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Research paper

Single Molecule Arrays for ultra-sensitive detection of rat cytokines in serum

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ABSTRACT

Rats are used as animal models for many human diseases. Cytokines can serve as biomarkers indicative of these diseases or disease states. Techniques for measuring cytokine expression levels often do not provide the sensitivity needed to measure these biomarkers in biological fluids because the concentrations of many cytokines are below the detection limits of conventional methods. In this paper, we present ultra-sensitive digital immunoassays using Single Molecule Arrays (Simoa) for seven rat cytokines: TNF-α, IL-10, IL-17F, GM-CSF, IFN-γ, IL-4, and IL-1α. These ultra-sensitive immunoassays have limits of detection (LODs) in the femtomolar range and provide the ability to measure rat cytokines in serum below the LODs of conventional immunoassays. We also measured these cytokines in healthy rat serum to obtain baseline levels. The ability to measure cytokines present at low concentrations in rat serum will facilitate future studies of disease using rats as animal models.

1. Introduction

Cytokines are cell signaling proteins that mediate the immune system's response to infection and inflammation (Borish and Steinke, 2003). Many cytokines are secreted into the circulation and can be measured in various biological fluids including blood, saliva, and urine. Changes in cytokine levels in these biological fluids have been used to identify physiological or pathological states. Thus, cytokines can be highly informative biomarkers for monitoring disease diagnosis, prognosis, progression, and response to therapy. Different patterns of cytokine expression have been previously implicated in various diseases such as cancers (Lin and Karin, 2007; Lippitz, 2013), infectious diseases (Turner et al., 2014), and autoimmune diseases (Chun et al., 2007).

Cytokine levels in various biological fluids have been extensively studied in animal models of disease. Rat models in particular have been used to study cytokine levels in diseases such as brain injury (Woodroofe et al., 1991) cardiac disease (Deten et al., 2002), and cancer (Carbó et al., 2000). In order to evaluate the significance of cytokine modulation in pathological conditions, it is necessary to establish a physiological range of these proteins in healthy animals.

The gold standard method for measuring cytokines in biological fluids, and particularly serum, is the enzyme linked immunosorbent assay (ELISA). However, due to their naturally low abundance, multiple cytokines in healthy samples are below the detection limit of ELISA (Anderson, 2010). In many cases, cytokines can only be measured when there is a robust physiological response and their concentrations reach a measureable level, for instance when a disease has progressed to an acute stage. Consequently, a more sensitive approach is required to enable detection and precise quantification of cytokines in biological fluids.

In this paper, we employ a digital immunoassay approach utilizing Single Molecule Arrays (Simoa) to overcome the detection limit barrier (Rissin et al., 2010; Rissin et al., 2008; Rissin et al., 2011). This method is a bead-based sandwich ELISA in which single protein molecules are first captured on beads and then detected inside femtoliter-sized wells. More specifically, antibodies that recognize a specific protein target are first conjugated to paramagnetic beads. A large excess of these capture beads is then added to a sample containing low copy numbers of the target analyte, ensuring that there is either zero or one target protein molecule bound per bead. A second biotinylated detection antibody binds to the captured target protein molecule, and the immunocomplex is then labeled with an enzyme conjugate, streptavidin-β-galactosidase (SβG), to form an enzyme-labeled immunocomplex. The enzyme-labeled beads are then resuspended in a fluorogenic substrate solution and loaded onto an array of femtoliter-sized wells in which each well is able to hold only one bead. The wells are sealed with oil and the fluorescent product of the enzyme-substrate reaction is confined to a 50 fL volume, thereby producing a high local concentration that is easily detected by a charge coupled device (CCD) camera. The dynamic range can be expanded beyond the digital range by using the average fluorescence intensity of the active beads for higher target concentrations. For both the digital and analog ranges, the average number of enzyme molecules per bead (AEB) is used as the unit of measurement. Simoa immunoassays typically have detection limits in the low

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femtomolar range and a dynamic range that spans four orders of magnitude. Simoa immunoassays have been previously developed to measure human cytokines in serum and plasma (Song et al., 2011; Rivnak et al., 2015; Wu et al., 2015).

Here we present seven ultra-sensitive Simoa immunoassays for seven rat cytokines: TNF-α, IL-10, IL-17F, GM-CSF, IFN-γ, IL-4, and IL-1α. The LODs of these assays are between 1.0 fM and 71.8 fM, which are considerably lower than the LODs of conventional ELISAs. Using our ultra-sensitive Simoa immunoassays, we measured and established baseline concentrations of these cytokines in healthy rat serum samples.

2. Methods

2.1. Preparation of antibody coated capture beads

Capture antibodies were reconstituted and stored according to the instructions provided by the manufacturer. Antibody catalog numbers are provided in Table S1. The antibody was buffer exchanged to remove the storage buffer by first adding 0.13 mg of antibody solution to an Amicon filter (50 K, EMD Millipore). Bead Conjugation Buffer (Quanterix) was then added to the filter up to a total volume of 500 μL. The filter device was centrifuged at 14,000 × *g* for 5 min. The effluent was discarded and the process was repeated twice. The filter was inverted into a new tube and centrifuged at 1000 × *g* for 2 min. The filter was rinsed with 50 μL of Bead Conjugation Buffer and centrifuged at 1000 × *g* for 2 min. The concentration of the antibody was measured using a NanoDrop 2000 spectrophotometer. The antibody was diluted to 0.5 mg/mL in Bead Conjugation Buffer and stored on ice until ready for use. 2.8×10^8 carboxylated, 2.7 μm, paramagnetic beads (Quanterix) were transferred into a microtube and washed three times with 200 μL of Bead Wash Buffer (Quanterix). The beads were then washed two times with 200 μL of Bead Conjugation Buffer and re-suspended in 190 μL of Bead Conjugation Buffer. Fresh 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (ThermoFisher) was reconstituted in 1 mL of Bead Conjugation Buffer just prior to use. To activate the beads, 10 μL of EDC were added to the bead suspension to give a final concentration of 0.5 mg/mL and a final volume of 200 μL. The beads were then placed on a rotator (HulaMixer, ThermoFisher) for 30 min. The activated beads were then washed with 200 μL of Bead Conjugation Buffer. 200 μL of capture antibody solution was then added to the beads, vortexed, and placed on the rotator for 120 min for conjugation. The antibody-conjugated beads were then washed two times with 200 μL of Bead Wash Buffer. The beads were then blocked with 200 μL of Bead Blocking Buffer (Quanterix) and placed on the rotator for 30 min. The beads were washed with 200 μL of Bead Wash Buffer, washed with 200 μL of Bead Diluent (Quanterix), and re-suspended in 200 μL of Bead Diluent. The beads were counted using a Beckman Coulter multi-sizer and stored at 4 °C for up to 6 months.

2.2. Rat serum samples

20 individual male and 20 individual female Wistar Hannover rat (5–8 weeks) serum samples were purchased from BioreclamationIVT. Samples were aliquoted by the vendor and stored at − 80 °C. Samples did not undergo more than one freeze-thaw cycle. Samples were diluted in Q-block buffer (1X PBS (Sigma), 25% Newborn Calf Serum (ThermoFisher), 0.01% Tween 20 (Sigma), 5 mM EDTA (Sigma), 0.15% Proclin 300 (Sigma)).

2.3. Preparation of reagents and assay setup for Simoa assay

Antibody-coated capture beads were diluted in ELISA buffer (2.66X PBS, 2.66% Tween 20, 13.3 mM EDTA, 4% Proclin 300) to a concentration of 26,000 beads/μL. Biotinylated detector antibodies were diluted in Detector Diluent (Quanterix) to the desired concentration. Detector antibody concentrations are provided in Table S2. Streptavidin-β-galactosidase (SβG) Concentrate (Quanterix) was diluted to 150 pM in SβG Diluent (Quanterix). Recombinant protein standards (R & D Systems) were serially diluted to desired concentrations in Q-block buffer. The reagents including beads, detector, and SβG were placed in plastic bottles (Quanterix). The samples, including calibrators and serum samples, were loaded onto a 96-well plate (Quanterix). All reagents (capture beads, detector antibodies, SβG, enzyme substrate Resorufin β-D-galactopyranoside (RGP), Wash Buffer 1, Wash Buffer 2, and Simoa Sealing Oil) were purchased from Quanterix and loaded onto the Simoa HD-1 Analyzer (Quanterix) based on the manufacturer's instructions. Either three or two step assay configurations were chosen. Additional information on assay configuration and incubation times for each cytokine assay is provided in Table S2. In a three-step assay configuration, 25 μL of bead solution and 100 μL of sample were pipetted into a reaction cuvette and allowed to incubate. The beads were then pelleted with a magnet and the supernatant was removed. Following several washes, 100 uL of detector antibody was added and incubated. The beads were then pelleted with a magnet and the supernatant was removed. Following a series of washes, 100 μL of SβG was added and incubated. The beads were washed, re-suspended in RGP solution, and loaded onto the array. In a two-step assay configuration, 25 μL of bead solution, 100 μL of sample, and 20 μL of detector antibody were pipetted into a reaction cuvette and allowed to incubate. The beads were then pelleted with a magnet, the supernatant was removed. Following several washes, 100 μL of SβG was added and incubated. The beads were washed, re-suspended in RGP solution, and loaded onto the array. The array was then sealed with oil and imaged. Images of the arrays were analyzed and AEB (average enzyme per bead) values were calculated by the software in the HD-1 Analyzer. Detailed information on the Simoa HD-1 Analyzer has been previously reported (Wilson et al., 2015).

2.4. Data analysis

Rat serum samples along with calibration curves were measured using the Simoa HD-1 Analyzer. The calibration curves were fit using a 4PL fit with $1/y^2$ weighting factor. The calibration curves were used to determine concentrations of the unknown rat serum samples. This analysis was done automatically using the software provided by Quanterix with the Simoa HD-1 Analyzer. All measurements were performed in triplicate. The limit of detection (LOD) of each assay was calculated as three standard deviations (SDs) above the background.

3. Results and discussion

3.1. Ultra-sensitive immunoassay development

Ultra-sensitive immunoassays were developed for seven rat cytokines: TNF-α, IL-10, IL-17F, GM-CSF, IFN-γ, IL-4, and IL-1α. The assay development process entailed screening and selecting antibody pairs and optimizing assay conditions such as labeling reagent concentrations and incubation times. Each assay was evaluated by spiking recombinant protein into buffer consisting of 25% newborn calf serum. Calibration curves for these seven rat cytokine assays are shown in Fig. 1A. Error bars represent the standard deviation for triplicate measurements. As demonstrated in detail in Fig. 1B, coefficients of variation (CVs) for most of the calibrators are less than 20%, indicating good assay precision. Background signals of these assays range from 0.003 to 0.03 AEB due to different levels of non-specific interactions of labeling reagents with the beads. Some assays with similar background levels have higher signals at a given protein concentration, which is due to higher capturing and labeling efficiencies of the antibodies. For example, the GM-CSF and IL-10 Simoa assays have similar background levels, but the signal at the highest measured concentration is higher in the GM-CSF assay compared to the IL-10 assay. As previously shown, antibody-

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