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Research paper

Development of quantitative suspension array assays for six immunoglobulin isotypes and subclasses to multiple *Plasmodium falciparum* antigens

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ABSTRACT

Background: Quantitative suspension arrays are powerful immunoassays to measure antibodies against multiple antigens in large numbers of samples in a short time and using few microliters. To identify antigen targets of immunity for vaccine development against complex microbes like *Plasmodium falciparum*, such technology allows the characterization of the magnitude and antigenic specificity of Ig isotypes and subclasses that are important for functional responses. However, standardized assays are not widely available.

Methods: We developed six quantitative suspension array assays to measure IgG1, IgG2, IgG3, IgG4, IgM and IgE specific to multiple *P. falciparum* antigens. Secondary and tertiary antibodies, as well as human purified antibodies for standard curves, were tested among several commercially available sources. Positive and negative controls included plasmas from malaria hyper-immune African adults and from malaria-naïve European adults, respectively. Reagents were selected and optimal antibody and test sample dilutions established according to sensitivity, specificity and performance of the standard curves. The variability between replicates and plates was assessed with 30 test samples and controls.

Results: Assays were able to detect *P. falciparum* antigen-specific antibodies for all isotypes and subclasses in samples from malaria-exposed individuals, with low background signal in blank wells. Levels detected in malaria-naïve individuals were overall low except for IgM. For the IgG2 and IgE assays, a triple sandwich was required for sensitivity. Standard curves with 5-parameter logistic fit were successfully obtained in all assays. The coefficients of variation for measurements performed in different days were all < 30%, and < 5% when comparing duplicates from the same plate.

Conclusion: The isotype/subclass assays developed here were sensitive, specific, reproducible and of adequate quantification dynamic range. They allow performing detailed immuno-profiling to large panels of *P. falciparum* antigens to address naturally- and vaccine-induced Ig responses and elucidate correlates of malaria protection, and could also be applied to other antigenic panels.

1. Introduction

In the assessment of humoral immunity against complex infections such as malaria, still affecting 91 countries with 216 million cases and 445,000 deaths in 2016 (WHO, 2017), it is key to have immunoassays that can reliably measure multiple immunoglobulins (Ig) and antigens in a mid-high throughput miniaturized manner. Antigen and isotype/subclass targets of naturally-acquired immunity (Doolan et al., 2009; Moncunill et al., 2013) need to be identified to characterize mechanisms of protection and find ways to induce them through vaccination.

Most malaria sero-epidemiological and vaccine studies only measure antigen-specific IgG (Rogier et al., 2017; Fouda et al., 2006), since in the 60's it was established that transfer of purified IgG can control *P. falciparum* infection (Cohen et al., 1961). Nevertheless, antigenic targets of protection are unknown, and diverse Ig isotypes and subclasses are generated in response to malaria infection (Tongren et al., 2006; Scopel et al., 2006; Branch et al., 1998; Perlmann et al., 1997). These various isotypes could be differentially elicited by antigens and have different effector functions, some of them being protective while others not (Gunn and Alter, 2016; Chung et al., 2015; Ackerman et al., 2016). It is generally known that IgG1 and IgG3, both considered cytophilic antibodies, are the main subclasses generated

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against *P. falciparum* antigens (Bouharoun-Tayoun and Druilhe, 1992; Bouharoun-Tayoun et al., 1995; Cavanagh et al., 2001), but their relevance and function needs to be better studied. The most accepted mechanism by which IgG1 and IgG3 may protect against *P. falciparum* infection is through their ability to fix complement and mediate opsonic phagocytosis (Boyle et al., 2015; Osier et al., 2014). However, it needs to be better established whether non-cytophilic IgG2 and IgG4 antibodies, despite being present at low levels in exposed individuals, could be induced in detriment of cytophilic subclasses considered as protective, and to what extent their increase could be associated with risk of malaria. Furthermore, the role of IgM and IgE in malaria immunity has been less studied and merits more attention according to recent data associating those responses to protection (Arama et al., 2015) or risk (Rinchai et al., 2015; Calissano et al., 2003; Perlmann et al., 2000), respectively. Therefore, an appropriate understanding of the magnitude and antigenic specificity of each of the Ig isotypes and subclasses is very important for the development of a new generation of effective vaccines.

Traditionally, the measurement of specific antibodies has been done by the enzyme-linked immunosorbent assay (ELISA) (Israelsson et al., 2008; Ahmed Ismail et al., 2014; Medeiros et al., 2013). Although this classical technique has been very useful over the years, it demands significant amount of time, the use of relatively large sample volumes and, importantly, only allows quantifying antibodies against a single antigen at a time. A mid-high throughput multiplex alternative technique is the quantitative suspension array technology (qSAT), particularly suited for complex parasites like *P. falciparum* that is estimated to contain around 5000 proteins, many of which are polymorphic and/or variant, and stage-specific. qSAT has several advantages compared to ELISA already demonstrated in many studies in diverse research areas (Brown et al., 2012; Smits et al., 2012; Perraut et al., 2014; Basile et al., 2013). For example, qSAT allows working with 5 or less microliters of plasma, serum or saliva, and simultaneously quantify up to 500 different proteins/antibodies, peptides, RNA or DNA fragments in a single well. In addition, the qSAT is a very flexible platform that allows different antibody sandwiches, representing a perfect tool to assess the levels of different Ig isotypes and subclasses in large numbers of samples.

In this study we have developed 6 different qSAT assays to measure antigen-specific IgG subclasses (1 to 4), IgM, and IgE using several panels of minimum 6 to 10 *P. falciparum* antigens. For this purpose, several antibody sandwiches were tested to choose the optimal combination for each isotype/subclass assay. In addition, isotype/subclass specific singleplex standard curves were developed to select optimal sample dilutions for data analysis and to calibrate the assay. The variability of the assays between replicates and plates was also evaluated.

2. Material and methods

2.1. Human samples

A plasma pool made of 22 samples from malaria hyper-immune adults from Manhiça, Mozambique (Aranda et al., 2005), was used as positive control. Fourteen individual plasma samples from European adults never exposed to malaria were used as negative controls. Test samples from 30 malaria-exposed individuals, adults and children, collected in the context of different immunological studies (Aguilar et al., 2012; Campo et al., 2011; Nhabomba et al., 2014), were assayed in the setting up and assessment of the different assays.

Written informed consent was obtained from participants before sample collection; in the case of children the informed consent was obtained from parents or guardians.

Approval for the protocols was obtained from the Hospital Clínic of Barcelona Ethics Review Committee and the National Mozambican Ethics Review Committee.

2.2. P. falciparum recombinant antigens

A primary multiplex panel including 10 recombinant proteins with a broad range of immunogenicities was initially established to set up the IgG_{1-4} and IgM assays using the Luminex xMAP™ technology (Luminex Corp., Austin, Texas) (Campo et al., 2011). The antigens were selected based on their important role as candidate vaccines, and for being representative of the different phases of the parasite life cycle. The panel included 4 pre-erythrocytic antigens: cell-traversal protein for ookinetes and sporozoites (CelTOS) (Kariu et al., 2006), liver-stage antigen 1 (LSA-1) (Hillier et al., 2005), sporozoite surface protein 2 (SSP2, also known as TRAP) (Rogers et al., 1992) and circumsporozoite surface protein (CSP) (Plassmeyer et al., 2009); and 6 erythrocytic antigens: apical membrane antigen 1 (AMA-1) from 3D7 and FVO strains (Kocken et al., 2002; Reddy et al., 2015; Dutta et al., 2007), merozoite surface protein 1 ($MSP\text{-}1_{42}$) from 3D7 and FVO strains (Angov et al., 2003; Angov et al., 2008), fragment II of region II of the 175 kDa erythrocyte binding protein (EBA-175 or PfF2) (Pandey et al., 2002), and Duffy binding-like alpha (DBL-α) domain of *Pf*EMP-1 (Mayor et al., 2009). *P. falciparum* AMA-1 and MSP-1 are polymorphic proteins, and the two most studied strains are 3D7 and FVO. Antigens based on primary sequences from both strains have been developed as vaccine candidates because of the strain-specific nature of antibody responses to many malarial antigens. Experimental vaccines based on only one genotype of these proteins have been tested in field trials showing different degree of protection depending on the circulating strain (Laurens et al., 2013; Otsyula et al., 2013). As antibody responses to polymorphic proteins may vary in different populations, we included AMA-1 and $MSP\text{-}1_{42}$ from both strains in the panel to have a broader repertoire and check whether they elicited different IgM and IgG subclass responses. Bovine serine albumin (BSA)-coupled beads were also included in the multiplex for background determination. The pre-erythrocytic antigens were expressed in *Pichia Pastoris* and provided by Protein Potential, LLC (Rockville, Maryland, USA). AMA-1 3D7, EBA-175 and DBL-α were provided by the International Centre for Genetic Engineering and Biotechnology (ICGEB, India). AMA-1 FVO and $MSP\text{-}1_{42}$ 3D7 and FVO were provided by the Walter Reed Army Institute of Research (WRAIR, USA). The BSA was purchased to Sigma-Aldrich.

A secondary panel was used to set up an antigen-specific IgE assay, including those antigens showing some IgE reactivity in previous tests using a positive pool (data not shown). The antigens included in the IgE panel were: the Exported Protein 1 (EXP-1, Protein Potential), Merozoite Surface Protein 3 (MSP-3 3D7, ICGEB), Merozoite Surface Protein 2 type CH150 (MSP-2 CH150, Edinburgh University, UK), CSP full length (Protein Potential), NANP repeat region (NANP, WRAIR) and C-term region (C-term, WRAIR).

2.3. Coupling of recombinant antigens to microspheres

MAGPLEX 6.5 µm COOH-microspheres were purchased from Luminex Corporation (Austin, TX). The beads stock was gently resuspended on a rotary shaker for 30 min, followed by soft vortexing for 1 min and sonicated for 30 s. The amount of beads to be coupled to each antigen was calculated assuming the use of 1000 beads per region and per sample, and a maximum of $2.5 \cdot 10^6$ beads in 250 µL reaction volume. The beads were washed twice with 250 µL of distilled water in a concentration of 10,000 beads/µL by short vortex and sonication for 20 s, and using a magnetic separator (Life Technologies, 12321d). Next, the beads were resuspended in 80 µL of activation buffer, 100 mM monobasic sodium phosphate (Sigma, S2554), pH 6.2 by vortex and sonication for 20 s. To activate the beads for cross-linking to proteins, 10 µL of 50 mg/mL sulfo-*N*-hydroxysulfosuccinimide (Thermo Fisher Scientific, 24,525) and 10 µL of 50 mg/mL 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimidehydrochloride (Thermo Fisher Scientific, 22,981) were simultaneously added to the reaction tubes, mixed gently by vortexing and incubated for 20 min, at room temperature (RT), in a