COMMENTARY

Detection, phenotyping and quantification of dengue virus-specific B cells using fluorescent probes

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ABSTRACT

Dengue viruses are some of the most important mosquito-borne pathogens worldwide. They cause illness in 50–100 million individuals per year and have a significant global health impact in low- and middle-income countries. It is important to improve our understanding of the humoral response to dengue virus, as antibodies (Abs) are associated with protection from or susceptibility to severe dengue disease. In recent years, significant advances have been made toward identifying Ab targets and evaluating the functional properties of Abs. However, much less is known about the cellular source of Abs, B cells, in part because the reagents to phenotype and characterize antigen-specific B cells have been challenging to develop. Here, we discuss our recent experience with developing and using fluorescent viruses to probe the B cell response to dengue virus. We present the strengths and weaknesses of flow cytometric analysis of antigen-specific B cells and discuss the use of these probes to phenotype and characterize specific B cells during and after natural infection and in ongoing dengue vaccine trials.

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Introduction

There is a consensus that antibodies (Abs) are important for the resolution of viremia and long-term immunity against dengue virus (DENV), a complex of 4 serotypes, DENV-1, DENV-2, DENV-3, and DENV-4.¹ Prior B cell immunity is also widely acknowledged as a key determinant of susceptibility to severe disease, dengue hemorrhagic fever, because the Abs secreted by B cells have the potential for pathological effects on subsequent DENV infections.² The induction of a strong neutralizing Ab response to all 4 serotypes of DENV following vaccination is a major goal of vaccine developers since Abs can work with high specificity, avidity, and targeted functionality to eliminate the pathogen upon reencounter. Analyses of such responses have traditionally measured Ab titers in the serum. The information gained from those measurements has been very useful, but there has been much less focus on the cell subset, namely B cells, that produces antibodies.

A review of early attempts at identifying and isolating antigen-specific B cells has already been performed.³ More recent efforts to detect antigen-specific B cells have largely relied on ELISPOT assays using intact virus or recombinant proteins to assess antibody-secreting cells directly *ex vivo* or after short-term polyclonal *in vitro* activation.^{4,5} While these studies have yielded information about the proportion of antigen-specific B cells in patients and/or vaccinees, the ELISPOT assay does not allow for the isolation and downstream analysis of particular cells of interest. Flow cytometry-based approaches can sort B cells with plasmablast

(CD27+CD38++) or memory B cell (CD27+IgD-) phenotypes. Bulk sorting these populations allows for global analysis of the Ab repertoire, at the expense of information regarding the antigen specificity of Abs (outside of the analysis of cell culture supernatants). Single cell sorts have been useful to isolate and generate recombinant monoclonal Abs; however, this approach does not account for the heterogeneous mixture of Abs and subsets of B cells available to respond to a subsequent DENV infection with a homologous or heterologous DENV serotype.

Most recently, we and others have developed fluorescently-labeled dengue virus (FL-DENV) or recombinant DENV protein reagents to directly identify and characterize antigen-specific B cells.^{6–8} This approach leverages the power of multiparametric flow cytometry and allows sorting of antigen-specific cells, and important subsets of those cells, to study them in greater detail. We will discuss the progress made and challenges with using fluorescent antigen to identify and characterize DENV-specific B cells using flow cytometry. Furthermore, we will summarize recent efforts to use these reagents to track B cells during and after natural dengue infection and discuss how these probes would be informative for evaluating B cell responses in ongoing dengue vaccine trials. A better understanding of how B cells respond to and are altered by DENV natural infection and vaccination will help define protective DENV immunity and therefore inform approaches that evaluate the effectiveness of candidate DENV vaccines.

Challenges with isolating antigen-specific B cells

Upon initial exposure to a pathogen, naïve B cells are activated and secrete immunoglobulin (Ig)M before establishing germinal centers in which they undergo affinity maturation and class-switch recombination to produce other types of Abs, including IgA and IgG.⁹ A large burst of short-lived antibody-secreting cells, plasmablasts, circulate in the blood, typically peaking within 2 weeks of antigen exposure. Long-lived Ab-secreting cells, plasma cells, migrate to the bone marrow to produce the serum Abs that comprise the first line of defense against a secondary infection. Memory B cells are also generated and circulate poised to rapidly secrete Ab upon re-exposure to dengue. These memory B cells exist in the circulation at low frequencies and do not actively secrete Ab unless activated.⁴

The isolation of antigen-specific B cells has been challenging partly because the frequency of cells specific for any particular antigen is extremely low. Because of their relatively small numbers, most of the techniques for antigen-specific B cell isolation have used expansion *in vitro* to increase the number of specific B cells. As early as the 1980s, the detection of B cells specific for a model antigen such as ovalbumin or hapten using flow cytometry was done successfully.³ B cells express antigen-specific cell surface receptors (BcR) that bind to native, non-processed antigen. A major limitation in isolating antigen-specific B cells has been the non-specific binding of reagents to B cells, which impacts the purity of the isolated population.¹⁰ Detection of pathogen-specific B cells is further complicated by the fact that the precise locations of the B cell epitopes on pathogens are often unknown. In addition, many B cell epitopes on DENV are comprised of complex conformational structures not mimicked by linear peptides or recombinant proteins.¹¹ A potential problem associated with the use of labeled dengue viruses or antigens is their inherent ability to adhere to host cells. For viruses that attach to B cells and utilize their normal cell-entry mechanism, this could mimic labeling of specific B cells via BcR-binding.

Choice of dengue antigen

Considerable effort has been made to understand which proteins are recognized by Abs elicited in humans by DENV infection or vaccination.¹² Abs specific for the envelope (E), pre-membrane (prM) protein and non-structural protein 1 (NS1) with poor, moderate or potent neutralizing activity have been isolated in dengue immune individuals during and after primary or secondary dengue infection.¹³ The E protein is composed of 3 ectodomains: Domains I and II combined (DI-II) contain the hinge region; Domain III (DIII) contains both lateral ridge and A-strand epitopes thought to be a target of many neutralizing Abs in mice and humans.¹² Abs elicited in humans preferentially recognize glycosylated epitopes on the E protein. The expression system used to produce recombinant dengue proteins is therefore important, as proteins can be variably glycosylated in *E. coli*, yeast, *Drosophila* and mammalian expression systems, impacting their ability to be recognized by Abs.¹⁴ Several Abs recognize the prM protein and immature forms of DENV.^{12,13} While the NS1 protein is strongly recognized by Abs, much less is known about particular NS1 epitopes. A

number of human monoclonal Abs from DENV immune donors bind complex quaternary epitopes present only on mature viruses and not on the E protein produced as a soluble recombinant protein.¹¹

Direct comparison of monomeric versus tetrameric labeled tetanus antigen showed that a substantial fraction of memory B cells can be missed depending on the antigenic structure used for detection¹⁵; similar observations were made with HIV protein monomers.¹⁶ Therefore generating recombinant tetramers of E DIII, E DI-II and NS1 dengue proteins are likely to be more sensitive than monomers and increase the avidity of binding of DENV specific reagents to the BcR. Cox et al. used biotin-tagged DENV-2-80E to sort specific memory B cells from a dengue immune donor and generated 9 monoclonal Abs that were further characterized.⁸ While B cells typically recognize conformational epitopes, some linear epitopes are also recognized by Abs. Newman et al. developed a tetrameric fluorochrome-labeled form of the R4A peptide (R4Atetramer), which mimics double-stranded DNA, and used the reagent to identify autoreactive, antigen-specific B cells.¹⁷ There are many linear peptides on dengue proteins that have been recognized by Abs.¹⁸ Fluorochrome-labeled tetrameric peptides may be helpful in identifying and phenotyping subsets of B cells that recognize linear epitopes. We used amine labeling of infectious DENV to identify DENV-specific memory B cells in DENV-immune individuals.⁶ We identified a small frequency of memory B cells from DENV immune but not naïve individuals that bound fluorescently labeled (Alexa Fluor) AF DENV and demonstrated that sorted AF DENV+ memory B cells could be activated to secrete DENV-specific Abs.

Specificity of binding of probes to the BcR

An important consideration for B cell detection reagents is the specificity of binding of the fluorescently labeled probe (protein, virus or peptide) to the B cell receptor.^{3,10,16} It is helpful to include an IgG marker in the staining strategy as the resulting staining pattern will consist of a diagonally-shaped population, representing the roughly 1:1 ratio between the IgG of the BcR and its cognate antigen. It is also recommended that the same probe be conjugated to 2 different dyes such that B cells that recognize the dyes will fall in the single positive quadrants while B cells that specifically recognize the antigen will fall in the double-positive quadrant of a flow plot when equimolar concentrations are used. An additional control is to compete out the fluorescence from a labeled probe in a titration curve experiment using an unlabeled version of the same probe.

Choice of labeling reagents, controls, and optimizing staining

We have used amine reactive dyes such as Alexa Fluor to successfully label DENV preparations (FL-DENV) as was initially demonstrated by Zhang et al..¹⁹ We found that at high concentrations of FL-DENV both memory and naïve B cells from DENV naïve individuals bound FL-DENV, and in many cases, it was not possible to distinguish the low frequency of DENV+ memory B cells in immune individuals from those in naïve individuals.⁶ It is often necessary to use sub-saturating

concentrations of FL-DENV to achieve cleaner backgrounds. There are many types of negative controls that can also be used.^{3,10,20} First, it is helpful to have cells from a subject who is not expected to have the antigen-specific B cells of interest, such as from a donor who is seronegative for DENV. Second, the use of an irrelevant probe or negative control (for example, FL-Vero supernatant from the same cell source in which DENV was propagated) was very helpful for us to reduce the background staining.^{6,7} It is also useful to include "fluorescence minus one" (FMO) controls, in which all of the stains are used except the FL-DENV.

Data acquisition and limits of detection

Memory B cells to a specific antigen represent a very low frequency among CD19+ B cells which means that one must acquire a large number of events on the flow cytometer to have confidence in the data. The appropriate number of events depends on the frequency of CD19+ B cells and memory B cells (often characterized as CD27+ CD19+ cells) in PBMC. In our experience, the frequency of memory B cells is quite varied in different donors and so we acquire data files containing as many lymphocytes as possible.⁷ In clinical samples, this has been a challenge as we have access to limited numbers of PBMC during acute infection. However, as we have recently shown, the frequencies of FL-DENV+ memory B cells are much higher during acute infection compared with convalescence. The limits of detection of the FL-DENV staining method are dependent on several factors, which include (1) the quality of the fluorescent reagent and the intensity of its signal; (2) the quality and source of the PBMC sample; (3) the background staining on memory B cells from naïve donors; and (4) the number of events acquired.

Applications of fluorescent probes

Use of FL-DENV to study antigen-specific B cells after natural infection

In the absence of readily available antigen-specific B cell reagents, studies of natural dengue infection have either focused on the plasmablast response within the first 2 weeks post-infection or the memory B cell response. Memory B cells circulate months to years post-infection and require activation to initiate Ab secretion. While plasmablasts are thought to be primarily antigen-specific early after antigenic challenge,^{21,22} there is also evidence of polyclonal activation of B cells during acute dengue.²³ Compared to other febrile illness (OFI) patients, dengue patients had higher numbers of Abs directed against non-dengue pathogens, such as polio,²⁴ and in vitro studies demonstrated that DENV can activate B cells in the absence of overt infection.²⁵ These findings complicate the analysis of the plasmablast cell population but may explain why we see high "background" staining with our fluorescent DENV probes particularly in the naïve B cell compartment (data not shown). In contrast to the high numbers of plasmablasts in the periphery, memory B cells circulate at very low frequencies and the relative proportion of antigen-specific cells is much lower.

The relationship between plasmablasts and memory B cells, and to serum Abs to DENV, is beginning to be explored. Some

studies have found a correlation between serum Ab titers and the frequency of circulating Ab-secreting cells in dengue patients,^{22,24} while others did not^{21,26}; one study found a correlation in secondary but not primary infections.²⁷ The picture that appears to be developing is that plasmablasts and memory B cells comprise different populations of cells which partially overlap. Plasmablasts were shown to produce a predominance of E-specific Abs, whereas memory B cell-derived Abs targeted a broader array of proteins, including prM and NS1.^{21,28} BcR repertoire analysis has yielded conflicting results, with one group demonstrating low levels of somatic hypermutation (SHM) in acute plasmablasts from dengue patients,²⁹ suggesting polyclonal activation, and another study demonstrating high levels of SHM suggestive of antigen selection.³⁰ Neither of those studies was able to sort DENV-specific cells directly. Recently, fluorescently tagged DENV were used to sort individual antigen-specific memory B cells weeks to several months after the onset of fever.²⁸ The BcR repertoire of memory B cells differed significantly compared with the BcR repertoire of the plasmablast population. As the antigen-specific plasmablast response may not represent the total plasmablast population,²⁶ examining the DENV-specific plasmablast repertoire is warranted.

A recent study by Ellebedy et al. identified a population of activated B cells (ABCs) that, in the cases of Influenza and Ebola virus, was related to memory B cells.²⁰ These ABCs were present early after infection, but differed from plasmablasts by way of surface marker expression (CD38^{lo-int}CD20^{hi} [ABCs] vs. CD38^{hi}CD20^{neg} [plasmablasts]), proliferation (Ki67+ vs. Ki67++), and Ab production; ABCs needed activation to produce Ab whereas plasmablasts secreted Ab directly ex vivo. Longitudinal analysis of PBMC using fluorescent antigens identified ABCs in the recall response to Influenza as well as the primary response to Ebola, demonstrating the value of similar reagents to help understand DENV-specific B cell responses in primary vs. secondary infections.

We used probes with different specificities (serotypes DENV-1 and -2) on separate fluorochromes to both verify individual probe specificity and gain information about the immunologic profiles of our patient population.^{6,7} A combination of probes, each with a distinct specificity, is expected to yield flow cytometric plots that demonstrate a mutually exclusive binding pattern for each probe or in the case of DENV an interesting serotype-cross-reactive profile. We detected DENV-specific plasmablasts and memory B cells in the circulation during acute primary and secondary DENV infection and tracked the transition of plasmablasts into long-lived memory B cells.⁷ We are currently evaluating fluorescent probes for all the serotypes of DENV, but the challenges associated with generating 4 distinctly labeled DENV viruses are not trivial.

Use of FL-DENV to study antigen-specific B cells after vaccination

Phase 2 and 3 trials of a tetravalent DENV vaccine by Sanofi Pasteur demonstrated the failure of neutralizing Ab titers to predict protection from all 4 serotypes of DENV.³¹ These studies highlighted the importance of studying antigen-specific B cells to determine their contribution to protection from disease

and/or infection. In contrast to the small but growing number of studies in natural infection, there are currently no published studies that have assessed the frequency and phenotype of DENV-specific B cells generated after vaccination. The use of FL-DENV would make it possible to isolate and characterize the total population of DENV-specific B cells induced by vaccination. Comparison of the BcR repertoire (bulk analysis) between vaccine-induced and infection-generated B cells is important, as is the analysis of paired Ig heavy and light chains (single cell analysis) for the detailed characterization of monoclonal Abs. Such investigations could address several outstanding questions, including the longevity of serotype-specific as compared with serotype-cross-reactive and/or flavivirus cross-reactive Abs and their respective roles in prevention or exacerbation of disease. We also have a limited understanding of the contribution of different isotypes of Ab (e.g., IgM, IgA, or different subclasses of IgG) to DENV immunity and whether different vaccine products influence the isotypes of Igs that are generated. The correlation between circulating serum Abs and memory B cell-derived Abs remains unclear. It will be important to distinguish between B cells that produce cross-reactive, poorly neutralizing Abs from those that produce serotype-specific, potentially neutralizing Abs and to determine how to generate a strong neutralizing response to all 4 serotypes of DENV.

Vaccine studies provide a unique opportunity to collect large volumes of samples and offer a clearer definition of the kinetics of responses compared with natural infection, since we know when the vaccine was administered. Partially protective, serotype-cross-reactive immunity generated by primary DENV infection appears to wane over time leaving subjects potentially susceptible to secondary infection. Another advantage of studying DENV-specific immunity generated in the context of vaccination is the ability to control complicating factors, such as pre-existing dengue or other flavivirus immune responses (and the timing of pre-exposure events). One major question that will need to be addressed, however, is how tetravalent immunization (which is the leading dengue vaccine strategy) compares to monovalent natural infection.

Concluding remarks

As prior DENV immunity is implicated in the exacerbation of severe disease, the reactivation of serotype-cross-reactive memory B cells is likely to play a role in determining the clinical outcome of a given patient. Thus it is important to study DENV-specific B cells, to track their differentiation from naïve through effector and memory phases, and to characterize any potential deviations from “normal” protective profiles that may occur along the way. With several vaccine candidates and at least 2 human challenge models in various stages of development for dengue, we are uniquely situated in time to be able to better define protective anti-DENV immunity.

Disclaimer

The views expressed in this manuscript are the personal views of the author(s) and do not necessarily represent the views of the U.S. Army or the U.S. Department of Defense.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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