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Research paper

Optimization and qualification of a multiplex bead array to assess cytokine and chemokine production by vaccine-specific cells

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ABSTRACT

The magnitude and functional phenotype (e.g. proliferation, immune stimulation) of vaccine-induced T-cell responses are likely to be critical in defining responses that can control pathogenic challenge. Current multi-parameter flow cytometric techniques may not be sufficient to measure all of these different functions, since characterizing T-cell responses by flow cytometry is presently limited to concurrent measurement of at most 10 cytokines/chemokines. Here, we describe extensive studies conducted using standardized GCLP procedures to optimize and qualitatively/quantitatively qualify a multiplex bead array (MBA) performed on supernatant collected from stimulated peripheral blood mononuclear cells (PBMC) to assess 12 cytokines and chemokines of interest. Our optimized MBA shows good precision (intra-assay, inter-day, inter-technician; coefficients of variation <30%) and linearity for most of the analytes studied. We also developed positivity criteria that allow us to define a response as positive or negative with a high degree of confidence. In conclusion, we provide a detailed description of the qualification of an MBA, which permits quantitative and qualitative evaluation of vaccine-induced immunogenicity and analysis of immune correlates of protection. This assay provides an excellent complement to the existing repertoire of assays for assessing immunogenicity in HIV vaccine clinical trials.

1. Introduction

Vaccines offer the most effective and durable intervention for infectious diseases, and vigorous efforts are underway to develop vaccines for the major global health threats, including HIV, malaria and tuberculosis. T-cell responses are multifaceted and often include the simultaneous production of multiple cytokines/chemokines, which indicate varying capacities for specific immune functions such as proliferation, immune stimulation, and cytotoxic potential. The magnitude and functional phenotype of the vaccine-induced T-cell responses are likely to be critical in defining responses that can control pathogenic challenge. Current multi-parameter flow cytometric techniques may not be sufficient to measure all of these different functions, since characterizing cellular responses by standardized flow cytometric methods is presently limited to concurrent measurement of at most ten intracellular cytokines/chemokines and

Abbreviations: 5PL, Five parameter log-logistic curve; HVTN, HIV Vaccine Trials Network; ICS, intracellular cytokine staining; LLOD, lower limit of detection; LOD, limit of detection; LOQ, limit of quantitation;MBA, multiplex bead array; PTE, potential T-cell epitope; SOP, standard operating procedure; ULOD, upper limit of detection; GCLP, good clinical laboratory practices; MFI, median fluorescence intensity.

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selected functional markers (Horton et al., 2007; McElrath et al., 2008). An alternate approach to functional characterization is to measure concentrations of cytokines secreted in supernatant of peripheral blood mononuclear cells (PBMC) or other cell sources following ex vivo stimulation. Traditionally these can be measured by enzyme-linked immunosorbent assays (ELISAs), but relatively few cytokines can be measured conveniently since separate ELISAs are required for each cytokine.

Recently, a number of new technologies have been developed or improved that allow simultaneous measurement of multiple cytokines and a commonly-used format is the multiplex bead array (MBA) assay. The ability to measure a broad array of cytokine/chemokines using small sample volumes has allowed new insight into disease pathogenesis (Lanteri et al., 2009; Stacey et al., 2009; Pine et al., 2011). Although the precision and reproducibility of the MBA assay have been examined in several studies, the suitability of such an assay for assessment of vaccine-induced T-cell immunogenicity performed under good clinical lab practices (GCLP) and over a period of time rather than in a single batch is not documented in these studies.

In a study of multianalyte bead-based (Luminex) kits, World Health Organization (WHO) cytokine standards were assayed at the same expected concentrations as the standards provided with each kit, but WHO and kit standards often yielded very different absolute concentrations (Nechansky et al., 2008). In addition, multiple studies have compared regular-sensitivity multiplex assays with each other (Khan et al., 2004; Kofoed et al., 2006; Djoba Siawaya et al., 2008) or with ELISAs (Liu et al., 2005; Richens et al., 2010). These comparison studies have shown variable agreement among assays and have indicated that absolute cytokine concentrations differ across testing platforms.

In context of the increasing use of multiplexed kits in biomedical research and their potential application for surrogate markers in clinical trials, the results of these studies indicate that such data have to be interpreted with caution. We sought to characterize the performance of the MBA assay when performed using standardized GCLP procedures in order to minimize the variability of data generated. Thus, we performed qualification experiments for an MBA assay that simultaneously measures up to 42 secreted factors. While this assay is more typically used to directly measure cytokine/chemokine concentrations in serum and plasma, we applied this technology to measure analyte concentrations in supernatant collected from ex vivo antigen-stimulated PBMC.

By using a multiplex bead array to explore cytokine/chemokine responses, we can concurrently measure factors linked to a variety of immune functions, such as Th1, Th2, pro-inflammatory, regulatory, and chemotactic responses. Since the correlates of protection against HIV-1 infection/progression are not fully known, and since subtle differences in HIV-1 specific T cells may have profound effects on the capacity to prevent infection or to control subsequent HIV-1 disease course if infected, this information may provide useful insight in characterizing cellular responses in future vaccine trials. These data may influence the advancement of vaccines focused on inducing cellular responses to phase III efficacy trials and thus require characterization of validation parameters typically assessed when analytical assays are validated or qualified. These eight parameters are detailed by the International Conference on Harmonization and the US Food and Drug Administration (ICH, 1996; FDA, 2001) and include: (1) specificity/selectivity, (2) accuracy, (3) precision, (4) detection limit, (5) quantitation limit, (6) linearity, (7) range and (8) robustness. Here we describe the optimization and qualification of the stimulated-PBMC multiplex bead array assay designed to allow qualitative and quantitative evaluation of vaccine-induced responses. We also show an example of use of the assay to measure immunogenicity in a candidate HIV vaccine trial.

2. Material and methods

2.1. Study participants

Optimization and qualification experiments were performed on supernatants collected from ex vivo antigen-stimulated PBMC. PBMC used for the *optimization* of the multiplex bead array were collected by leukapheresis from three individuals enrolled in the HVTN 068 clinical trial (De Rosa et al., 2011), at a time point approximately 1 year after first immunization. These individuals received two doses of a recombinant Ad5-vectored vaccine encoding HIV-specific antigens. PBMC used for the *quantitative qualification* (precision, linearity, accuracy) experiments were collected by leukapheresis from HIV-seronegative individuals with a known T-cell response to CMV from the Seattle Assay Control (SAC) cohort, thereby providing sufficient cryopreserved PBMC from a single time point for all qualification studies. For *qualitative qualification* (specificity) experiments, PBMC were collected from thirty individuals (20 vaccine and 10 placebo recipients) randomly selected from participants enrolled in the RV144 clinical trial conducted in Thailand (Rerks-Ngarm et al., 2009) at baseline and at peak immunogenicity (week 26, 2 weeks following final immunization). The relevant Institutional Review Boards for each study approved the protocols, and prior to enrollment all volunteers provided written consent after being informed of the nature and possible consequences of the studies.

2.2. PBMC sample processing

PBMC were isolated and cryopreserved either from whole blood or from a leukapheresis product within 8 h of venipuncture using standard procedures as previously described (Bull et al., 2007). For assay use, PBMC were thawed and rested overnight at 37 °C/5% CO_2 in R10 [RPMI 1640 (GibcoBRL, Carlsbad, CA) containing 10% FCS (Gemini Bioproducts, West Sacramento, CA), 2 mM L-glutamine (GibcoBRL), 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate] prior to stimulation. A minimum cell viability of 66% measured after overnight resting on the day following the thaw was required for use.

2.3. PBMC stimulations

PBMC were stimulated to assess ex vivo responses to: (1) a pool of CMV 15-mer peptides overlapping by 11 amino acids spanning the entire pp65 protein; or (2) pools of HIV-1 15-mer peptides (synthesized by Biosynthesis, Lewisville, TX) overlapping by 11 amino acids spanning Env (clade AE 92TH023) or Gag (clade B LAI, based on the Env and Gag protein sequences encoded by the RV144 ALVAC-HIV [vCP1521] vaccine). All peptide pools were used at a final concentration