



# Approaches to Interrogating the Human Memory B-Cell and Memory-Derived Antibody Repertoire Following Dengue Virus Infection

Zoe L. Lyski and William B. Messer\*

Department of Molecular Microbiology and Immunology, Oregon Health and Sciences University, Portland, OR, United States

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### \*Correspondence:

William B. Messer  
messer@ohsu.edu

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Memory B-cells (MBCs) are potential antibody secreting immune cells that differentiate and mature following host exposure to a pathogen. Following differentiation, MBCs remain in peripheral circulation after recovery and are poised to secrete antigen-specific antibodies if and when they are re-exposed to their cognate antigen. Consequently, MBCs form the founder population and provide one of the first lines of pathogen-specific defense against reinfection. The role MBCs play is complicated for viruses that are heterologous, such as dengue virus (DENV), which exist as antigenically different serotypes. On second infection with a different serotype, MBCs from initial dengue infection rapidly proliferate and secrete antibodies: many of these MBC derived antibodies will be cross-reactive and weakly neutralizing, while some antibodies may recognize epitopes conserved across serotypes and have the capacity to broadly neutralize 2 or more serotypes. It is also possible that a new population of MBCs and antibodies specific for the second virus serotype need to arise for long-term broader immunity to develop. Methods to interrogate and track memory B cell responses are important for evaluating both natural immunity and vaccine response. However, the low abundance of MBCs for any specific pathogen makes it challenging to interrogate frequency, specificity, and breadth for the pathogen of interest. This review discusses current approaches that have been used to interrogate the memory B cell immune response against viral pathogens in general and DENV specifically. Including strengths, limitations, and future directions. Single-cell approaches could help uncover the DENV specific MBC antibody repertoire, and improved methods for isolating DENV specific monoclonal antibodies from human peripheral blood cells would allow for a functional analysis of the anti-DENV repertoire.

**Keywords:** dengue, virus, hybridoma, B-cell immortalization, ELISPOT, flow cytometry, long-term immunity, monoclonal antibody

## INTRODUCTION

Neutralizing antibody responses play a critical role in anti-viral immunity—controlling and preventing infection, and are an important aim of vaccination. During initial infection, naïve host B-cells, specific to the infecting antigen, proliferate and differentiate into short-lived plasmablasts that secrete antibodies at a high rate to combat the existing infection. Following viral clearance,

two distinct layers of humoral immunity remain to protect against repeat infection with the same antigen—antibodies in the sera, constitutively produced by long lived plasma cells (LLPCs) and memory B-cells (MBC) primed to expand and secrete antibodies upon antigen re-exposure.

LLPCs, are terminally differentiated, non-dividing cells that reside in the bone marrow and produce antibodies for years to decades (1–3) providing protection against repeat infections with the same antigen (4, 5). These antibodies are typically assessed by *in vitro* neutralization and binding assays, and in many cases regarded as correlates of protection against viral pathogens (6).

MBCs make up the second line of antibody-mediated defense, providing protection by rapidly activating, proliferating and secreting antibodies in response to cognate antigen. Once regarded as a backup to LLPC-derived antibodies, the specificity and breadth of potential MBC responses are increasingly appreciated, especially with regard to protection against heterogeneous but antigenically related viral pathogens, such as influenza, different serotypes of DENV, and viral escape mutants (5, 7).

Naïve B-cells, MBC precursors, originate in the bone marrow before migrating to the spleen where they undergo further differentiation, redistribute to lymph nodes, and await antigen encounter. Recent work in mice suggests that MBCs originate in low affinity germinal center compartments within peripheral lymph nodes (5), which might contribute to more broadly reactive Ig receptors and increase the breadth of recall responses (5, 7). MBCs form a heterogeneous population, and it is thought that they preferentially differentiate upon reinfection. Specifically, IgG MBCs favor differentiating into plasmablasts whereas IgM MBCs preferentially re-enter germinal centers to undergo further rounds of affinity maturation (5, 8–10).

MBCs can be identified by their B-cell receptor (BCR), a membrane bound immunoglobulin (Ig) identical to the antibody they secrete upon activation. Upon reinfection, the recall response is rapid, dominated by high affinity isotype switched antibodies, IgG, IgA, or IgE, depending on pathogen. This recall response leads to the generation of new antigen-specific LLPCs and MBCs (5).

Although human MBCs have been characterized for many important viral pathogens, including HIV (11), RSV (12), influenza (13), human DENV MBC derived antibodies were not fully characterized until 2010 (14, 15). Mosquito-transmitted DENV is responsible for ~100 million symptomatic cases and 35,000 deaths annually (16) making it the most common and serious vector-borne disease affecting humans. DENV is an enveloped positive sense RNA virus, that circulates as four distinct serotypes (DENV 1-4), with 60–85% shared sequence homology (17). Repeat infections often occur in DENV endemic regions—Asia, Latin America, Africa, and parts of Oceania. Population growth, increased global travel, and spread of the vector have led to increasing epidemics. DENV infection causes symptoms that include high fever and rash. A portion of patients (~500,000 per year) develop severe dengue—referred to as dengue shock syndrome (DSS) or dengue hemorrhagic fever (DHF)—which can further progress to organ failure and death. First (1°) infection with one serotype is

thought to provide life-long protection against that serotype, but only short-lived protection against heterologous infection (18). Secondary (2°) infection with a different serotype can lead to broader protection, up to all four serotypes, but comes at a greater risk of serious disease during acute infection, through a process of antibody dependent enhancement (19), which occurs when sub-neutralizing antibodies bind to virus and facilitate uptake into cells via Fc receptors (20). The mechanism by which subsequent broader immunity develops is incompletely understood: while it is known that immediately following second infection the antibody response contains a large proportion of cross-reactive antibodies that can neutralize both viruses (21) the relative contribution of type specific, weakly cross-neutralizing and broadly neutralizing antibodies to long-term immunity is unclear, and may depend on virus maturation state (22). Further complicating the hypothesized role of broadly cross-neutralizing antibodies is the recent finding by Raut et al. (23) that *in vitro* neutralization assays using mature and partially mature tissue-culture derived DENV1 over-estimated by almost 15-fold the potency of heterotypic neutralizing antibodies when compared to neutralizing potency against the same fully mature DENV1 circulating in humans. Deeper understanding of the diversity and epitope-specificity of 1° MBCs could lead to the development of subunit vaccines that preferentially elicit potentially neutralizing antibodies against all 4 DENV serotypes while avoiding potentially enhancing antibodies.

Analyses of human antibody response to DENV infection have traditionally characterized serum antibodies, a product of LLPCs through virus neutralization, binding, and enhancement assays (24). More recently, greater focus has been put on characterizing B-cells that produce these antibodies. Historically, methods to interrogate antigen-specific B-cells, particularly in humans, have been challenging to develop (25, 26). However, complex, studying individual and population MBCs and the monoclonal antibodies (mAbs) they produce is an area of critical importance. Such studies allow for better understanding of the nature of human immune response to pathogens such as DENV, and are expected to lead to more rationally designed vaccines.

Over several decades, methods to interrogate antigen-specific MBCs have had several useful functions: identifying subset of MBCs available to respond to repeat infections (27, 28), tracking MBCs prior to and after vaccination or booster (6), isolating and characterizing human mAbs following natural infection or vaccination (29), and analyzing memory-derived antibody repertoires (28). Only recently have these methods been employed in the DENV field. Here we review the leading approaches for characterizing human DENV MBCs, evaluating their strengths, limitations and potential for further contribution to the field (summarized in Table 1).

## Limiting Dilution Assay (LDA)

The LDA was first used to detect virus-specific MBCs in mice over 20 years ago (32). This approach allows the frequency and specificity of rare antigen-specific MBCs in circulation to be

**TABLE 1** | Summary of human DENV-specific monoclonal antibodies isolated from immune donors.

References	Method	Efficiency	#Donors	mAbs isolated	Key findings
Schieffelin et al. (15)	B-cell immortalization (EBV)	N/A	1 > 2 years post infection	3	DENV-specific MBCs were in circulation >2 years post exposure 2% of cultures were DENV2 specific All isolated mAbs were IgG1
Beltramello et al. (14)	B-cell immortalization (EBV) with CpG Screened and cloned by limiting dilution.	6.5–14%	5 3-1° 2-2° 200 days to >8 years post- infection	70	All of the isolated mAbs were IgG: 68 IgG1, 1 IgG3, 1 IgG4 13 mAbs recognized EDIII and were the most potently neutralizing of all the mAbs isolated (5 serotype specific, 8 cross reactive) 34 mAbs recognized DI/DII they were highly cross reactive and less neutralizing, 6 mAbs recognized PrM, 11 mAbs recognized non-structural proteins, and 1 recognized capsid
Smith et al. (27)	B cells immortalized (EBV) with CpG and CHK2. Screened by ELISA. Positive wells fused to generate hybridomas.	> 10-fold increase in the # of successful human hybridomas generated	12 6-1° 6-2° 4-24 years post infection	37	29/37 isolated mAbs recognized E protein, 26 were IgG1 and 3 were IgG2 26/37 isolated mAbs were cross reactive, most bound to EDI/II 5- isolated mAbs EDIII specific (1C7, 1M23, 2J20, 1B23, 1M19) all cross reactive 3-mAbs had moderate to strong neutralizing potency against at least one serotype (2D22, 5J7, 2J20) 8- isolated mAbs PrM specific, mAbs exhibited enhancing properties
Smith et al. (30)	B-cell immortalization (EBV) CpG and CHK2 Screened by ELISA and neutralization then fused to generate hybridomas	N/A	3 2-1° 1-2° 1-9 years post- infection	50	Most potently neutralizing mAbs bound to EDIII (1M7) or complex (1F4) epitopes on intact virions. DENV specific MBC frequency similar between primary and secondary donors at 14–18 DENV specific MBC per thousand B cells. 15 of the isolated mAbs were non-neutralizing, and bound to rE or PrM.
Cox et al. (25)	FACS using E (DENV2-80E) and dual labeled secondary antibodies Isolated double positive MBCs	20 million PBMCs, 148 DENV E+ MBCs sorted.	1 from an endemic region Serum neutralized all 4 serotypes	9	DENV E specific MBCs are present in naturally infected donors. Authors isolated and characterized DENV neutralizing mAbs from MBCs against envelope domain I and the fusion loop. Of the sorted MBCs following 2 week stimulation in culture 64% were positive for IgG, 20% were positive for DENV by ELISA, 8% secreted DENV2 specific mAbs Of the 9 mAbs isolated 1-non-neutralizing, 3-serotype-specific, 2-neutralized 2–3 serotypes 3-neutralized 4 serotypes.
Appanna et al. (28)	FACS using fluorescently labeled DENV3. Isolated DENV positive MBCs	N/A	4 2°	19	40–60% of the DENV-specific MBCs sorted bound to DENV Most mAbs isolated bound to complex epitopes, 24.4% bound to PrM and 17.8% bound to rE Majority were cross reactive and weakly neutralizing
Nivarthi et al. (31)	B-cell immortalization (EBV) CpG and CHK2 followed by fusion	N/A	2 1° DENV- 4	8	Frequency of DENV-specific B-cells in circulation 0.19–0.2%. Of the 8 mAbs isolated, 2 neutralized DENV-4 and recognize regions on EDI/EDII hinge.

enumerated. PBMCs or enriched B-cells are stimulated *ex vivo* with a mitogen cocktail along with non-proliferating feeder cells. With this approach MBCs become antigen-secreting cells. The cells can be enumerated by ELISpot (described later) or secreted antibodies assayed by antigen-specific ELISA. This approach has been used to determine the frequency of viral-specific MBCs in humans following vaccination or natural infection (6, 33–35).

## Strengths and Limitations

Non-specific stimulation of human MBCs allows for the characterization of multiple antigen-specific MBC derived antibodies from a single PBMC sample (6). Antibody containing supernatant or MBCs can be used for a wide range of assays including: ELISpot, ELISA, and neutralization. The major limitations of this approach are that the cells are not

immortalized therefore longevity is limited, surface BCR is down-regulated, and single antigen-specific MBCs clones cannot be identified and subjected to downstream sequencing and cloning.

## Enzyme-Linked Immunosorbent Spot Assay (ELISpot)

Provides a sensitive and specific tool to detect antigen-specific MBCs. First described over 35 years ago (36) as a method for quantifying rare B and T cells and is still widely used today, as it is sensitive enough to detect a single antigen-specific cell. Plasmablasts can be studied directly *ex vivo*, but MBCs must be stimulated to become antibody-secreting cells. Membrane-bound antigen enables binding of mAbs secreted by B-cells. Bound antibody is detected using a secondary antibody and a colorimetric substrate, resulting in colored spots on the membrane that can be easily enumerated using imaging software. Advances in ELISpot technology have allowed researchers to detect different isotypes of MBCs that recognize multiple epitopes and multiple antigens (37). Recently developed multifunctional FluoroSpot assays allow enumeration of cross-reactive and type-specific DENV and Zika MBCs following natural infection and vaccination (19, 38). This allows researchers to determine serotype specificity on a single-cell basis, rather than polyclonal level.

## Strengths and Limitations

ELISpot is highly sensitive and allows for the enumeration of rare cells of interest—frequency, specificity, and antibody isotype can be determined. The major limitation is that it does not allow for isolation and downstream analysis—Functional properties of antibodies, such as neutralization cannot be assessed, and cells' BCRs of interest cannot be sequenced or cloned for mAb production.

## Hybridoma Approaches

The use of hybridomas to immortalize MBCs was first described over 40 years ago (39). Hybridomas are made by fusing a myeloma cell with a B-cell from an immunized or naturally infected individual, the resulting hybrid cell secretes mAbs specific to their antigen. Technical advances have made it feasible to generate hybridomas from human peripheral blood MBCs (40). These advances include expanding B-cells prior to fusion, finding new human myeloma cells to fuse with, and improved fusion techniques including electrical cytofusion (41). Using optimized techniques Yu et al. (41), fusion efficiency improved from 0.001 (40) to 0.43% (41) which enabled them to isolate neutralizing mAbs against RSV and influenza from human peripheral MBCs. Hybridoma technology is a well-established and indispensable platform for generating high-quality mAbs and has been used to produce mAbs against a wide range of viral antigens including DENV.

## Strengths and Limitations

Major advantages of this approach include pairing of BCR heavy and light chains (42), native constant region of the mAb expressed allowing Fc-mediated effector functions, such

as enhancement to be accessed (40). Finally, the hybridoma products are stable in culture and can be frozen for future use.

One major limitation of this approach is extremely low fusion efficiency. Consequently, traditional hybridoma strategies are not as well suited for identifying rare antigen-specific MBCs that circulate in low numbers in the periphery of immune donors, as overall only a small amount of the total B cell repertoire is captured.

The second major limitation has been the challenge of making human, rather than mouse, derived hybridomas. Work by Wahala et al. (43) found that humans and mice recognize distinct and different epitopes on the DENV virion following immunization in mice or natural infection in humans. Nearly all neutralizing antibodies found in humans after natural infection recognize complex quaternary epitopes on the surface of whole virions (44, 45), in contrast to the DENV neutralizing antibody response in mice, where the majority of neutralizing antibodies recognize a single domain region, domain III, on the envelope glycoprotein (43).

## B-Cell Immortalization

MBC immortalization can be achieved through transforming peripheral MBCs using Epstein Barr Virus (EBV), or through expression of BCL-6, and BCL-XL. This results in stable cell lines that express BCR on the surface and secrete antibodies, making them a useful tool in the generation of human mAbs and has become a leading approach in characterizing DENV-specific MBCs in humans.

EBV transformation for B-cell cultures was developed in the 1970's Steinitz et al. (46) when normal human B-cells were infected with EBV, a lymphotropic herpesvirus, transforming MBCs into stable antibody secreting cell lines. Supernatants can be screened for specificity to antigen of choice and serial dilution down to a single cell enables this method to be applied to mAb production. Many groups have utilized and continue to utilize EBV immortalization to isolate human mAbs against a wide variety of human pathogens, including HIV (47), SARS coronavirus (48), Influenza (49), RSV (50), and DENV (14, 15, 51).

Another technique employed to immortalize MBCs is through forced expression of BCL-6 (required for GC formation) and BCL-XL (anti-apoptotic Bcl-2 protein family). Both are expressed in GC B-cells, and by introducing these genes into peripheral blood MBCs and culturing with CD40L and IL-21, they become highly proliferating with surface and secreted Ig (52). BCL-6 + BCL-XL transduced cells express *AICDA*, encoding the enzyme activation-induced cytidine deaminase (AID), at the same levels as isolated tonsil derived GC B-cells, but not normally expressed in peripheral MBCs or plasma cells. AID mediates somatic hypermutation (SHM) and class-switch recombination (CSR) and therefore increases diversity of the BCR. AID is functional in these cells and low levels of SHM is observed in the Ig genes of expanded B-cells. These cells can be maintained for prolonged periods of time in culture to allow for mAb production (53). Using this approach, researchers have identified neutralizing mAbs in humans that recognize RSV (52), Hepatitis C virus (54), influenza (55), and DENV (56).

## Strengths and Limitations

Immortalized B-cells have a plasmablast-like phenotype, with secretory and membrane-bound Ig, which makes them a powerful tool for discovery and characterization of mAbs. Probes that bind to BCRs of interest enable the isolation of antigen-specific B-cells from a polyclonal population. Immortalized cells are stable and can be frozen for future use. The presence of AID and the potential for SHM can be utilized to generate clones that have higher or lower affinities than the parental clone, allowing for a method of affinity maturation in culture (53).

Transformation efficiency for BCL-6+BCL-XL is 60–80% in humans (53), and EBV transformation have improved from 10 to >30% with the addition of TLR agonists, typically CpG or R848 (50, 57). This approach requires significant numbers of cells to yield few cells of interest. Because cells proliferate with this approach, frequency of particular antigen-specific MBCs cannot be enumerated.

Using EBV-transformed human B-cells to generate human hybridomas can increase efficiency by as much as 25-fold compared to that of using untransformed PBMCs. Therefore, Investigators often utilize a combination approach of EBV immortalization followed by fusion to isolate human DENV-specific mAbs from naturally infected or vaccinated donors (28, 55).

## Antigen—Specific Flow Cytometry

Flow cytometry-based approaches have been used to enumerate antigen specific MBCs against model antigens in mice (58) and humans (33). However, viral antigen-specific flow cytometry has been utilized more recently, Weitkamp et al. (59) identified human rotavirus specific B-cells, Scheid et al. (60) characterized low-frequency HIV specific MBCs in humans and Woda et al. (61) characterized DENV-specific MBCs in human immune donors. Recognizing the complex and quaternary nature of DENV neutralizing epitopes (45) Authors Woda and Mathew (26) and Appanna et al. (28) used fluorescently labeled whole DENV virus (62) as a probe to detect DENV-specific MBCs in immune donors while Cox et al. (25) used biotinylated DENV envelope protein as a probe along with dual labeled streptavidin antibodies to identify DENV envelope-specific MBCs. This method enabled researchers to isolate 8 DENV-neutralizing mAbs from a single donor (Table 1).

## Strengths and Limitations

Antigen choice is important, DENV neutralizing epitopes are comprised of complex conformational structures and not recapitulated by simple linear peptides or recombinant proteins (45). However, whole viruses are inherently sticky and adheres to host cells. To tackle this non-specific binding (26, 61) utilized fluorescently labeled Vero cell supernatant as well as dual labeled probes to decrease background (63). A major strength of this approach is the possibility of tracking multiple serotypes of DENV-specific MBCs prior to and post infection or vaccination (61) as well as the potential for single cell sorting antigen-specific MBCs for downstream assays such as immortalization, sequencing, or cloning (25).

## FUTURE DIRECTIONS

An important early advancement in the field of human mAb generation was the advent of single-cell RT-PCR approaches (64) that allow for sequencing, cloning, and characterization of each BCR from individually sorted MBCs. This approach remains useful when the population of interest represents a large proportion of total cells in population (plasmablasts during acute infection), or when a valid probe or screening approach exists to identify MBCs of interest prior to sequencing. In addition to generating mAbs, sequencing of the BCR provides information about B-cell clonal evolution during infection. While groundbreaking, this single-cell approach is time and resource intensive as it requires heavy and light chains to undergo PCR, sequencing, and cloning independently and remain correctly paired for transfection into expression plasmids.

This single-cell approach provides a glimpse into the overall antibody repertoire, which has a potential diversity of more than  $1 \times 10^{13}$  in humans, but high throughput methods that capture the entire antigen-specific MBC repertoire recently developed with other pathogens would be expected to advance the DENV field as well. High-throughput droplet microfluidic approaches (65) allow for individual partitioning of single B-cells, that are individually barcoded and allow for paired sequencing of Ig heavy and light chains from a single B cell captured within a droplet. From this, a complete Ig library can be generated, as well this approach allows for simultaneous sequencing of barcoded Ig genes with the possibility of co-expressed functional genes to fully understand the pathogen specific MBC repertoire. MAb that are generated from these antibody gene sequences allow for a functional analysis of the repertoire.

Another high throughput approach (66, 67) recently used to isolate mAbs from humans involves using microfluidics to partition individual cells then physically link heavy and light mRNAs and perform overlap extension PCR to generate a continuous heavy-light chain amplicon for cloning into a yeast display system for Fab or IgG which allows screening for antigen specificity and affinity by FACS. Through this approach researchers were able to isolate broadly neutralizing antibodies against HIV, Ebola, and influenza.

The ability to fully interrogate the MBC response established after natural infection to viral antigens will allow researchers to durably and comprehensively interrogate vaccine responses to further understand the differences between natural and vaccine derived immunity.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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