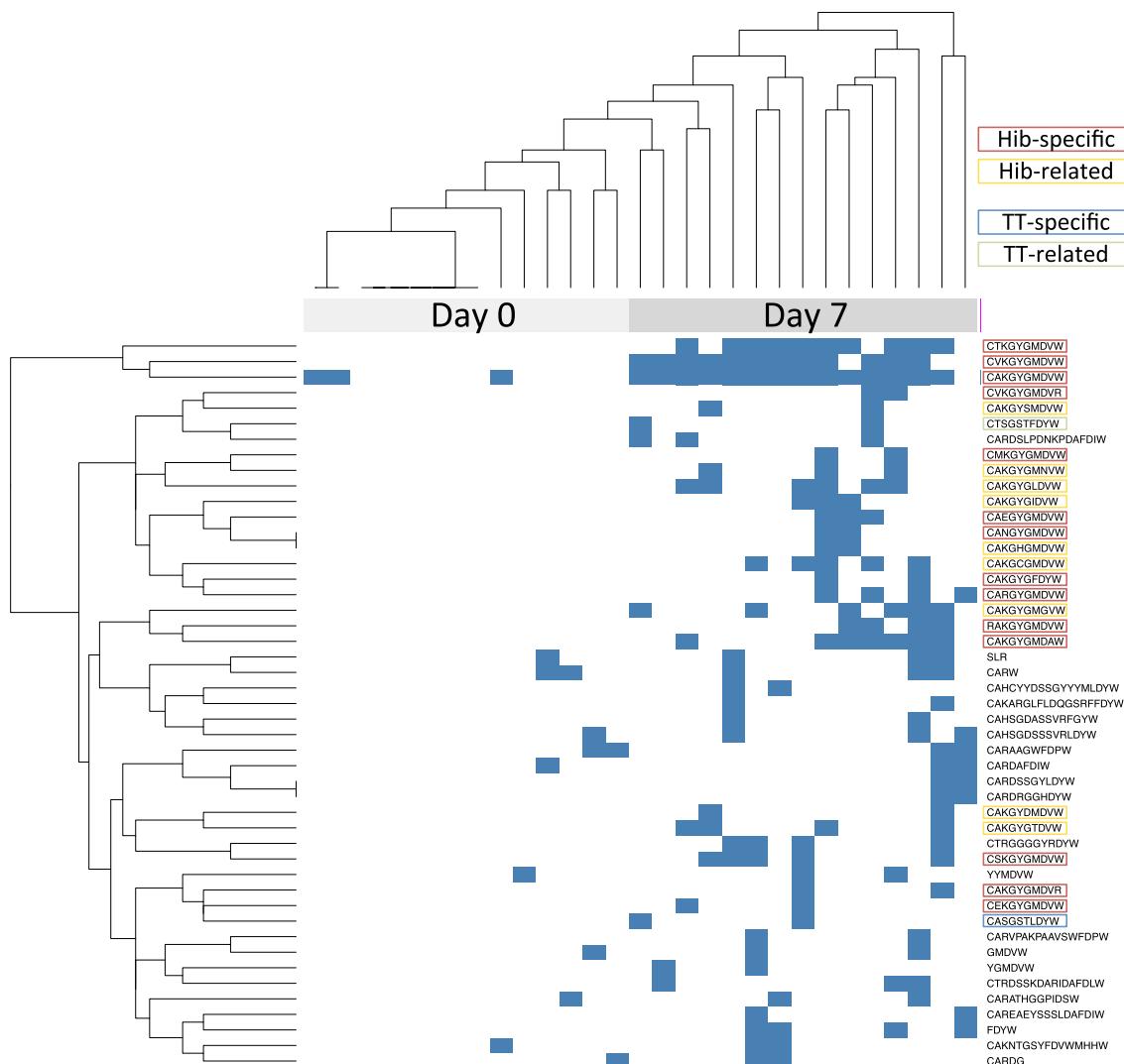


FIGURE 2. Heat map and unsupervised hierarchical clustering of shared CDR3 amino acid sequences. Individual samples (columns) were assessed for the presence of shared CDR3 amino acid sequences (rows). A blue square indicates that the CDR3 amino acid sequence of at least one sequence exactly matched the query string.

resembling known Hib motifs and that also were enriched in postvaccination samples (Fig. 2, Supplemental Fig. 3A). Although it is possible that these “novel” Hib-specific sequences are the result of PCR or 454 sequencing errors, it seems unlikely that similar errors would have occurred in separate samples from two participants. Sequences containing those additional motifs were rare in the current study (72/8099 shared sequences); therefore, it is likely that previous attempts using low-resolution techniques may have missed these sequences. Such previous methods were used to describe a limited number of mAbs directed against a variety of vaccine Ags and in response to natural infection (21, 24, 36–38, 41, 47–54). NGS allows capture of the whole breadth of the BCR repertoire by comparing sequences between time points and across individuals.

In the current study, although dominated by previously described sequences using V3-23 and J6 gene segments, Hib-specific sequences also were found to be encoded by a variety of V segments (Supplemental Fig. 3B). Furthermore, similar CDR3 amino acid sequences were encoded by similar V and J genes among several individuals (Supplemental Fig. 4A), suggesting that the Hib-specific Ab response is broader than previously acknowledged both within and between individuals and making it unlikely that germline allelic polymorphisms have a great impact on the

overall quantity and quality of anti-Hib Abs (55). These results also suggest that anti-Hib Abs can harbor a range of CDR1 and CDR2 sequences, as previously demonstrated in the immune response to a variety of Ags in mice (5).

We further expanded the sequence analysis, aiming to identify and characterize sequences targeting the TT protein contained in the vaccine given to study participants. Only 1 of 47 shared CDR3 amino acid sequences was identical to previously described TT-specific sequences. The lack of TT-specific sequences in the public repertoire may be the result of TT Ag complexity and the targeting of many more epitopes of this protein (compared with a polysaccharide Ag with repeating units) by B cells. However, by comparing the dataset with three published sources (36–38) and allowing for minimal mismatches between the CDR3 amino acid sequences, many more sequences closely resembling TT-specific sequences were identified (Fig. 4). Approximately 55% of the sequences related to previously known CDR3 amino acid sequences were shared among participants postvaccination, highlighting the convergence of the Ag-specific BCR repertoire. In contrast, almost none of the sequences resembled any of the 91 previously described H1N1 influenza-specific sequences (40, 41), both before and after vaccination (Fig. 5). Few MenC-specific

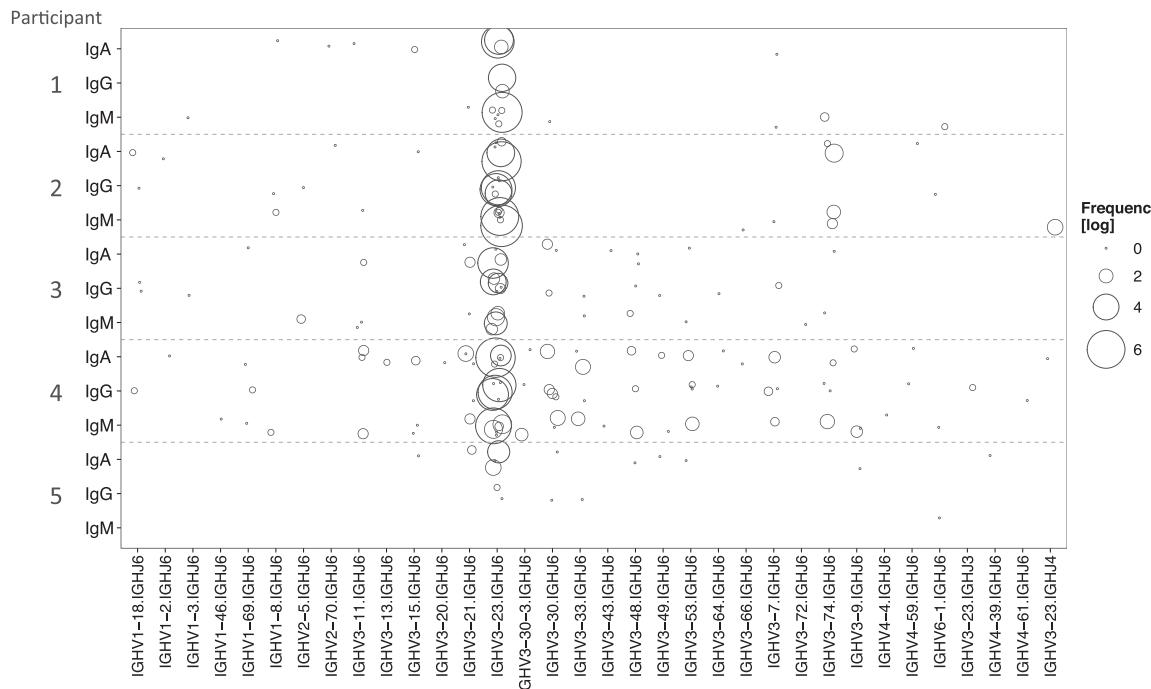


FIGURE 3. VJ gene usage of known Hib-specific sequences by individual participants according to isotype at day 7 postvaccination. For each participant, the VJ usage of Hib-specific sequences is shown by isotype, with the size of the circle representing the number of sequences of a particular VJ combination on the log scale. Participants showed similar VJ usage of Hib-specific CDR3 amino acid sequences across isotypes.

sequences have been identified, primarily in mice, which may explain why only three MenC-related sequences were found in the dataset (Fig. 5), all of which were shared among study participants; however, their frequencies were lower at day 7 than at baseline. It is possible that some of the day-7 shared sequences of unknown specificity are targeting the MenC polysaccharide, but we were unable to confirm this because of the lack of pre-existing MenC data. Using a single-component vaccine may help to identify MenC-specific sequences.

Mutational analysis of Hib- and TT-specific sequences within the dataset revealed that, in general, TT sequences are more mutated than Hib sequences. V gene mutations increased significantly for all

specificities between baseline and postvaccination samples, further indicating that these sequences are generated by vaccine-containing Ags. The differences in mutation between Hib and TT might be due to the nature of the Ag: protein versus polysaccharide. However, it is noteworthy that the volunteers in this study may have received as many as five doses of tetanus vaccine through the U.K. immunization program; however, most of the participants were unlikely to have received any Hib vaccine because they were born before this immunization program commenced. Hib is a frequent colonizing organism in healthy children (56), but the nature of B cell priming following Hib carriage is unclear.

CDR3 AA sequence	Reference	Length	No. of (related) sequences at baseline	No. of (related) sequences 7 days after vaccination	No. of participants with (related) sequences at baseline	No. of participants with (related) sequences 7 days after vaccination
CARDYFGGSVYYFDYW	Poulsen 2011	17	0	22	0	3
CARDYFGGSIYYFDYW	Poulsen 2011	17	0	30	0	3
CASGNTLDYW	Poulsen 2011	10	0	17	0	2
CATGRTLDYW	Poulsen 2011	10	0	35	0	2
CARSVVPATRAFDWF	Poulsen 2011	15	0	2	0	2
CARQTDNWFDPW	Poulsen 2011	12	5	80	2	3
CARDYSSPYYYFDYW	Poulsen 2011	14	3	10	2	2
CARDYFGSGPIYYFDHW	Poulsen 2011	17	0	5	0	2
CARDYGGSGSHYYFDYW	Poulsen 2011	17	0	14	0	3
CASGSTLDYW	Poulsen 2011	10	0	21	0	2
CATGNTLDYW	Poulsen 2011	10	0	46	0	2
CTSGVTFDYW	Poulsen 2011	10	2	10	1	2
CARRHYCSSTSCYDAFDIW	Poulsen 2011	19	0	2	0	2
ARHADNWFDP	Frolich 2010	10	1	4	0	3
ARQADNWFDP	Frolich 2010	10	0	71	0	3
AFTADNWFDP	Frolich 2010	10	0	3	0	2
CATGVTLDYW	Dekosky 2013	10	0	34	0	2
CVTGVTLDYW	Dekosky 2013	10	0	10	0	2
CATGFTLDYW	Dekosky 2013	10	0	34	0	2
CATGVTPDYW	Dekosky 2013	10	0	10	0	2
CATGITLDYW	Dekosky 2013	10	0	41	0	2

FIGURE 4. Known TT-specific CDR3 amino acid sequences that are found with minor changes in the dataset and are shared by at least two study participants. Shown are sequences in the dataset that are related to previously identified TT-specific sequences, along with information about CDR3 amino acid length, pre- and postvaccine frequency, and the number of participants sharing related sequences at baseline and at day 7 following vaccination.

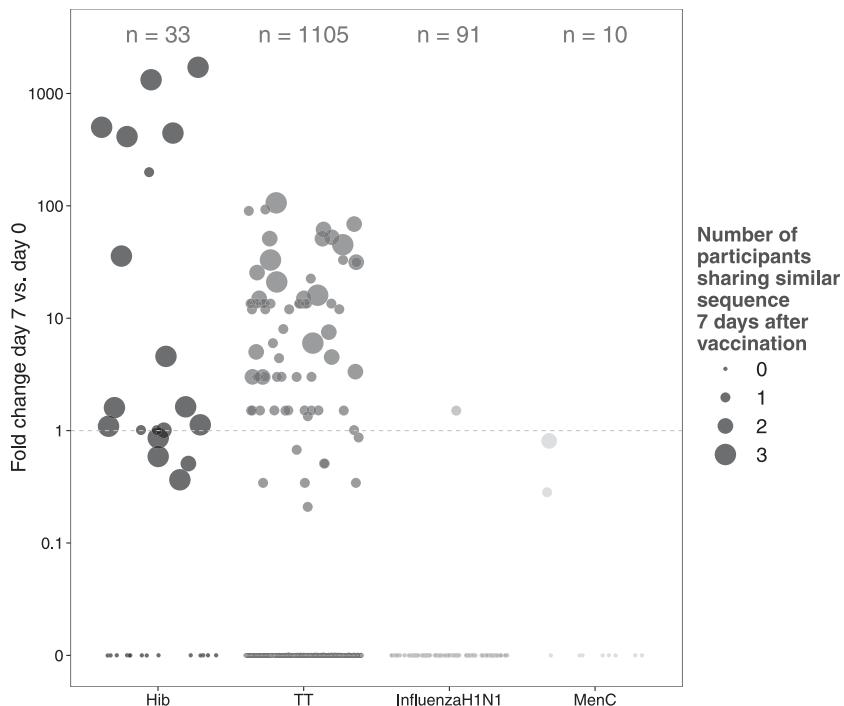


FIGURE 5. Fold change in frequencies at day 7 compared with baseline for sequences that were closely related to those previously described to be specific for Hib, TT, H1N1 influenza, and MenC, as well as the number of participants sharing these sequences. Enrichment of these sequences was calculated as fold changes between post- and pre-vaccination frequencies; for sequences not present at baseline, fold change was calculated as 1.5 times the frequency postvaccination.

Postvaccination plasma cell samples were more oligoclonal than baseline CD19⁺ samples for all isotypes (Supplemental Fig. 1B), consistent with the plasma cell population being enriched for Ag-specific cells. In contrast to previous work (11, 12), we were unable to detect a difference between the clonality of IgA, IgG, and IgM sequences when adjusted for the total number of sequences/sample (Supplemental Fig. 1C). Briney et al. (12) calculated the contribution of the 50 most common VDJ combinations for the overall repertoire in three B cell subsets that had been isolated by flow cytometry but did not adjust for the total number of sequences observed in each sample, which may have biased this result. Wu et al. (11) similarly reported on clone size distributions of different B cell subsets and found that switched memory

B cells, in particular, showed larger clone expansions than did naive B cells. Relatively few sequences/sample were considered in the latter study, and this may have been due to prior sorting of B cell subsets by flow cytometry. We performed cDNA synthesis and PCR amplification directly on bulk cell populations (CD19⁺ B cells or plasma cells), which required less in vitro manipulation. In the current study, sequences were compared before and after receipt of a highly immunogenic protein-polysaccharide vaccine. IgA and IgM repertoires obtained from CD19⁺ B cells were similar before and after vaccination, whereas the clonality of plasma cell samples was increased for IgA and IgM sequences compared with CD19⁺ B cell baseline samples. Interestingly, this difference in clonality between plasma and CD19⁺ B cell samples at

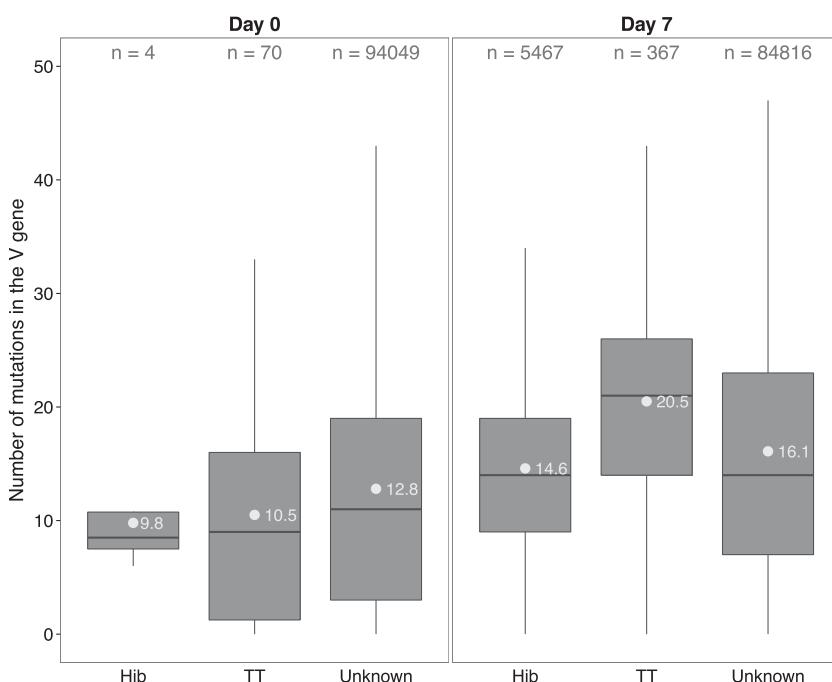


FIGURE 6. Number of V gene mutations by Ag specificity. Shown are boxplots of numbers of V gene mutations in sequences identified as Hib- or TT-specific compared with sequences of unknown specificity at baseline (*left*) and at 7 d postvaccination (*right*). *n* indicates the number of sequences/group of sequences. Gray dots with numbers represent the average mutations within each group of sequences.

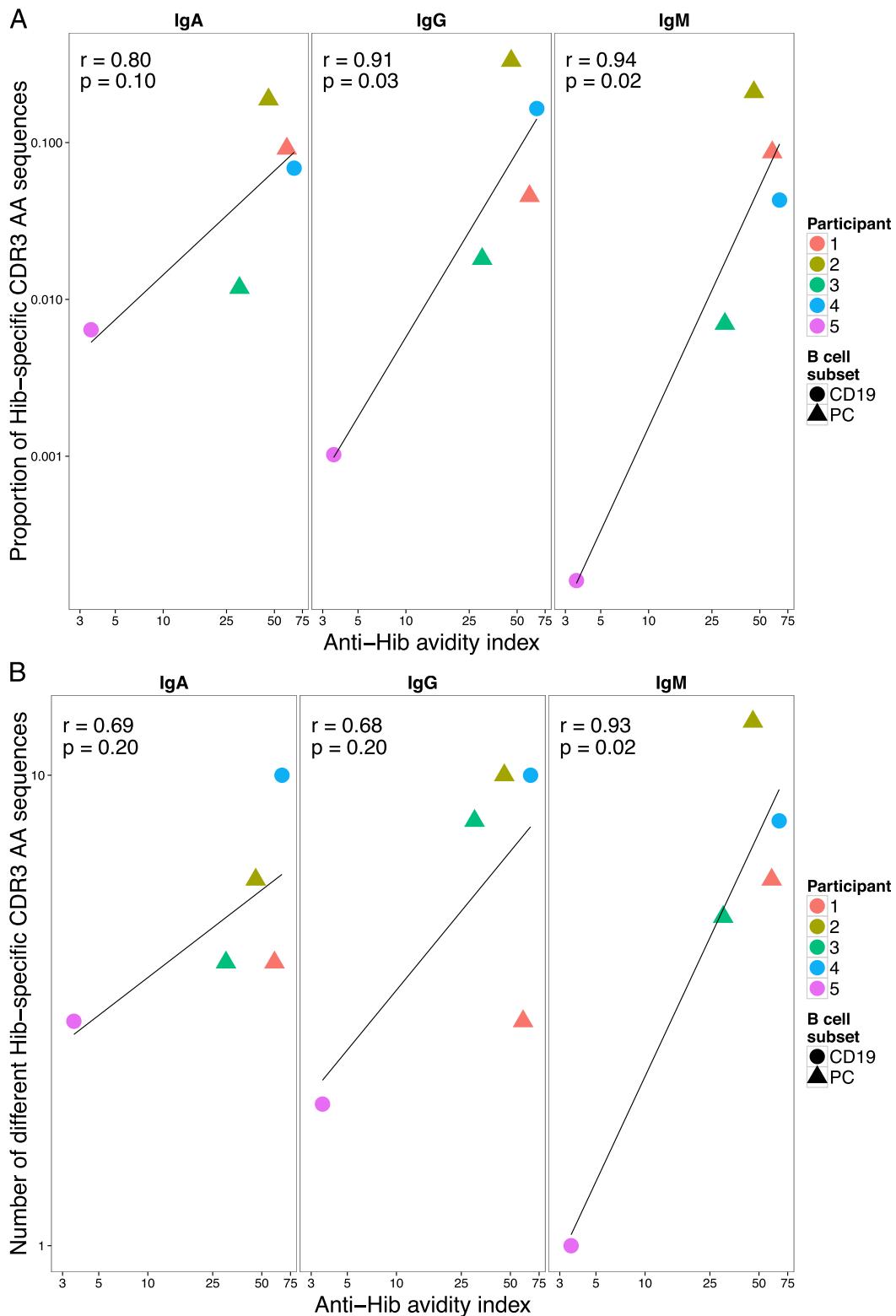


FIGURE 7. Correlation between anti-Hib avidity indices and frequency of Hib-specific sequences (**A**) or number of different Hib-specific CDR3 amino acid sequences 7 d after vaccination (**B**). Filled circles and triangles represent results of day-7 plasma cell (PC) and total B cell ($CD19^+$) samples. Pearson's correlation coefficient and the corresponding p value for each calculation are shown in the upper left corner of each graph; the line through the data points represents the regression line.

day 7 was not found in IgG sequences, indicating that the IgG sequence pool of $CD19^+$ B cells is dominated by newly generated (oligoclonal) Ag-experienced sequences. In addition, plasma cells are actively secreting cells containing vast amounts of BCR mRNA, resulting in overrepresentation of these sequences in the rep-

ertoire data, which may be more pronounced in the pool of IgG sequences.

For many vaccines, alternative methods to measure immunogenicity are desirable because current laboratory tests are too variable, difficult, or time-consuming to perform on a large scale or are not

available. High-throughput BCR sequencing data were compared with Hib immunogenicity data in the present study. The proportion of Hib-specific sequences in each sample correlated with anti-Hib avidity indices but not with total anti-PRP Ab concentration (Fig. 4, Supplemental Fig. 4D). The Ab data seem to suggest inverse relationships between the frequencies of Hib sequences and anti-PRP Ab concentration for total B cell and plasma cell populations (Supplemental Fig. 4D), but the numbers for each B cell population are small, and more data are needed to resolve this question. The relative number of Hib-specific sequences, as well as the number of different Hib-specific CDR3 amino acid sequences, correlated well with the anti-Hib avidity index postvaccination. Thus, the expansion of closely related sequences sharing a similar length (in this case 10 aa) seems to be a feature of a more pronounced immune response; therefore, it may serve as another characteristic in the future to identify “good responders.” Ab avidity represents a measure of the amount of functional Ab generated by the vaccine. Hib-specific sequences included mainly IgA₂ and IgG₂ sequences (Supplemental Fig. 4B), which is consistent with previous work demonstrating that IgG₂ anti-Hib Abs are the predominant IgG subclass in adult sera (57). Sequences that were identified as TT specific (i.e., identical to or related to previously known TT sequences) were enriched for IgA₁ and IgG₁ sequences postvaccination (Supplemental Fig. 4C), which is in line with published data (58–60) and further confirms the validity of this approach.

In conclusion, using high-throughput sequencing of the H chain B cell repertoire in the peripheral blood of participants following immunization with a Hib-MenC-TT glycoconjugate vaccine, we were able to confirm that the analysis of the public BCR repertoire postimmunization identifies BCRs enriched for vaccine-specific sequences. The identification of previously described Hib- and TT-specific sequences (and sequences related to them) through analysis of the public repertoire demonstrates convergence of CDR3 amino acid sequences in response to Ag stimulation. We linked Hib-specific CDR3 amino acid frequencies to functional anti-Hib Ab data, suggesting that the Hib-specific repertoire is a specific marker of the immune response to the Hib polysaccharide. To our knowledge, this study provides the first confirmation that the convergent BCR repertoire postimmunization can be used as a way of rapidly identifying Ag-specific sequences without sorting Ag-specific B cells. High-throughput methods to detect paired H and L chains have been described; when combined with such analyses, they will allow the production of functional Abs from such data.

Disclosures

The authors have no financial conflicts of interests.

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