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Single-Cell Analysis of B Cell/Antibody Cross-Reactivity Using a Novel Multicolor FluoroSpot Assay

Alexandros Hadjilaou,¹ Angela M. Green, Josefina Coloma, and Eva Harris

Dengue is a major public health problem globally. It is caused by four antigenically distinct serotypes of dengue virus (DENV1–4), and although serotype-specific and strongly neutralizing cross-reactive immune responses against the four DENV serotypes are thought to be protective, subneutralizing Abs can contribute to increased disease severity upon secondary infection with a different DENV serotype. Understanding the breadth of the immune response in natural DENV infections and in vaccinees is crucial for determining the correlates of protection or disease severity. Transformation of B cell populations to generate mAbs and ELISPOT assays have been used to determine B cell and Ab specificity to DENV; however, both methods have technical limitations. We therefore modified the conventional ELISPOT to develop a Quad-Color FluoroSpot to provide a means of examining B cell/Ab serotype specificity and cross-reactivity on a single-cell basis. Abs secreted by B cells are captured by an Fc-specific Ab on a filter plate. Subsequently, standardized concentrations of all four DENV serotypes are added to allow equal stoichiometry for Ag binding. After washing, the spots, representing individual B cells, are visualized using four fluorescently labeled DENV serotype-specific detection mAbs. This method can be used to better understand the breadth and magnitude of B cell responses following primary and secondary DENV infection or vaccination and their role as immune correlates of protection from subsequent DENV infections. Furthermore, the Quad-Color FluoroSpot assay can be applied to other diseases caused by multiple pathogen serotypes in which determining the serotype or subtype-specific B cell response is important. The Journal of Immunology, 2015, 195: 000–000.

Materials and Methods

DENV propagation and purification

*Aedes albopictus* C6/36 cells were infected with the four DENV serotypes at a multiplicity of infection of 0.01 in RPMI 1640 supplemented with 2% FBS (Life Technologies), 1% penicillin/streptomycin, and 1% HEPES. Cellular supernatant was collected 5–8 d postinfection and concentrated at a multiplicity of infection of 0.01 in RPMI 1640 supplemented with 2% FBS (Life Technologies), 1% penicillin/streptomycin, and 1% HEPES. Cellular supernatant was collected 5–8 d postinfection and concentrated using 100-kDa Amicon filter units (Millipore) by centrifuging at 3250 × g for 20 min at 4°C. The concentrated supernatant containing the virus was layered on a discontinuous 20/55% OptiPrep (Sigma-Aldrich) density gradient and ultracentrifuged at 3250 × g for 20 min at 4°C. The concentrated supernatant containing the virus was layered on a discontinuous 20/55% OptiPrep (Sigma-Aldrich) density gradient and ultracentrifuged at 3250 × g for 20 min at 4°C. The concentrated supernatant containing the virus was layered on a discontinuous 20/55% OptiPrep (Sigma-Aldrich) density gradient and ultracentrifuged at 3250 × g for 20 min at 4°C. The concentrated supernatant containing the virus was layered on a discontinuous 20/55% OptiPrep (Sigma-Aldrich) density gradient and ultracentrifuged at 3250 × g for 20 min at 4°C. The concentrated supernatant containing the virus was layered on a discontinuous 20/55% OptiPrep (Sigma-Aldrich) density gradient and ultracentrifuged at 3250 × g for 20 min at 4°C. The concentrated supernatant containing the virus was layered on a discontinuous 20/55% OptiPrep (Sigma-Aldrich) density gradient and ultracentrifuged at 3250 × g for 20 min at 4°C.
DENV serotype-specific detection mAbs were tested for their reactivity against the purified DENV virions via ELISA. AP, alkaline phosphatase.

Concentration as determined by BCA. Shown is a BCA dilution curve with equal amounts of each purified DENV serotype. (C) of the four purified DENV serotypes (DENV1 N1265-04, DENV2 N172-06, DENV3 N2845.6-09, and DENV4 N703-99) was standardized by protein concentration.

Characterization of detection mAbs by ELISA

A qualitative ELISA was used to assess the serotype specificity of the detection mAbs (anti-DENV1 E95, anti-DENV2 E96, anti-DENV3 S7J, and anti-DENV4 E88) prior to quantum dot (Qdot) conjugation (Qdot 565, 705, and 525, respectively). Briefly, plates were coated with a 1:100 dilution of the OptiPrep-purified DENV in coating buffer at 37˚C for 2 h. After three washes with PBS-0.05% Tween (PBS-T), plates were blocked with blocking buffer (5% milk in PBS-T) for 2 h at 37˚C. Detection mAbs were incubated with the primary Ab at a 1:1000 dilution in blocking buffer at 37˚C for 2 h. After three washes with PBS-T, the plates were incubated with either anti-human-HRP or anti-mouse-HRP secondary Ab depending on the origin of the primary Ab (human versus mouse) at 37˚C for 2 h. Plates were then washed three times with PBS-T and developed with 3',5',5'-tetramethylbenzidine (Sigma-Aldrich).

Conjugation of mAbs to Qdots

Purified mAbs (100 μg) were conjugated enzymatically to Qdots (Live Technologies) via the N-acetylgalacosamine of the glycosylation chain on the Fc region of the mAb after hydrolysis of a galactose residue. SiteClick technology (Invitrogen) was used according to the manufacturer’s instructions. Briefly, after the Tris buffer was exchanged with the Ab preparation buffer, galactosidase was used to cleave the galactose residue on the glycosylation chain, leaving the N-acetylgalacosamine exposed. Subsequently, β-1,4-galactosyltransferase was used to attach an azide group (N-azidoacetylgalactosamine) to the cleaved galactose moiety. Microcentrifugal filter units (m.w. cutoff 50 kDa) were used to purify the azide-modified mAbs from the enzymes, and the mAbs were incubated with dibenzocyclooctynol-modified Qdots overnight at room temperature. The conjugated mAbs were then ready for use. For Qdots 625 and 700, the manufacturer offers a microcentrifugal filter of 300 kDa to remove unconjugated Qdots and mAbs.

Confirmation of conjugation efficiency by flow cytometry

Flow cytometry was used to test the conjugation efficiency of the detection mAbs. For this purpose, Raji cells stably transfected to express dendritic cell–specific ICAM3-grabbing nonintegrin were infected with each of the DENV serotypes separately in infection media (RPMI 1640 supplemented with 2% FBS, 1% penicillin/streptomycin, and 1% HEPES) for 48 h. Cells were then centrifuged and washed with FACS buffer (1× PBS, 0.5% BSA, and 0.02% sodium azide). Washed cells were then fixed with 2% paraformaldehyde for 30 min and permeabilized on the Fc region of the mAb after hydrolysis of a galactose residue. SiteClick technology (Invitrogen) was used according to the manufacturer’s instructions. Briefly, after the Tris buffer was exchanged with the Ab preparation buffer, galactosidase was used to cleave the galactose residue on the glycosylation chain, leaving the N-acetylgalacosamine exposed. Subsequently, β-1,4-galactosyltransferase was used to attach an azide group (N-azidoacetylgalactosamine) to the cleaved galactose moiety.

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FIGURE 1. Development of the QCF. (A) Schematic comparison of ELISPOT and QCF. (B) Standardization of Ag concentration. The concentration of the four purified DENV serotypes (DENV1 N1265-04, DENV2 N172-06, DENV3 N2845.6-09, and DENV4 N703-99) was standardized by protein concentration as determined by BCA. Shown is a BCA dilution curve with equal amounts of each purified DENV serotype. (C) Detection mAb specificity. DENV serotype-specific detection mAbs were tested for their reactivity against the purified DENV virions via ELISA. AP, alkaline phosphatase.
washes with permeabilization buffer and two washes with FACS buffer were performed. Samples were analyzed on a BD LSR Fortessa flow cytometer (BD Biosciences). Analysis was performed using FlowJo 8.8.7 software (Tree Star).

**Quad-Color FluoroSpot**

The membrane of a sterile IPFL clear 96-well plate (Millipore) was activated with 50 μl/well 70% ethanol for 2 min. After three washes with PBS, the plates were incubated with 2 μg/well Fcγ fragment-specific anti-human IgG (The Jackson Laboratory) overnight at 4°C. Wells were blocked with 200 μl RPMI 1640 supplemented with 10% PBS for 2 h at 37°C after the wells were washed three times with PBS. Human hybridomas, EBV-transformed B cells, or activated PBMCs from DENV-positive or -negative individuals were counted and plated in a total of eight serial dilutions of 1:2. PBMCs were activated with IL-2 (1000 U/ml)/R848 (2.5 μg/ml) for 5 d. Wells were incubated at 37°C in 5% CO₂ for 48 h to allow sufficient Ab deposition to occur. After the incubation, cells were washed away with 200 μl PBS-T 0.05% three times with a final PBS rinse. All four OptiPrep-purified DENV serotypes were plated at a final concentration of 1 μg/serotype/well for 1 h at 37°C. After the DENV incubation, wells were washed three times with 200 μl PBS-T 0.05%, with a final PBS rinse. The four Qdot-conjugated, serotype-specific mAbs (50 μl/well at a 1 μg/ml final concentration for each detection mAb) were added to each well and incubated for 1 h at 37°C. Finally, the plate was washed two to three times with PBS and once with double-distilled H₂O and dried using a 96-well MultiScreen Vacuum Manifold (Millipore). Visualization of the fluorescent spots was performed using a CTL ImmunoSpot S6 Micro-Analyzer (Cellular Technology Limited). Specifically, four different emission filters are used, each designated to one serotype. Each emission filter, and therefore serotype, is read by the CTL reader individually, and a merged picture is generated that is the combination of all four images. The software that is installed in the reader counts the spots for each filter/serotype individually. Subsequently, the software identifies the spots that are found in one filter (type-specific) or more than one filter (bi-, tri-, or tetra-serotype specific) through pixel colocalization. To control for background, two negative controls were used. First, stimulated PBMCs from each DENV-positive Nicaraguan sample were processed as above; however, after PBMC incubation and Ab deposition through IgG binding, Qdot-conjugated serotype-specific detection mAbs were added directly without the DENV Ag incubation step. In all cases, no reactivity (background) was observed. In addition, stimulated PBMCs from DENV-negative Oakland Blood Bank donors were used as a negative (background) control. In this instance, the complete QCF procedure was followed, including incubation with DENV Ag. However, because the donors were DENV negative, this control also showed no reactivity.

To determine total IgG-secreting cells, PBMCs were plated in 1:2 serial dilutions of the untreated (ex vivo) or stimulated (in vitro) cells starting from 60,000 PBMCs/well. Wells were coated with anti-human IgG (Fc-specific) Ab. Following cell incubation and Ab deposition, IgG-positive spots were visualized using a polyclonal PE-labeled anti-human IgG detection mAb (H+L). Spots were then counted using the CTL reader.

**Results**

**Modification of the conventional ELISPOT**

The QCF is a modified version of an ELISPOT that uses purified virus from all four DENV serotypes and fluorophore-linked serotype-specific mAbs to identify to which DENV serotype(s) Abs from Ab-secreting cells (ASCs) bind. Flipping the configuration of the assay from Ag coated on the plate followed by detection of captured Abs (conventional ELISPOT), to the capture of Abs secreted by B cells on the plate followed by detection of Ag specificity (QCF) enables determination of serotype-specificity on a single-cell basis, rather than only at the cell-population level (Fig. 1A). Specifically, eight serial 1:2 dilutions of ASCs consisting of either EBV-transformed B cells/hybridomas (1 × 10⁷ in the first well) or activated PBMCs from dengue patients or DENV-immune blood-bank donors (1 × 10⁶ stimulated PBMCs) are incubated for 48 h in a sterile IPFL clear 96-well plate that has been activated, bound with anti-human Fc-specific IgG, and blocked. This captures ASC-secreted Abs, after which the cells are removed via washes. The wells are then incubated with purified virions from all four serotypes in equal concentration (1 μg/serotype/well). Spot specificity is determined by the addition of fluorophore-linked serotype-specific detection mAbs. Excess Ab is removed, and spots are visualized on a fluorescent ELISPOT reader.

Several key technical considerations of this assay are as follows. First, the Ab on the plate membrane used to capture the Abs secreted by ASCs is an Fc-specific anti-human IgG that binds the Fc region of the secreted Ab, leaving its V region accessible for binding virus. Second, equal amounts of each Ag (purified DENV) are used to allow binding to the captured Ab. Third, a majority of the Ab response is directed against the surface of the DENV virion—envelope (E) and premembrane, and many human neutralizing Abs bind to quaternary epitopes on the assembled virion.

**FIGURE 2.** Detection of DENV-specific cells from human DENV serotype cross-reactive and DENV2-specific ASCs. (A) D11C is an EBV-transformed human DENV cross-reactive B cell line and was used as the ASC in the QCF. Four filters were used for detection by a CTL Analyzer, each specific to the Qdot conjugated to the serotype-specific mAbs. The merged filters show the serotype-cross-reactivity of D11C. (B) Serotype-cross-reactive ASCs were detected via QCF using the human hybridoma 1G17. The figure shows detection of ASC reactivity to each DENV serotype, and the merged image shows the cross-reactivity of all the ASCs. (C) Serotype-specific ASCs were detected using 2D22, a human DENV2-specific hybridoma. The single filters of each serotype and the merged filters show reactivity only with DENV2.
that are not found on recombinant monomeric E (7, 13, 14). Furthermore, using virions instead of recombinant E increases the brightness of the spots due to amplification (up to 180 E protein epitopes per virion, with occupancy determined by each mAb) (15). DENV was propagated in A. albopictus cells (C6/36) and purified via a discontinuous OptiPrep density gradient (16). SDS-PAGE and Coomassie blue staining confirmed the purity of the DENV serotype preparations (Supplemental Fig. 1), and total protein concentration for each batch of Ag was measured by bicinchoninic acid (BCA) (Fig. 1B). By using equal in-excess protein concentrations of the four DENV virions, the Ag specificity of the ASCs is determined by the affinity and avidity of the Ab secreted without bias toward any DENV serotype.

**Optimization of the DENV QCF**

We initially tried to visualize DENV-specific spots by direct or indirect fluorescent labeling of the captured DENV. Directly labeling the purified DENV with protein-amine binding dyes (e.g., Alexa Fluor) or membrane dyes (e.g., DiO, DiI) was not bright enough for visualization in the QCF (data not shown). Therefore, we screened by ELISA a panel of mAbs to identify those with serotype-specific reactivity. Three murine mAbs, all binding E domain III (anti-DENV1 E95, anti-DENV2 E96, and anti-DENV4 E88) (17–19) and one human E domain I/II–directed mAb (anti-DENV3 5J7) (14, 20), were selected. The epitopes targeted are maturation insensitive; thus, the maturation state of the virion should not affect binding of the detection mAbs (20, 21).

**FIGURE 3.** Analysis of single DENV serotypes or detection mAbs. (A) Captured Abs secreted by D11C were incubated with all four DENV serotypes and detected using each individual serotype-specific mAb separately. (B) Conversely, Abs secreted by D11C were incubated with a single DENV serotype per well and detected with all four serotype-specific mAbs. (C and D) Spots were enumerated and compared with the total number of IgG-secreting cells and the number of spots detected when all four serotypes and four mAbs were used simultaneously. No significant differences were observed, as determined using the Friedman test.
serotype specificity of each Ab was assessed by ELISA and flow
cytometry, confirming no cross-reactivity between the different
DENV serotypes (Fig. 1C). Qdots (22) are tunable nanocrystals
(10–30 nm) for which fluorescent properties can be altered by
size modification. Their bright fluorescence and minimal spectral
overlap made Qdots an attractive candidate for the QCF, so the
two serotype-specific mAbs were conjugated to four distinct
Qdots (Supplemental Fig. 2).

To assess the ability of the QCF to distinguish the ASC serotype
specificity, we first used a human serotype-specific anti-DENV2
hybridoma (mAb 2D22) and human cross-reactive pan-DENV
EBV-transformed B cells or hybridomas (mAbs D11C and
1G17). When incubated with the four DENV virions and all four
erserotype-specific detection mAbs, the QCF distinguished between
serotype-specific and cross-reactive ASCs (Fig. 2). To rule out
steric hindrance or interference among the four detecting mAbs
when used simultaneously, captured Abs secreted by ASCs (pan-
DENV D11C) were incubated with all four DENV serotypes and
detected using each individual serotype-specific detection mAb
separately (Fig. 3A). Conversely, to determine whether addition
of four viruses simultaneously hindered determination of the
specificity of the ASCs, captured Abs were incubated with a single
DENV serotype per well and detected with all four serotype-
specific detection mAbs (Fig. 3B). Testing parallel wells plated
with equivalent numbers of cells, the number of spots detected
with either the four DENV serotypes separately or four individ-
ual detection mAbs was comparable to the total number of IgG-
secreting cells and to the number of spots detected when all four
serotypes and four mAbs were used simultaneously (Fig. 3C, 3D).
Thus, inclusion of all four DENV serotypes and all four serotype-
specific mAbs in a single well did not interfere with the sensitivity
or serotype-specificity of the assay.

Detection of DENV serotype-specific and cross-reactive
responses in stimulated human PBMCs

We first sought to determine optimal stimulation conditions to
activate MBCs into ASCs. PBMCs from six different donors from
the Nicaraguan National Blood Center were stimulated under three
different conditions: 1) CD40L/IL-21/BAFF; b) CD40L/IL-21; and
3) IL-2/R848 for either 3 or 5 d in vitro. Following stimulation, the
total number of IgG-secreting cells was evaluated via FluoroSpot.
Five days of in vitro stimulation with IL-2/R848 consistently
yielded the highest number of IgG-positive ASCs in every donor
(Fig. 4A). We then evaluated the ability of the QCF to determine
DENV-specific responses from DENV-exposed or DENV-naive
blood donors from Nicaragua or California, respectively. PBMCs

FIGURE 4. DENV-specific responses in DENV-exposed and DENV-naive blood bank donors. (A) Optimization of MBC stimulation. PBMCs from
Nicaraguan blood bank donors (n = 6) were activated, and the number of IgG + spots per million activated PBMCs was measured. CD40L feeder cells/IL-21
(100 ng/ml), CD40L feeder cells/IL-21 (100 ng/ml)/BAFF (100 ng/ml), or IL-2 (1000 U/ml)/R848 (2.5 μg/ml) were used for activation. Two-way repeated-
measures ANOVA revealed a significant difference between the number of IgG + ASCs obtained in each condition. Bonferroni posttest determined sig-
nificant pairwise comparisons. ***p < 0.001. PBMCs from DENV-exposed Nicaraguan blood bank donors (NBD #97 and #156) (B and C) and
a DENV-negative blood bank donor from the Oakland Red Cross (OBD #625) (D) were stimulated with IL-2 (1000 U/ml)/R848 (2.5 μg/ml) for 5 d in vitro.
PMBCs were plated in serial 1:2 dilutions starting from 1 × 10^6 cells. Stimulation was confirmed by IgG + detection (data not shown). The QCF result
shows DENV-specific spots in Nicaraguan blood donors (B and C) and no DENV reactivity in the DENV-naive blood donor (D).
from blood bank donors were stimulated for 5 d with IL-2 and R848 and analyzed by QCF. Both serotype-specific and cross-reactive MBCs were observed in the MBC population from DENV-exposed blood donors (Fig. 4B, 4C), whereas no DENV-reactive spots were seen in the DENV-naive control (Fig. 4D).

We next sought to assess whether the QCF could be used to detect MBC and PB/PC responses in primary human PBMCs from DENV-infected individuals during either acute- or convalescent-phase time points in participants in our Hospital-based Dengue Study in Nicaragua (11, 23). PBMCs from a DENV-positive patient (secondary DENV3) were plated directly ex vivo and assessed via QCF to determine the PB/PC response. Cells were serially diluted as above, and DENV-specific ASCs were detected with the four DENV serotypes and four DENV serotype-specific detection mAbs. The Nicaraguan PB/PCs ex vivo were serotype cross-reactive (Fig. 5A). Next, convalescent PBMCs from a patient with primary DENV3 infection, a patient with secondary DENV3 infection, and a DENV-negative blood bank donor from Oakland as a negative control were stimulated in vitro with IL-2/ R848 for 5 d and analyzed via QCF. MBCs from the primary convalescent sample revealed a predominantly serotype-specific response against DENV3 (Fig. 5B), consistent with previous reports (10), whereas the secondary convalescent sample displayed more serotype cross-reactive MBCs (Fig. 5C). The number of B cells specific for one, two, three, or four DENV serotypes was enumerated (Fig. 5). The DENV-negative control displayed no B cell reactivity to DENV (data not shown).

Discussion

We have developed a novel modification of the conventional ELISPOT, the QCF, to detect DENV serotype-specific and cross-reactive B cell responses on a single-cell basis. Using DENV-specific human EBV-transformed B cells, we showed that visualization of ASCs with all four DENV viruses and all four DENV-specific mAbs was as sensitive as detection with a single virus or a single detection mAb. Furthermore, the QCF was able to quantify serotype-specific and cross-reactive single B cells from the PBMCs of DENV-exposed blood donors and DENV-infected patients, in both PB/PCs and MBCs.

The correlates of protection against DENV infection and/or severe disease are not well understood, but are thought to be associated with a strongly neutralizing Ab response. However, infection with one DENV serotype imparts long-term protection against reinfection with the same serotype but not against infection from heterologous serotypes, and secondary infection with a different serotype increases the risk for developing severe disease, thought to be due at least in part to Ab-dependent enhancement (24). Recent studies have provided valuable information about MBC specificity at a B cell population level by conventional ELISPOT (10, 11) and by using human mAbs generated from DENV-exposed individuals (4, 5, 7, 14). Although informative, these studies have not addressed how Ab and MBC serotype-specific or cross-reactive responses develop over time and mature after primary or secondary natural DENV infection on a single-cell basis. It is critical to understand how the MBC response evolves and what constitutes immune correlates of protection in natural DENV infections. Thus, it is imperative to better understand B cell and Ab responses to natural DENV infections, which will in turn facilitate evaluation of vaccine candidates in clinical trials. The QCF technology will enable an in-depth analysis of the generation and evolution of the specificity of B cell responses in primary and secondary DENV infections as well as vaccinees and will permit B cell serotype specificity or cross-reactivity to be evaluated as a potential immune correlate of protection in natural DENV infections and in trials of candidate dengue vaccines.

Finally, the QCF can be adapted to assess other diseases in which identifying the serotype- or subtype-specific B cell response is
paramount. In addition to DENV, other infections such as influenza (25), HIV (26, 27), and severe acute respiratory syndrome-coronavirus (28) have been reported to generate Ag-specific Ab responses that can enhance infection and disease progression. In the case of HIV and influenza, it has been hypothesized that vaccination with the wrong virus could lead to enhanced infection within the vaccinated population (26). Thus, understanding the specificity and cross-reactivity of B cells generated by a vaccination protocol against different viral subpopulations could be vital to predicting vaccination safety and efficacy; the QCF is a tool that could be used to facilitate this analysis.

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Disclosures
The authors have no financial conflicts of interest.

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