REPORT

mAbs 7:4, 707–718; July/August 2015; Published with license by Taylor & Francis Group, LLC

Efficient generation of monoclonal antibodies from single rhesus macaque antibody secreting cells

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Keywords: nonhuman primate, antibody secreting cell, single cell PCR, monoclonal antibody, IG, vaccine, dengue virus

Nonhuman primates (NHPs) are used as a preclinical model for vaccine development, and the antibody profiles to experimental vaccines in NHPs can provide critical information for both vaccine design and translation to clinical efficacy. However, an efficient protocol for generating monoclonal antibodies from single antibody secreting cells of NHPs is currently lacking. In this study we established a robust protocol for cloning immunoglobulin (IG) variable domain genes from single rhesus macaque (*Macaca mulatta*) antibody secreting cells. A sorting strategy was developed using a panel of molecular markers (CD3, CD19, CD20, surface IgG, intracellular IgG, CD27, Ki67 and CD38) to identify the kinetics of B cell response after vaccination. Specific primers for the rhesus macaque IG genes were designed and validated using cDNA isolated from macaque peripheral blood mononuclear cells. Cloning efficiency was averaged at 90% for variable heavy (VH) and light (VL) domains, and 78.5% of the clones (n = 335) were matched VH and VL pairs. Sequence analysis revealed that diverse IGHV subgroups (for VH) and IGKV and IGLV subgroups (for VL) were represented in the cloned antibodies. The protocol was tested in a study using an experimental dengue vaccine candidate. About 26.6% of the monoclonal antibodies cloned from the vaccinated rhesus macaques react with the dengue vaccine antigens. These results validate the protocol for cloning monoclonal antibodies in response to vaccination from single macaque antibody secreting cells, which have general applicability for determining monoclonal antibody profiles in response to other immunogens or vaccine studies of interest in NHPs.

Introduction

Nonhuman primates (NHPs) are widely used in preclinical studies for development of human vaccines. They can also be used to gain confidence in defining vaccine images and their ability to elicit the desired immune response, derived from reverse vaccinology approaches. Vaccine efficacy evaluation has historically relied on the immune response to experimental vaccine in NHPs including rhesus macaque.[1-4] Antibody elicitation is one of the key attributes of immune response to vaccines. However, due to technical limitations, antibody response to vaccination is measured as overall serum binding and/or as the development of a functional titer such as neutralization to the vaccines or targeted viruses. While serum antibody titers provide a general humoral response to experimental vaccines, polyclonal antibodies are of little value for defining the critical components of the host humoral response and this is particularly true for pathogens with sero-type variants and complex antigens. Profiling monoclonal antibodies (mAbs) generated from vaccinated NHPs can reveal crucial aspects of the immunologic response to a vaccine such as antigen epitopes for generating high affinity, neutralizing, cross-reactive antibodies, and inter-relationship of binding and neutralizing antibodies to analyze in vivo maturation of responses post vaccination.

Cloning of immunoglobulins (IG) or antibodies from infected or immunized rhesus macaques has mostly been

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Submitted: 04/02/2015; Revised: 04/24/2015; Accepted: 05/04/2015
http://dx.doi.org/10.1080/19420862.2015.1051440
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accomplished using methods which include phage display, immortalization of B cells, or single cell cloning of memory B cells.[5-8] Antibody secreting cells originating from activated memory B cells possess unique properties including their ability to produce large amounts of IG in response to ongoing infection or immunization, and are enriched in specificity for the antigens of interest. Therefore, antigen-specific IG can be isolated from single antibody secreting cells efficiently without antigen pre-screening. This is a useful approach in evaluation of candidate vaccines for which the antigens are not well defined.

Methods for cloning human IG from human antibody secreting cells have been developed,[9-15] and several protocols for cloning monoclonal antibodies from NHPs plasma/memory B cells have also been reported.[7,9,16] Here we report a robust and efficient process for cloning IG from single rhesus macaques antibody secreting cells that achieves higher cloning efficiency than that previously reported,[7] and validate the method with an experimental dengue vaccine. This methodology is expected to have general application for studies of IG in response to experimental vaccines using preclinical NHPs models.

Results

Immunization of rhesus macaques and single cell sorting of antibody secreting cells

A tetravalent live attenuated dengue virus followed by a boost with recombinant dengue virus envelope glycoprotein was used for rhesus macaque (*Macaca mulatta*) immunization, described in details in the methods.

In order to determine the peak of the humoral response for optimal peripheral blood mononuclear cells (PMBCs) sampling, we carried out a time course fluorescence activated cell sorter (FACS) analysis of macaque immunized with the *MMR II* vaccine of live attenuated measles, mumps and rubella virus vaccine. The PBMCs were collected from days 4, 5, 6, and 7 post *MMR II* vaccination and analyzed by FACS for cell surface markers (CD19, surface IgG (sIgG), CD138) as well as intracellular IgG (IC IgG) and proliferation Ki67 markers. At day 7, a unique population was clearly observed, which was IC IgG and Ki67 positive (**Fig. 1A**). Based on this observation, we subsequently focused on the PBMCs isolation on day 7 post vaccination. Blood samples (10 ml per subject) were collected from rhesus macaques on day 7 following boosting with the dengue vaccine and PMBCs were freshly isolated. To develop a gating strategy for isolation of macaque antibody secreting cells from PBMCs, antibodies for detection of a panel of surface markers (CD3, CD19, CD20, sIgG, CD27 and CD38) were selected based on the NHP database [http://www.nhpreagents.org/NHP/ReagentBySpecies.aspx?Species=9] (**Table 1**). Single staining of CD3, CD19, CD20, sIgG and CD38 revealed distinct subpopulations: 58.6% for CD3 positive cells, 25.2% for CD19 positive cells, 25.9% for CD20 positive cells, 12.4% for positive surface IgG cells, and 73.21% for CD38 positive cells. However, most PBMCs (93.9%) showed positive staining for CD27 in reference to the fluorescence minus one (FMO) control (**Fig. S1**). To determine if CD27 cell marker is necessary for macaque antibody secreting cells sorting, PBMCs (1×10^7 per sample) were stained with the antibody cocktail, (CD3, CD19, CD20, sIgG, CD27 and CD38). Populations with (strategy 1) $CD3^-/CD19^{low\ to\ +}/CD20^{-\ to\ low}/\ sIgG^-/CD38^+\ /CD27^+$, and (strategy 2) $CD3^-/CD19^{low\ to\ +}/CD20^{-\ to\ low}/sIgG^-/CD38^+/CD27^-$ were sorted into 96-well plates for antibody genes cloning (**Fig. 1B**). We isolated and expressed the IG from the 2 strategies in order to detect their binding specificity to the dengue virus envelope glycoprotein. There was no significant difference in percentages of antigen-specific antibody secreting cells for the 2 sorting strategies: 7.1% (11 out of 154 mAbs tested) gated in strategy 1 (CD27 positive population) and 8.4 % (12 out of 142 mAbs tested) gated in strategy 2 (CD27 negative population). Therefore, we target the antibody secreting cells as $CD3^-$ / $CD19^{low\ to\ +}$ / $CD20^{-\ to\ low}$/ $sIgG^-$/ $CD38^+$ / $CD27^{-\ or\ +}$ cells.

It is well established that antibody secreting cells are vulnerable to experimental procedures including freeze-thaw.[17,18] We compared the properties of single antibody secreting cells sorted from fresh and cryopreserved PBMCs. The percentage of antibody secreting cells isolated from cryopreserved PBMCs was approximately half of that obtained from freshly prepared samples, and cloning efficiency also showed a significant decline using cryopreserved PBMCs (**Fig. S2**). These results suggest that although frozen blood samples can be used for antibody secreting cell sorting, fresh samples are a more reliable source for cloning IG genes from antibody secreting cells.

Primer design and validation

Although the human and rhesus macaque genomes are highly conserved with an overall sequence identity of ~93%,[19] primers designed for cloning human IG genes are not directly applicable to macaque.[7,20] Based on previous studies of macaque immunoglobulin gene sequences[5,19-31] and IMGT/GENE-DB (388 Macaca mulatta IG genes),[32] we designed a total of 11 heavy chain primers, 9 kappa chain primers, and 8 lambda chain primers for the first round PCR of the variable genes (**Table 2**). The first round PCR forward primers are based on the leader sequences and the reverse primers cover the adjunct IG constant (C) region. The 5′- leader region primers were designed to span the artificially spliced leader (L-PART1+L-PART2) which corresponds to the L-REGION found in cDNA, in order to avoid the amplification of genomic DNA (**Fig. 2A**). For the second round nested PCR, 14 heavy (IGH) primers, 14 kappa (IGK) primers and 14 lambda (IGL) primers were used (**Table 3**). These IGH, IGK and IGL primers were designed based on the sequences of the framework region 1 (FR1) of the variable (V) regions for the 5′ primers, and on the sequences of the joining (J) regions for the 3′ primers (**Fig. 2A**). The nested PCR primers contain a 15-nucleotide (nt) extension which overlaps with the IgG expression vector. This design allows the direct in-frame cloning of the VH (V-D-J-REGION) or VL (V-J-REGION) domain genes[33] into the full-length IgG expression vectors using the In-Fusion cloning.

First, we validated the primers using cDNA isolated from rhesus macaques PBMC. As shown in **Figure 2B**, specific DNA bands were amplified by all primer sets. It was demonstrated previously that

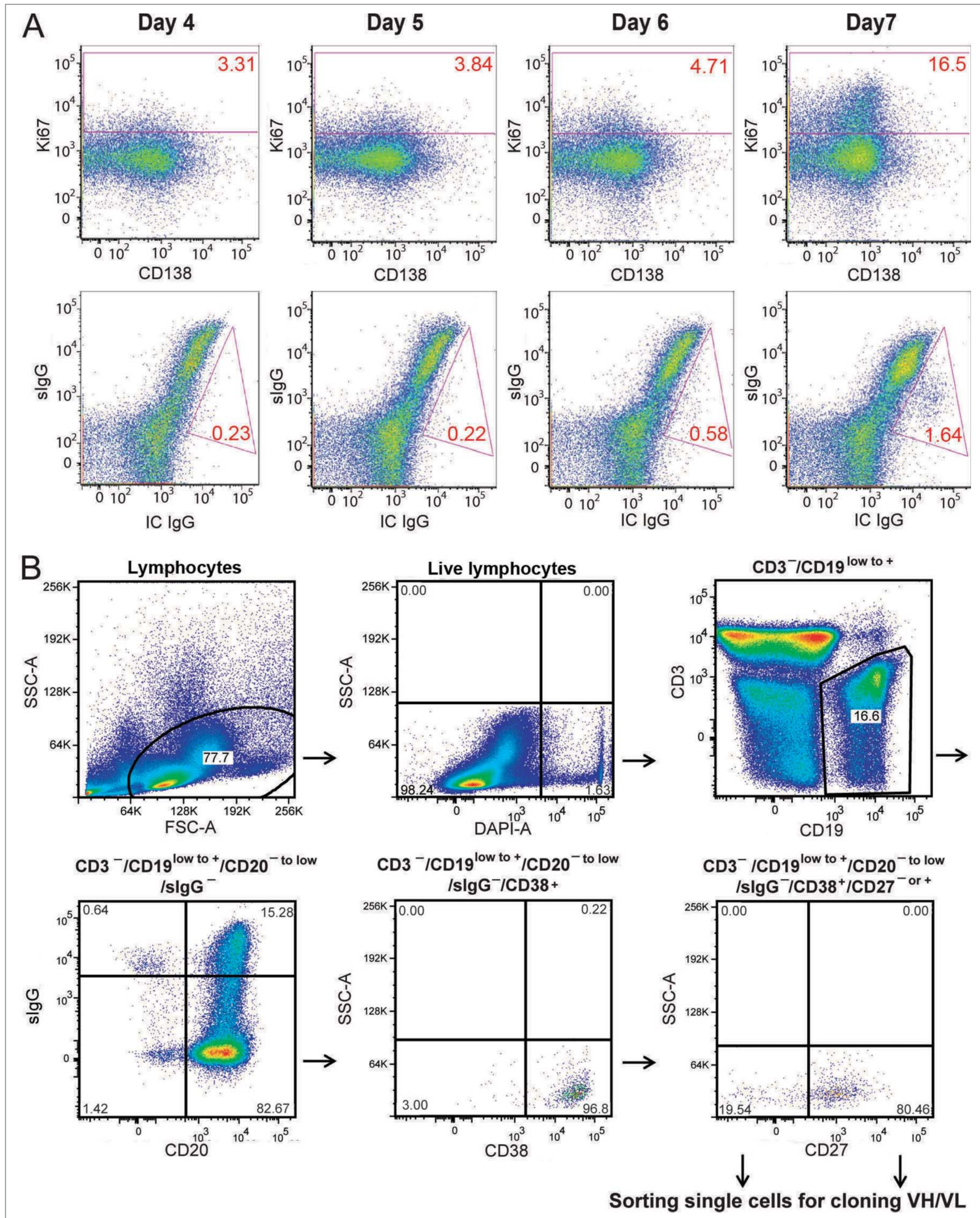

Figure 1. Analysis of antibody secreting cells dynamic change post immunization, and sorting of single antibody secreting cells by flow cytometry. (**A**) The peak of antibody secreting cells after boost was determined by up-regulated expression of Ki67 and intracellular IgG (IC IgG) by flow cytometry. (**B**) Antibody secreting cells ($CD3^{-}/CD19^{low\ to\ +}/CD20^{-\ to\ low}/IgG^{-}$ $/CD38^{+}/$ $CD27^{-\ or\ +}$) were sorted as single cells in individual wells of 96-well plates containing cell lysis buffer. Sorting results are shown in the contour graphs. FSC-A, forward-scatter-area; DAPI 4′, 6-diamidino-2-phenylindole.

Table 1. Antibodies used for flow cytometry

Antigen	Clone	Fluorophore	Supplier
CD3	SP34–2	APC-Cy7	BD Biosciences
CD19	J3–119	APC	Beckman Coulter
CD20	L27	PerCP-Cy5.5	BD Biosciences
sIgG	G18–145	PE	BD Biosciences
CD27	1A4	PE-Cy7	Beckman Coulter
CD38	AT-1	FITC	Stemcell Technologies
Ki67	B56	PE	BD Biosciences
IC IgG	G18–145	BV605	BD Biosciences
CD138	DL-101	PE-Cy7	eBiosciences

IGKV4 to IGKV7 subgroups were of low gene abundance.[7] In our experiments, it was difficult to amplify V-KAPPA domains using the degenerate primers for IGKV4 to IGKV7 subgroups (data not shown). In contrast, primer pools for VKAPPA1, VKAPPA2, and VKAPPA3 can amplify the gene product (**Fig. 2B**). DNA sequencing revealed that the VKAPPA1/VKAPPA2/VKAPPA3 primer pool not only amplify the 3 IGKV1, IGKV2 and IGKV3 subgroups, but also amplify the IGKV4 to IGKV7 subgroups (**Fig. 3B**). This is most likely due to the high sequence homology among the IGKV subgroups and the high level of degeneracy of the VKAPPA1/VKAPPA2/VKAPPA3 primer pool. Similar results were observed in VH and V-LAMBDA domain cloning (**Fig. 3B**). The amount of cDNA synthesized from a single cell is very low, reported to be in the range of 10–30 pg.[34] In order to optimize conditions for amplification of such small amounts of cDNA from a single cell, we assessed the performance of PCR using cDNA over a range of 100 ng to 1 pg per reaction (25 μl) using pooled primers for heavy chain or light chain, and a pair of primers for β–actin amplification as positive control. As shown in **Figure 2C**, the endpoint template concentration for β–actin amplification is 1–5 pg, while visible PCR amplification on agarose gel required the minimum cDNA templates of 50 pg for heavy and kappa chains, and 100 pg for lambda chain due to relative low abundance of lambda cDNA in PBMCs. Considering that antibody secreting cells express higher level of IG mRNA than observed for PBMC, the results suggest that the antibody primer sets are capable of amplifying IG genes from a single antibody secreting cell.

Antibody sequence analysis

To detect the IG genes cloning efficiency from the single antibody secreting cells. We sorted the cells into 96-well plates from rhesus macaques immunized with the experimental dengue vaccine. Following cDNA synthesis, 2 rounds of PCR were performed. We used the housekeeping gene β-actin as a control to detect the percentage of wells containing sorted cell(s). Of the 352 wells, 335 wells showed β-actin signals, indicating that 95.2% of the wells contained sorted cell(s). For IG gene amplification and cloning, the first round PCR was performed with the 5′-leader and 3′-constant region primer sets and the second round PCR used the FR1 and J region primer sets. A ~400

Table 2. First round PCR primer list

		Primer name	Primer sequence
1st round heavy chain primer	5′ primer	RhLDRVH1A	5′TCSTCTCCACAGGCGCCCACTC
		RhLDRVH1B	5′TCCTCTMCRYAGGTGCCMASTC
		RhLDRVH1C	5′TCCTCTCCGCAGGGGCCCACTC
		RhLDRVH2	5′GTCCCGTCCTGGGTCTTGTC
		RhLDRVH3A	5′CTATTTTARRAGGTGTCCAGTG
		RhLDRVH3B	5′CTCTTTTGAAAGGTGTCCAGTG
		RhLDRVH3C	5′CTATWYTAAAAGGTGTCCAGTG
		RhLDRVH4	5′AGCTCCCAGATGGGTCYTGTCC
		RhLDRVH5	5′TCTCCCCCACAGGAGTCTGTGC
		RhLDRVH6	5′GGCCTCCCATGGGGTGTC
	3′ primer	Rh gamma-PCR1	5′GGACAGCCKGGAAGGTGTGC
1st round kappa chain primer	5′ primer	RhLDRVκ1	5′TCCAATYTCAGGTGCCARATGT
		RhLDRVκ2	5′ATTTCAGGATCCAGTGGGGAT
		RhLDRVκ3A	5′TCCAATTTCAGATACCACYGGA
		RhLDRVκ3B	5′TCCAATCTCAGRTACCRCCGGA
		RhLDRVκ4	5′TGGGTCTCGGTGCCCGTCAGG
		RhLDRVκ5	5′TGGATCTCTGGTGCCTGTGGG
		RhLDRVκ6	5′TGGATCTCTGATGCCAGGGCA
		RhLDRVκ7	5′TGTGCTCCAGGCTGCAATGGG
	3′ primer	Rh kappa-PCR1	5′GAGGCAGTTCCAGATTTCAA
1st round lambda chain primer	5′ primer	RhLDRVλ1A	5′TCTCTSACTTCCAGGGTCCTGG
		RhLDRVλ1B	5′TCTCCCRCTTCCAGGGTCCTGG
		RhLDRVλ2	5′CTCCCTCTTTCCAGGRTCCTGG
		RhLDRVλ3A	5′TCCTCTCTTGCAGGTTCCGTGG
		RhLDRVλ3B	5′TCTTTTCTTGCAGTCTCTGTGG
		RhLDRVλ4	5′CTCTGTTTTCAGGGTCTCTCTC
		RhLDRVλ5	5′CTGTGTTTGCAGGTTCCCTCTC
	3′ primer	Rh lambda-PCR1	5′CCGCGTACTTGTTGTTGCTCTGT

K = G + T, M = A + C, R = A + G, S = G + C, W = A + T, Y = C + T.

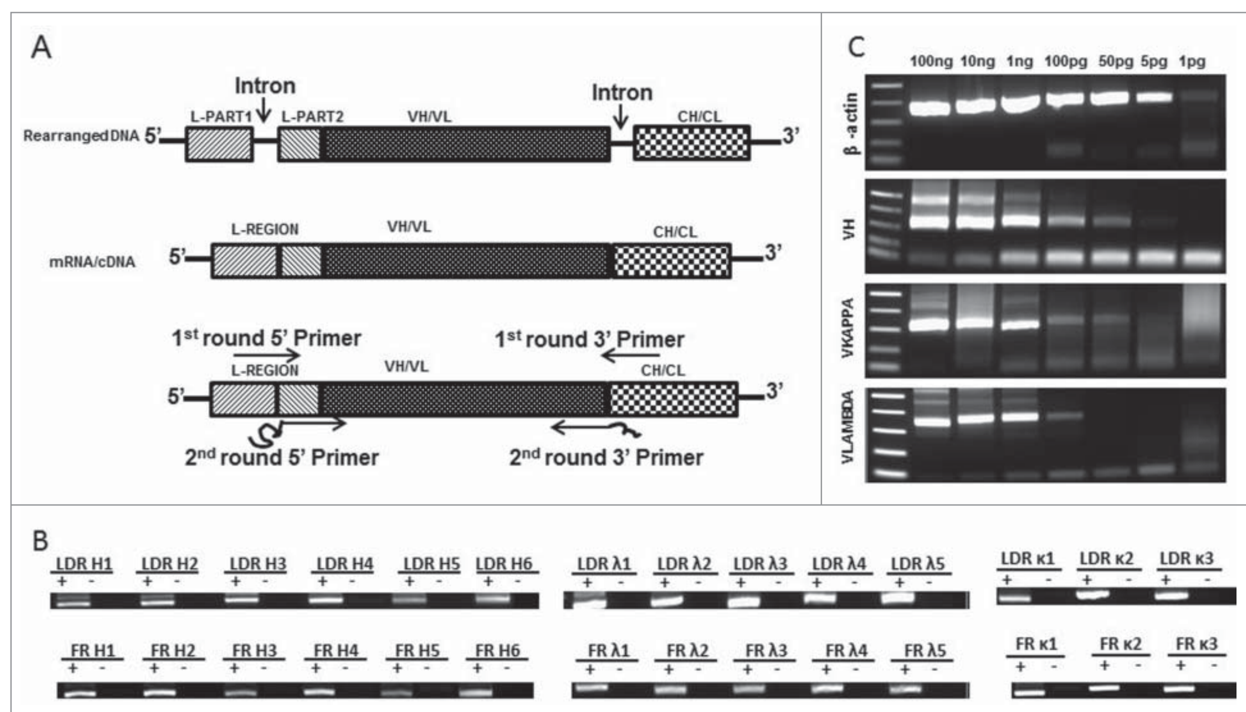

Figure 2. Design and validation of PCR primers for cloning IG variable domain genes from rhesus macaques. (**A**) In genomic DNA, the leader of the V gene is encoded by L-PART1 and L-PART2 separated by a short intron. The 5′ first round PCR primers were designed to hybridize with the L-REGION (L-PART1 and L-PART2 spliced in cDNA) in order to avoid the amplification of genomic DNA. The 3′ first round PCR primers are located in the constant (**C**) region proximal and downstream of the J region. The 5′ second round PCR primers start at the beginning of framework 1. The 3′ second round PCR primers align to the distal end of J region. The second round primers contained a 15-nucleotide extension which overlaps with the IgG expression vector sequence for direct In-Fusion cloning of the PCR products into the vectors. (**B**) The primer pools for each IG gene subgroup were tested with 50 ng cDNA templates from PBMCs and PCR products were visualized on 2% agarose gels. The expected size of the PCR products is between 300–500 bp. (**C**) Sensitivity of first round PCR primers for heavy, kappa or lambda chains were tested using 100 ng to 1 pg serial diluted cDNA templates.

base pair (bp) PCR product was expected for both the VH and VL (V-KAPPA or V-LAMBDA) domains (**Fig. 3A**). After cloning, we choose 5 colonies from each original PCR product for DNA sequencing. A total of 98% clones contained a single DNA sequence with a few exceptions having 2 sequences (∼2%). Of those clones containing 2 sequences, one was always an IG productive sequence (in-frame junction and no stop codons) whereas the other sequence often was an IG unproductive sequence (out-of-frame junction and/or stop codons). Of the 335 wells yielding positive β-actin bands, 302 wells (90%) showed positive amplification for heavy chain, while 299 wells (89%) resulted in light chain PCR products. Among the light chain products, 201 of them (67%) were kappa chains and 98 of them (33%) were lambda chains. Overall, a total of 263 wells produce both heavy and light chain products, which represents 78.5% of cloning efficiency among β-actin positive wells. Of the 263 light chains, 64% were kappa chains and 36% were lambda chains. It has been established that the ratio of kappa and lambda light chains in humans is approximately 2:1.[35] The observed suggests that the procedures employed in this study did not show bias in IG cloning, assuming that the ratio is similar in rhesus macaque.

The V gene identification of the VH and VL (V-KAPPA or V-LAMBDA) domains and the CDR3 length were analyzed using IMGT/V-QUEST (http://www.imgt.org) from IMGT®,[36] the international ImMunoGeneTics information system®.[37] **Figure 3B** shows the V subgroup distribution in 302 VH, 201 V-KAPPA and 98 V-LAMBDA. For the VH, IGHV4 is the most represented subgroup (39%) followed by IGHV3 (27%) and IGHV5 (18%). Four less represented subgroups (IGHV1, IGHV2, IGHV6 and IGHV7) constitute the remaining 16% of VH sequences. For the V-KAPPA, IGKV1 is the most represented subgroup (66%) followed by IGKV3 (17%) and IGKV2 (14%). The four less represented subgroups (IGKV4, IGKV5, IGKV6 and IGKV7) constitute the remaining 3% of the V-KAPPA sequences. For the V-LAMBDA, IGLV1 is the most represented subgroup (50%), followed by IGLV2 to IGLV5 with 10–11% each, IGLV6 to IGLV9 contain the remaining 10% sequences. The results are consistent with previous reports on distributions of the IGHV, IGKV and IGLV subgroups in rhesus macaques.[7,25,38,39] On the other hand, the VH CDR3 length ranged from 7–22 amino acids (AA) with about 60% of the clones in the 10–14 AA range. The majority (96%) of the V-KAPPA CDR3 are less than 9 AA in length. In contrast, the V-LAMBDA has longer CDR3 with 72% in the 10–14 AA range (**Fig. 3C**). These results are consistent with previous reports on distributions of VH, V-KAPPA and V-LAMBDA CDR3 length distribution for rhesus macaques.[40,41]

Table 3. Second round PCR primer list

		Primer name	Primer sequence
2nd round heavy chain primer	5′ primer	RhFRVH1A IF5	5′*ACAGGTGTCCACTCG*GAGGTCCAGCTGGTRCAGTC
		RhFRVH1B IF5	5′*ACAGGTGTCCACTCG*CAGGWGCAGCTGGTGCAGTC
		RhFRVH2 IF5	5′*ACAGGTGTCCACTCG*CAGGTGACCTTGAAGGAGTCTG
		RhFRVH3 IF5	5′*ACAGGTGTCCACTCG*GARGTGCAGYTGGTGGAGTCTG
		RhFRVH4A IF5	5′*ACAGGTGTCCACTCG*CAGSTGCAGCTGCAGGAGTCGG
		RhFRVH4B IF5	5′*ACAGGTGTCCACTCG*CAGCTGCAGCTGCAGCTGCAGG
		RhFRVH5 IF5	5′*ACAGGTGTCCACTCG*GAGGTGCAGCTGGTGCAGTCTG
		RhFRVH6 IF5	5′*ACAGGTGTCCACTCG*CAGGTGCAGCTGCAGGAGTCAG
	3′ primer	RhFRVH1 IF3	5′*GATGGGCCCTTGGTGGATGC*TGAGGAGACGGTGACCAGGGC
		RhFRVH2 IF3	5′*GATGGGCCCTTGGTGGATGC*TGAGGAGATGGTGATTGGGGT
		RhFRVH3 IF3	5′*GATGGGCCCTTGGTG*GATGCTGAAGAGACGGTGACCCTGAG
		RhFRVH4/5 IF3	5′*GATGGGCCCTTGGTGGATGC*TGAGGAGACGGTGACCAGGAC
		RhFRVH6 IF3	5′*GATGGGCCCTTGGTGGATGC*TGAGGAGACGGTGACCAGAAC
		RhFRVH7 IF3	5′*GATGGGCCCTTGGTGGATGC*TGAGGAGACGGTGACGACGAC
2nd round kappa chain primer	5′ primer	RhFRVκ1 IF5	5′*CTTACAGACGCTCGCTGC*GACATYCAGATGWCCCAGTCTC
		RhFRVκ2A IF5	5′*CTTACAGACGCTCGCTGC*GATAYTGTGATGAYCCAGACTC
		RhFRVκ2B IF5	5′*CTTACAGACGCTCGCTGC*GATGTTGYRATGACTCAGTCTC
		RhFRVκ3A IF5	5′*CTTACAGACGCTCGCTGC*GAAATWGTRATGACGCAGTCTC
		RhFRVκ3B IF5	5′*CTTACAGACGCTCGCTGC*CAAGTTATATTGACTCAGTCTC
		RhFRVκ4 IF5	5′*CTTACAGACGCTCG*CTGCCTGGATCTCTGGTGTCTGTGG
		RhFRVκ5 IF5	5′*CTTACAGACGCTCGCTGC*CCTTTGGATCTCTGMTGCCAGG
		RhFRVκ6 IF5	5′*CTTACAGACGCTCGCTGC*TGGGTTCCAGTCTCCAAGGG
		RhFRVκ7 IF5	5′*CTTACAGACGCTCGCTGC*TGTGCTCCAGGCTGCAATGG
	3′ primer	RhFRVκ1 IF3	5′*ATGGTGCAGCCACCGTACG*TTTGATCTCCAGCTT
		RhFRVκ2 IF3	5′*ATGGTGCAGCCACCGTACG*TTTGATTTCCACCTT
		RhFRVκ3 IF3	5′*ATGGTGCAGCCACCGTACG*TTTGATCTCCACTTT
		RhFRVκ4 IF3	5′*ATGGTGCAGCCACCGTACG*TTTGATATCCAGTTT
		RhFRVκ5 IF3	5′*ATGGTGCAGCCACCGTACG*TTTAATCTCCAGTCG
2nd round lambda chain primer	5′ primer	RhFRVλ1A IF5	5′*ACAGACGCTCGCTGC*CAGTCTGTGCTGACGCAGCCGC
		RhFRVλ1B F5	5′*ACAGACGCTCGCTGC*CAGTCTGTGCTGACWCAGCCWC
		RhFRVλ2A IF5	5′*ACAGACGCTCGCTGC*CAGKCTGCCCYGACTCAGCCTC
		RhFRVλ2B IF5	5′*ACAGACGCTCGCTGC*CAGTCTGCCCCGAYTCAGTCTC
		RhFRVλ3A IF5	5′*CTTACAGACGCTCGCTGC*TCCTCTGRGCTGACTCAGGAGC
		RhFRVλ3B IF5	5′*ACAGACGCTCGCTGC*TCCTATGAGCTGACACAGCCAC
		RhFRVλ4 IF5	5′*ACAGACGCTCGCTGC*CAGCCTGTGCTGACTCAGTCGC
		RhFRVλ5A IF5	5′*ACAGACGCTCGCTGC*AAGCCTATGCTGACTCAGCCGG
		RhFRVλ5B IF5	5′*ACAGACGCTCGCTGC*CAGCCTGTGCTGACYCAGCC
	3′ primer	RhFRVλ1 IF3	5′*GTTGGCCTTGGGCTG*TAGGACGGTCAGCCG
		RhFRVλ2 IF3	5′*GTTGGCCTTGGGCTG*TAGGACGGTCAGTCG
		RhFRVλ3 IF3	5′*GTTGGCCTTGGGCTG*TAGGACGGTGAGCCG
		RhFRVλ4 IF3	5′*GTTGGCCTTGGGCTG*GAGGACGGTCAGCCG
		RhFRVλ5 IF3	5′*GTTGGCCTTGGGCTG*TAGGACGGTCAGACG

The extension of 15 nucleotides is shown in italics (K = G + T, M = A + C, R = A + G, S = G + C, W = A + T, Y = C + T).

Antibody binding properties

In order to determine the proportions of cloned IG that can recognize dengue immunogens of the vaccine, we expressed 214 IG in chimeric form with human IGHG1 and IGKC constant region. Monoclonal antibodies secreted in the serum free culture medium were used for binder screening by ELISA. Of the 214 IG expressed, 58 cell culture supernatants showed positive antigen binding (26.6%) and the selection of a positive hit was defined as an assay response which was at least 2-fold above the negative control (**Fig. 4A**). Five representative antibodies displayed variability in antigen binding specificity with high affinity at the concentration of 5 ng/ml (**Fig. 4B**). Antibody D1, D4 bound to envelope glycoproteins of all 4 dengue virus strains; while D2 selectivity bound to dengue 3 and dengue 4 glycoproteins; antibodies D5 and D3 exhibited antigen binding specificity to dengue 1 and dengue 2 glycoproteins.

Protocol overview

Based on published studies describing a surge of human antibody secreting cells on day 7 after boost immunization,[9,15,42] and also our observations on the expression kinetics of proliferation Ki67 marker and intracellular IgG in PBMC from rhesus macaques after boost with the *MMR II* vaccine, we collected rhesus macaques blood samples 7 d after boost with a recombinant dengue vaccine. PBMCs were isolated freshly and stained with a panel of antibody reagents identifying cell markers in macaque. This overall FACS

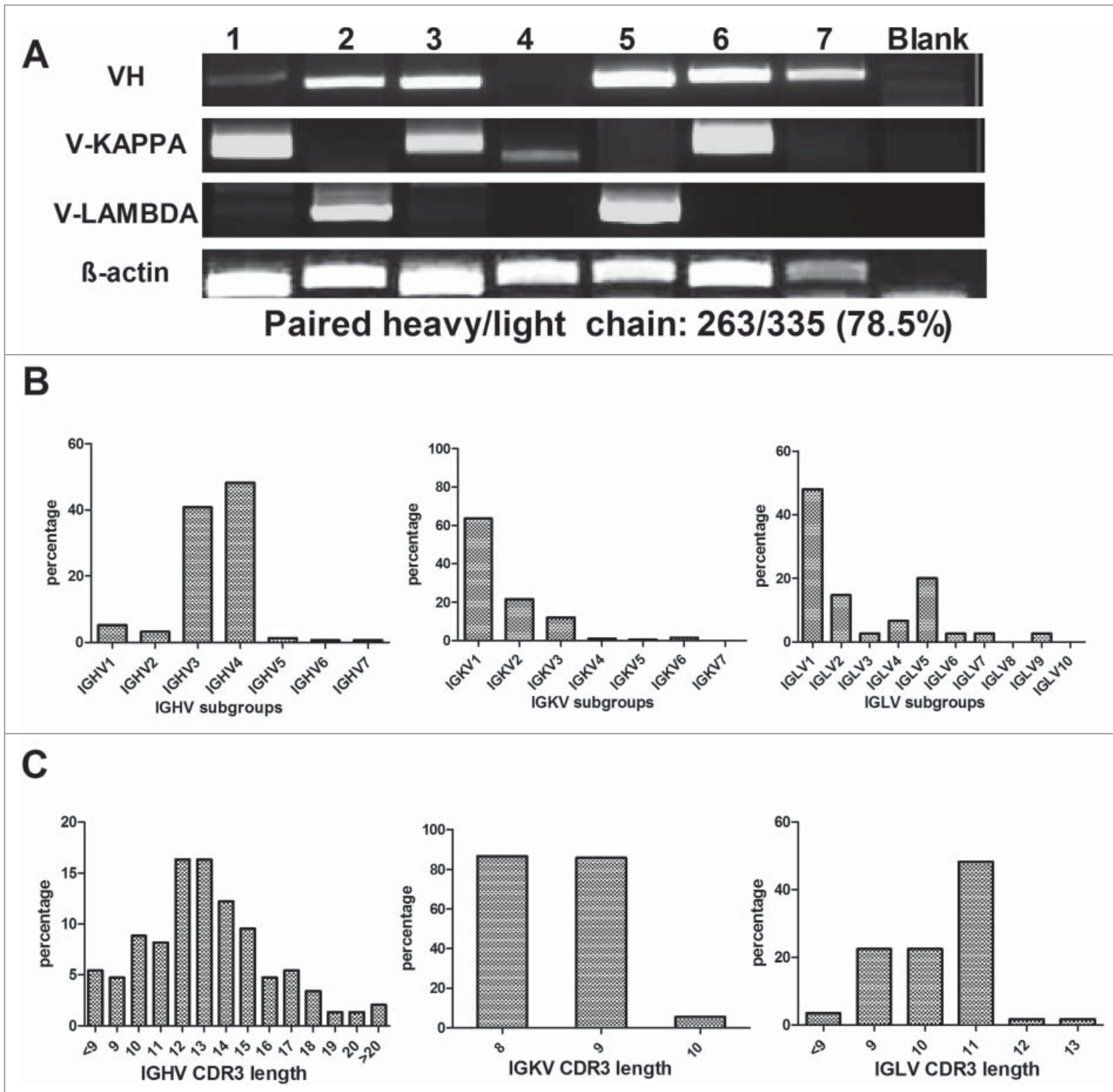

Figure 3. Cloning of IG variable domain genes from single antibody secreting cells. (**A**) Representative gel picture showing PCR products of VH, V-KAPPA or V-LAMBDA domain genes and β-actin amplified from single antibody secreting cells. (**B**) The IGHV, IGKV and IGLV subgroup distribution of IG cloned from single antibody secreting cells was analyzed using IMGT/V-QUEST. Histograms show average V gene subgroup usage of 302 heavy, 201 kappa, and 98 lambda chain sequences. (**C**) The CDR3 AA length distribution of 302 VH, 201 V-KAPPA and 98 V-LAMBDA.

gating and sorting strategy is outlined in **Figure 1B**. Single antibody secreting cells were sorted into 96-well plates containing cell lysis buffer using a cytometric cell sorter. Single cell RT-PCR was then carried out to amplify the variable domains using a set of customized specific primer pairs for rhesus macaques IG genes. The paired VH and V-KAPPA or V-LAMBDA were cloned into the IgG expression vector and the V domain of the resulting chimeric chains were sequenced. The paired H and L chains were expressed in HEK293F mammalian cells for the evaluation of antigen binding.

Discussion

The elicitation of an efficacious neutralizing antibody response following immunization is recognized as a key attribute in the design and success of experimental vaccines. Total serum antibody titer provides an early indication of the overall humoral response to vaccination, but has limited value for dissecting host antiviral antibody immunity at clonal levels. In contrast, understanding the antibody response at the clonal level can provide crucial information on antigen epitopes, binding affinity, cross-reactivity, infection enhancing or neutralizing activity, the ratio of binding and

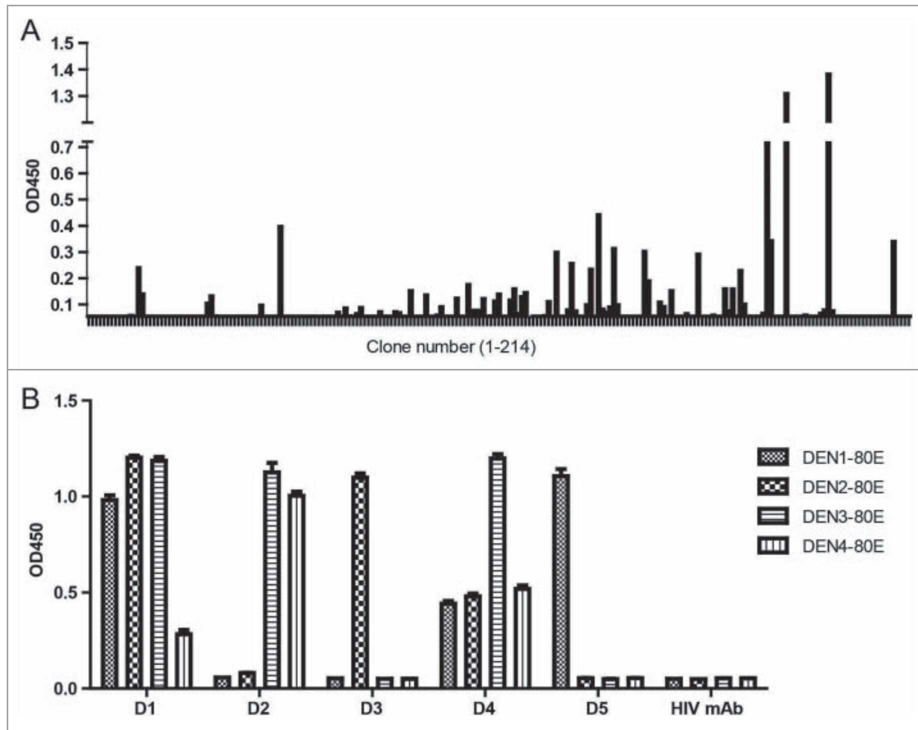

Figure 4. Antigen binding activity of mAbs cloned from single antibody secreting cells of rhesus macaques immunized with a dengue subunit vaccine candidate. (**A**) Recombinant dengue envelope protein was coated at a concentration of 1 μg/ml in 96-well plate. A total of 50 μl of IgG expression supernatants collected 7 d post transfection was diluted at 1:2 in PBS as primary antibody. HRP conjugated anti-human antibody was used as detection antibody, OD450 read was deducted by blank control. (**B**) Recombinant dengue envelope protein from 4 different strains was coated individually at a concentration of 1 μg/ml in 96-well plate. Five purified mAbs were used as primary antibody at the concentration of 5 ng/ml. Anti-HIV antibody was used as negative control. HRP conjugated anti-human antibody was used as secondary antibody. OD450 read was detected.

neutralizing antibodies, and in vivo affinity maturation in response to vaccination. NHPs are regarded as the "most close to human" preclinical animal model; however, a robust protocol for profiling comprehensive panels of monoclonal antibodies in vaccinated NHPs is currently lacking, even though profiling of antibody response to vaccination or natural viral infection at the monoclonal level in humans has rapidly advanced in recent years.[9,37,43-45] As a result of the availability of well-defined markers for human antibody secreting cells and memory B cells, large panels of IG can be directly cloned from these cells by single cell sorting and PCR strategies. Since NHP models are widely used in preclinical vaccine development, investigation of IG through profiling single antibody secreting cells during vaccination of NHPs can play significant role in the guidance of vaccine development and serve as a translational bridge to the clinic.

Cell surface IgG in memory B cells facilitate antigen specific cell sorting and the enrichment of positive clones.[46] However, when the neutralizing or binding epitopes of a pathogen are not well defined, it is especially useful to generate antibodies from antibody secreting cells which are specifically, but transiently, activated as part of immune responses, thus bypassing the antigen-baiting step used for the isolation of positive memory B cells.[10,46] However, the surge of antibody secreting cells requires immunogen stimulation, and it prevents the use of humans as a model during preclinical stage evaluation of vaccine efficacy due to safety and ethical considerations. NHP such as rhesus macaques are often used as a preclinical model in the safety and efficacy evaluation of experimental vaccines. Isolation of antibody sequences from rhesus macaques memory B cells as well as antibody secreting cells has been recently reported,[7,16,20,47] but the cloning efficiency, primer coverage, peak plasma cell timing and simplicity of the procedures are far from optimal.

The protocol that we have developed for the efficient generation of IG from macaque single antibody secreting cells includes 4 major steps: 1) vaccination of rhesus macaques; 2) single antibody secreting cell sorting from PBMCs at peak of plasma cell responses; 3) cloning IG V domain genes from single cells using rhesus macaques IG gene specific primers; and 4) cloning of paired VH and VL into an IgG expression vector, expression as chimeric H and L chains and screening of positive clones. Since the phenotype of rhesus macaques antibody secreting cell is still not well defined, we used markers defined for human antibody secreting cells in the macaque study. Even though rhesus macaques and human are evolutionarily closely related, antibodies developed for identifying human antibody secreting cell markers do not necessarily recognize the analogous rhesus macaque plasma cells. We selected CD3, CD19, CD20, surface and intracellular IgG, Ki67, CD38, and CD27 to define the macaque antibody secreting cells as well as their kinetics after immunization. We observed distinct populations of proliferating ($Ki67^{+}$), and intracellular IgG^{+} B cells that peaked at day 7 post immunizations. Our results using sorting strategies of $CD27^{+}$ or $CD27^{-}$ cells did not show large differences in the percentage of antigen specific antibody secreting cells. However, it has been reported that rhesus macaques B cells which are $CD3^{-}$/$CD19^{+}$/$CD20^{-}$/$CD38^{+}$/$CD27^{-}$ can secret IgG.[16] It is shown that the $CD27^{high}$ B cell population was not increased in response to the vaccination scheme used in their study,[48] suggesting that the $CD27^{high}$ population were not antibody secreting cells. A recent study by Silveria et al revealed that macaque plasmablasts were CD19 and CD27 negative.[47] These results contrast with our studies where the antibody secreting cells were $CD19^{low\ to\ +}$. The discrepancy between the 2 studies could be explained by the vaccination strategy, as we used live attenuated dengue virus as a priming antigen in contrast to the recombinant SIV gp140 used

by the Silveria et al.[47] Studies by Neumann et al. used cell markers ($CD19^{+}/CD20^{-}/CD38^{+/2+}/CD138^{2+}$) for activated memory B cells[18] which are more similar to the markers used in our study. Nevertheless, improved cell surface markers and their corresponding detecting antibodies need to be identified to better define the rhesus macaques antibody secreting cell populations. It is also noted that, the peak of cells were detected at day 4 after immunization.[47] And this faster kinetics of the IG expressing cells appearance could be attributed to the specific adjuvants employed in their studies. It is prudent to determine the time of peak IG expressing cell in the blood post vaccination before starting a large scale antibody cloning and characterization project.

Two other factors influencing the efficiency of IG gene recovery from single antibody secreting cells are cell freshness and broad coverage primers. Cryopreservation of PBMCs is well established for certain research applications; however, our study showed that PBMCs following cryopreservation resulted in a decreased percentage of sorted antibody secreting cells (**Fig. S2**) relative to fresh PBMCs. This finding is consistent with previously reported studies.[18,46] In addition, our observed cloning efficiency of 20% using the frozen antibody secreting cells was lower than the 55% obtained using cells sorted from fresh PBMCs. Two potential explanations for the decrease in cloning efficiency can be postulated: 1) cells which are actively expressing large amounts of antibody may be highly sensitive to freeze-thaw stresses as a result of their highly activated metabolic state; and 2) the freeze-thaw process selectively induces a higher degree of damage to antibody mRNA. Therefore, while it is feasible to obtain antibody secreting cells from frozen PBMC samples, fresh blood samples will provide a better source for isolation of antibody secreting cells.

We noted that both the heavy chain and light chain cloning percentages were approximately 90% while the paired heavy and light chain clones were >75% in the dengue vaccine study, which contrasts with 43% paired heavy and light chain clones reported.[47] In comparison to previous studies,[7,47] our PCR amplification efficiency of IG V domain genes was significantly higher than that observed with sorted human plasmablasts or macaques B cells. The higher amplification efficiency can be partially attributed to our primers which were designed to provide broader coverage. Sequence analysis confirmed that heavy chains in our study cover all 7 different IGHV subgroups, comparing to coverage of only IGHV1 to IGHV4 subgroups in other reported studies.[47]

In conclusion, we have established an efficient protocol to amplify IG variable domain genes from single sorted antibody secreting cells for evaluation of antibody profiles in response to vaccination in NHPs. Compared with previous studies,[7,9,47] our cloning efficiency is much higher. It is expected that better results will be achieved once the phenotype of rhesus macaques antibody secreting cells isolated following vaccination are more accurately characterized. The platform we have described will be valuable for evaluating the efficacy of experimental vaccines especially for those for which protective epitopes are not well defined. The protocol is also well suited to study the evolution of antibody repertoire and in vivo maturation in response to vaccination or natural infection in NHPs.

Materials and Methods

Animal immunization and PBMCs isolation

Indian rhesus macaques (*Macaca mulatta*) were housed at the New Iberia Research Center, New Iberia, LA. All animal procedures were carried out in accordance with the animal care and use guidelines and the protocols were approved by the Animal Welfare Committee (AWC) of the New Iberia Research Center. For the vaccine studies, 2 vaccine candidates, a tetravalent dengue live attenuated vaccine and a tetravalent recombinant dengue subunit vaccine were administered using different regimens. The live attenuated vaccine (gifted by Dr. Stephen S. Whitehead) comprised dengue types 1–4 (rDEN1-rDEN1Δ30–1545; rDEN2-rDEN2/4 Δ30(ME)-1495,7163; rDEN3-rDEN3Δ30/31–7164; and rDEN4-rDEN4 Δ30–7132,7163,8308) which were administered at 1×10^5 plaque forming units (pfu) each. The tetravalent dengue subunit vaccine (experimental vaccine of Merck and Co. Inc.) comprised 4 truncated envelope glycoproteins (DEN1–80E, DEN2–80E, DEN3–80E, DEN4–80E) from each of the dengue virus (DENV) serotypes and were administered at 10, 10, 10, 20 μg respectively. Four healthy adult, rhesus macaques of either sex, weighing more than 3 kg, and which were flavivirus (DENV 1, 2, 3, 4, and West Nile Virus) antibody-negative by ELISA, were utilized in this study for each group. All animals received the live attenuated vaccine subcutaneously at 0 weeks and then received subunit DEN-80E vaccine formulated with Alhydrogel™ adjuvant (Brenntag Biosector) intramuscularly at 16 weeks. All vaccines were administered at 0.5 mL per dose. Bleeds (10 ml) were taken 7 d after boost under sedation with 10 mg/kg ketamine, given intramuscularly. The mononuclear cell fraction was extracted by density-gradient centrifugation with Lymphocyte Separation Medium as described (Mediatech, Cat. no: 25–072-CI). After extensive washing, the isolated PBMCs were either preceded to staining for FACS sorting or frozen in fetal bovine serum supplemented with 10% DMSO (Sigma, Cat. no: C6164).

Single cell sorting of rhesus macaques antibody secreting cells by flow cytometry

For cell surface staining, fresh PBMCs were resuspended in 100 μl of FACS staining buffer (3% BSA in PBS) containing antibody reagents against CD3, CD19, CD20, sIgG, CD27, CD38, CD138 (**Table 1**), and incubated for 40 min at 4°C. For intracellular staining, cells were washed with FACS staining buffer, permeabilized with cytofix/cytoperm™ Kit (BD Biosciences, Cat. no: 554714), and then stained with anti-IC IgG and anti-Ki-67 antibodies (**Table 1**). For frozen PBMCs, cells were thawed and slowly resuspended in RPMI 1640 medium (Sigma, Cat. no: 8758) pre-warmed to 37°C supplemented with 10% FBS, and stained with antibodies cocktail. The stained cells were analyzed and sorted by a BD FACSAria II cell sorter (BD Biosciences) into 96-well plates containing 10 μl cell lysis buffers

according to the gating strategy as shown in **Figure 1B**. Row H of the 96-well plate was used as blank control, which contains only lysis buffer without sorted cells. The lysis buffer was obtained from the SuperScript™ III CellDirect cDNA Synthesis Kit (Invitrogen, Cat. no: 18080–300). The targeted sorting population was $CD3^{-}/CD19^{low\ to\ +}/CD20^{-\ to\ low}/\ sIgG^{-}/CD38^{+}/CD27^{-\ or\ +}$. The sorted cells were stored at −80°C.

Design and validation of RT-PCR primers

PCR primers were designed based on published rhesus macaques germline sequences and our previous study.[5,7,19-37] The primer design strategy is illustrated in **Figure 2A**. The leader sequence of the V gene is encoded by L-PART1 and L-PART2 separated by a short intron that is spliced out in the mRNA.[49] The first round PCR 5′- primer set, RhLDR primers, was designed to hybridize with the L-REGION (L-PART1 and L-PART2 spliced in cDNA). The 3′- primer are situated in the constant (C) region of the IGHG and IGKC genes and were designed to enable amplification of IgG genes as described by Anton M. Sholukh et al.[20] (**Table 2**) The second round PCR 5′- primer set, RhFR primers, was designed to align with the beginning of framework 1 (FR1) of the variable region with the addition of 15 nucleotides overlapping with the IgG expression vector sequence to facilitate In-Fusion PCR. The 3′- primer are situated in the end of J-REGION (**Table 3**).

To validate the leader region (RhLDR) and FR1 (RhFR) primers, total RNA was isolated from 1×10^6 PBMCs. The RNA was reverse transcribed into cDNA with Superscript™ III Reverse Transcriptase (Invitrogen, Cat. no: 18080044) using $oligo(dT)_{20}$ according to the manufacturer's protocol. PCR products were generated with the PrimeSTAR™ GXL DNA polymerase (Clonetech, Cat. no: R050Q).

Reverse transcription and single cell IG cloning

Reverse transcription was carried out using the SuperScript™ III Cell Direct cDNA Synthesis kit (Invitrogen, Cat. no: 18080–300) according to the manufacturer's instructions with modification. Briefly, the 96-well plates with single sorted cells were thawed, followed by the addition of 2 μl $Oligo(dT)_{20}$(50mM), 1 μl dNTP Mix (10 mM) and 8 μl water. Incubate the mixture at 70°C for 5 minutes. Place the plate on ice, and then add 6 μl 5X reverse transcription buffer, 40 U RNaseOUT™, 200 U Superscript™ III Reverse Transcriptase, and 1 μl DTT (0.1M) in a total volume of 30 μl and thorough mixing by pipetting. The reaction was performed as follows: 50 min at 50°C, 5 min at 85°C and finally cooling to 4°C. The cDNA was stored at −20°C.

IG variable domain genes were amplified from 3.5 μl cDNA by nested PCR. All PCR reactions were performed in 96-well plates in a volume of 25 μl per well containing 0.8 μM each primer mixture, 200 μM dNTP each, 5 μl 5X buffer and 1U PrimeSTAR™ GXL DNA polymerase (Clonetech, Cat. no: R050Q). The PCR program was initiated by 5 min incubation at 94°C followed by 35 cycles of 98°C, 10s; 55°C, 30s; and 68°C 30 s; and a final elongation step at 68°C for 5 min before cooling to 4°C. The second round Infusion PCR using 2.5 μl first round PCR product as DNA template was carried out under the same condition as in the first round. The second round PCR products were evaluated on 2% agarose gels and if positive (bands ~400 bp), bands were purified for cloning.

Cloning was carried out using the In-Fusion™ HD Cloning kit (Clontech, Cat. no: 638909). Briefly, 50 ng purified PCR products were mixed with 2 μl In-Fusion™ HD enzyme premix and 100 ng linearized vector. Water was added up to a total volume of 10 μl. The reaction was incubated for 15 min at 50°C, then placed on ice. About 4 μl of the product was used for TOP10 competent *E. coli* transformation. Five colonies for each product were picked for sequencing confirmation.

Antibody sequence evaluation and expression

IG V domain sequences were analyzed for the IGHV, IGKV and IGLV subgroup distribution and for their CDR3 length using IMGT/V-QUEST (http://www.imgt.org),[36] and the international ImMunoGeneTics information system®.[37] The method of antibody expression in mammalian cells and purification by Protein A has been described previously.[50,51] Briefly, equal molar amounts of heavy-chain plasmid and light-chain plasmid were co-transfected into 293F cells for transient expression with TrueFect™ reagent (United BioSystems, Cat. no: NF0866–3). The supernatants were harvested 7 d after transfection. Antibodies were purified with Protein A beads (Repligen, Cat. no: CA-PRI-0100) according to the manufacturer's instructions.

Antibody binding ELISA

Briefly, 1 μg/ml of recombinant dengue envelope protein (experimental vaccine of Merck and Co. Inc.) was coated on the 96-well plates at 4°C overnight. Plates were blocked with 3% BSA in PBS at room temperature for 2 hours. After washing with PBST (0.5% Tween-20 in PBS) for 3 times, 100 μl of diluted supernatants (1:2 in PBS) or purified antibodies at the concentration of 5 ng/ml were added to and incubated with the plates for 1.5 hours. Wash the plates with PBST 5 times and add horseradish peroxidase (HRP) coupled goat anti-human IgG (Sigma, Cat. no: A-0170) to the plates for 1 hour. The Assay was developed by Pierce™ TMB Substrates (Thermo, Cat. no: 34021) according to the manufacturer's instructions. Optical densities (OD) were measured at 450 nm. Positive binding was defined as at least 2-fold above OD of the negative control.

Disclosure of Potential Conflicts of Interest

This study was in part supported by grants from Merck and some of the coauthors are Merck scientists.

Acknowledgments

The live attenuated dengue viruses used for immunizing the animals were kindly provided by Dr. Stephen S. Whitehead in the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health.

Funding

This study was partially funded by grants from Merck Research Laboratories, the Texas Emerging Technology Fund, and the Welch Foundation Grant No. AU0042.

Supplemental Material

Supplemental data for this article can be accessed on the pub lisher's website.

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