Research paper

Development of a custom pentaplex sandwich immunoassay using Protein-G coupled beads for the Luminex® xMAP® platform

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ABSTRACT

Multiplex bead-based assays have many advantages over ELISA, particularly for the analyses of large quantities of samples and/or precious samples of limited volume. Although many commercial arrays covering multitudes of biologically significant analytes are available, occasionally the development of custom arrays is necessary. Here, the development of a custom pentaplex sandwich immunoassay using Protein G-coupled beads, for analysis using the Luminex® xMAP® platform, is described. This array was required for the measurement of candidate biomarkers of vaccine safety in small volumes of mouse sera. Optimisation of this assay required a stepwise approach: testing cross-reactivity of the antibody pairs, the development of an in-house serum diluent buffer as well as heat-inactivation of serum samples to prevent interference from matrix effects. We then demonstrate the use of this array to analyse inflammatory mediators in mouse serum after immunisation. The work described here exemplifies how Protein G-coupled beads offer a flexible and robust approach to develop custom multiplex immunoassays, which can be applied to a range of analytes from multiple species.

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1. Introduction

Vaccines are of global importance to public health because they are a cheap, safe and efficient means of combating infectious diseases, cancer and chronic diseases (Nossal, 2011). In order to accelerate the development and introduction of safer and more effective new vaccines, novel approaches to identify and predict adverse reactions to experimental vaccines early in the development cycle are essential (Kaufmann et al., 2014; Rappuoli et al., 2014). This will require the continued cooperation between the pharmaceutical, academic and regulatory sectors. BioVacSafe (Biomarkers for enhanced Vaccines immunoSafety) is an Innovative Medicine initiative (IMI)-funded consortium consisting of 19 partner organisations from leading European industrial, regulatory and academic institutions (www.biovacsafe.eu) (Lewis and Lythgoe, 2015). This 5-year project aims to identify biomarkers that will speed up, improve as well as reduce the cost of testing and monitoring vaccine safety, both before and after release to the market. As part of this project, many human and animal studies are being carried out and are generating a vast number of samples for analyses. xMAP® Technology is being employed as a high throughput approach to screen these samples for multiple potential biomarkers simultaneously, especially using small sample volumes.

The Luminex® xMAP® Technology (Houser, 2012; Vignali, 2000) is based on microscopic (5.6 or 6.5 µm) beads, known as microspheres, which are filled with different ratios of two or three dyes resulting in 500 unique fluorescent profiles or bead regions for the development of up to 500-plex assays. Analytes bound to the beads via "capture" molecules (e.g., antibodies, antigens, oligonucleotides, enzyme substrates, receptors, etc.) are detected by the reporter molecule phycoerythrin (PE) conjugated to streptavidin or a secondary or "detection" molecule (e.g., antibody). Multiplexing (i.e. simultaneous detection of many analytes in the same sample) is possible because one capture molecule to a specific analyte is attached to a specific bead region (i.e. a set of beads with the same fluorescent profile). Luminex® analyser instruments are essentially two laser flow cytometers: the first laser allows identification of the bead region by excitation of the bead dyes, whilst the second enables quantification of the analyte bound to the bead by excitation of the PE reporter molecule (Houser, 2012; Vignali, 2000). Analyte concentration is determined by measuring the median fluorescence intensity (MFI) of the reporter dye and interpolating from the standard curve.

It is not always possible to obtain a commercial xMAP® array that can detect all analytes of interest, and the use of multiple ELISAs is not a viable alternative for large numbers of small volume samples. Consequently, development of home-brew xMAP® assays is necessary. MagPlex® Microspheres are superparamagnetic beads with a surface

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Table 1

Catalogue numbers and stock concentrations of R&D systems DuoSet ELISA reagents utilised, as well as final concentrations of the capture antibodies used to coat the Protein G-coupled beads.

Mouse analyte	Cat. No.	Top standard (ng/ml)	Capture antibody (µg/ml)	Detection antibody (µg/ml)	Capture antibody coating concentration (µg/ml)
IL-2Ra	DY2438	0.5	144	108	2.88
TREM-1	DY1187	4	144	9	2.88
IL-1Ra	DY480	10	144	72	2.88
PTX3	DY2166	14	720	18	14.4
IP-10	DY466	4	360	108	7.2
CRP	DY1829	1.5	360	72	7.2
sTNFRII	DY4260	0.5	360	72	7.2

containing 100 million carboxyl groups on each bead, which facilitate covalent attachment of "capture" molecules during a two-step carbodiimide reaction (Staros et al., 1986). This chemistry involves activation of the carboxyl groups with the aid of EDC and sulfo-NHS to form amine reactive sulfo-NHS-ester intermediates. All proteins contain primary amine groups that readily form covalent amide bonds with activated carboxylated beads in the correct pH/ionic strength conditions. The most popular use of carboxylated xMAP® beads is as antibody-coupled reagents for use in sandwich immunoassays. However, the orientation of the coupled capture antibody is random (de Jager and Rijkers, 2006), and antibody immobilisation may mask antigen recognition epitopes (Schwenk et al., 2007). Consequently, only a fraction of the coupled antibodies will have the capacity to bind ligands.

In this study, Protein G-coupled MagPlex® Microspheres were generated as a universal reagent for the development of custom xMAP® arrays. Protein G is a cell wall protein of group G streptococci that binds immunoglobulins via Fc regions (Bjorck and Kronvall, 1984; Reis et al., 1984). Aside from ensuring the correct orientation of capture antibodies, the major advantage of coupling Protein G to xMAP® beads is the ease of producing "mix and match" bead regions for multiplex assays without further coupling reactions. Here, we describe the use of ELISA antibody pairs and Protein G-coupled beads for the development of a multiplex sandwich immunoassay for simultaneous quantification of interleukin 2 receptor alpha (IL-2Ra), IFN gamma inducible protein 10 (IP-10; also known as CXCL10), C-reactive protein (CRP), interleukin 1 receptor antagonist (IL-1Ra) and soluble Tumour Necrosis Factor Receptor II (sTNFRII) in small volumes of mouse sera. In determining optimal procedures for the multiplex assay, antibody cross-reactivity against Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) and Pentraxin 3

(PTX3) were identified, thereby ruling out their inclusion in the array. The analytes of interest are potential biomarkers of vaccine safety being investigated by the IMI-JU project BioVacSafe.

2. Materials and methods

2.1. Reagents, buffers and instruments

Bead coupling reactions were carried out using recombinant Protein G (AbD Serotec; 740601L), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; ThermoFisher Scientific) and N-hydroxysulfosuccinimide (Sulfo-NHS; ThermoFisher Scientific). Handheld tube (Life Technologies or Qiagen) or plate (Merck Millipore) magnetic separators were used when washing magnetic beads. Luminex analyses were carried out using a Bio-Plex®/LiquiChip 100 (Bio-Rad or Qiagen) instrument.

R&D systems Duoset® ELISA kits containing standards, capture antibodies and biotinylated detection antibodies (Table 1) were utilised. Also employed were biotinylated rabbit anti-Protein G (Abcam; ab7251), Streptavidin-PE (eBioscience; 12-4317-87, BD Pharmingen; 554,061, or Biolegend; 405245) as well as PE-conjugated species-specific secondary antibodies: anti-hamster IgG (R&D Systems; F0120), anti-goat IgG (R&D Systems; F0107) and anti-rat IgG (R&D Systems; F0105B). Commercial buffers utilised were General serum diluent (ImmunoChemistry Technologies; 647), Plasma sample diluent (ImmunoChemistry Technologies; 694) and Neptune diluent (ImmunoChemistry Technologies; 6124). The following buffers were also prepared: activation buffer [0.1 M Monobasic Sodium Phosphate, pH 6.2], PBS [pH 7.4; containing 138 mM NaCl, 2.7 mM KCl], PBS-TBN# [PBS, 0.1% IgG-free BSA (Jackson ImmunoResearch), 0.02% Tween-20, 0.05% Azide], PBS-BN# [PBS, 1% IgG-free BSA, 0.05% Azide], PBS-BSA [PBS, 1% BSA] and J Buffer [PBS, 20 mM Tris–HCl, 1% Goat Serum, 1% Sheep Serum, 0.05% Tween-20]. In addition, bovine serum albumin (BSA), foetal bovine serum (FBS; Gibco), normal mouse serum, non-fat dried milk powder (Panreac Applichem) and Tween-20 were employed as blocking agents in PBS, where appropriate. All buffers were filter-sterilised and stored at 4 °C. Unless otherwise stated, buffer reagents were obtained from Sigma Aldrich.

2.2. Coupling Protein G to MagPlex® microspheres

Protein G used in the coupling reactions was free of sodium azide, BSA, glycine, Tris and amine-containing additives. Prior to use, all buffers were brought to room temperature, EDC and Sulfo-NHS were desiccated at room temperature for approximately 1 h, and
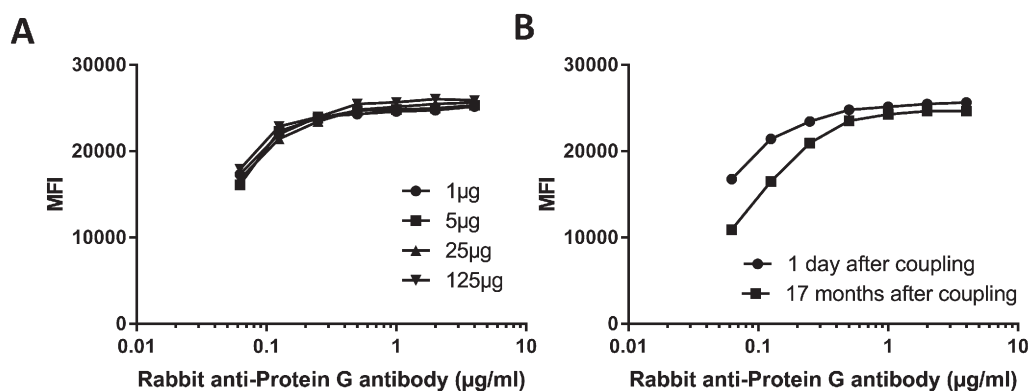

Fig. 1. Coupling and stability of Protein G on xMAP® beads. (A) MagPlex® Microspheres were conjugated to varying amounts of Protein G, ranging from 1 to 125 µg per 1.25×10^6 beads, in a carbodiimide reaction. Coupling was confirmed by labelling with dilutions of PE-conjugated rabbit anti-Protein G antibody. Optimal coupling is achieved when the curve saturates at ≥10,000 MFI. (B) The stability of the Protein G-coupled beads was determined by comparing coupling confirmations carried out 1 day or 17 months post coupling.

MagPlex® Microspheres (Bio-Plex Pro™ Magnetic COOH Beads; Bio-Rad; MC100XX) were vortexed for 30 s then sonicated for 15 s. For a 1× scale coupling reaction, 1.25×10^6 monodisperse carboxylated beads were washed and resuspended in 80 µl of activation buffer. USA

Scientific co-polymer microcentrifuge tubes (Cat No. 1415–2500) were used to prevent loss of beads resulting from sticking to the walls of microcentrifuge tubes. Sulfo-NHS then EDC (50 mg/ml in activation buffer; 10 µl of each) was added to the beads, followed by 20 min
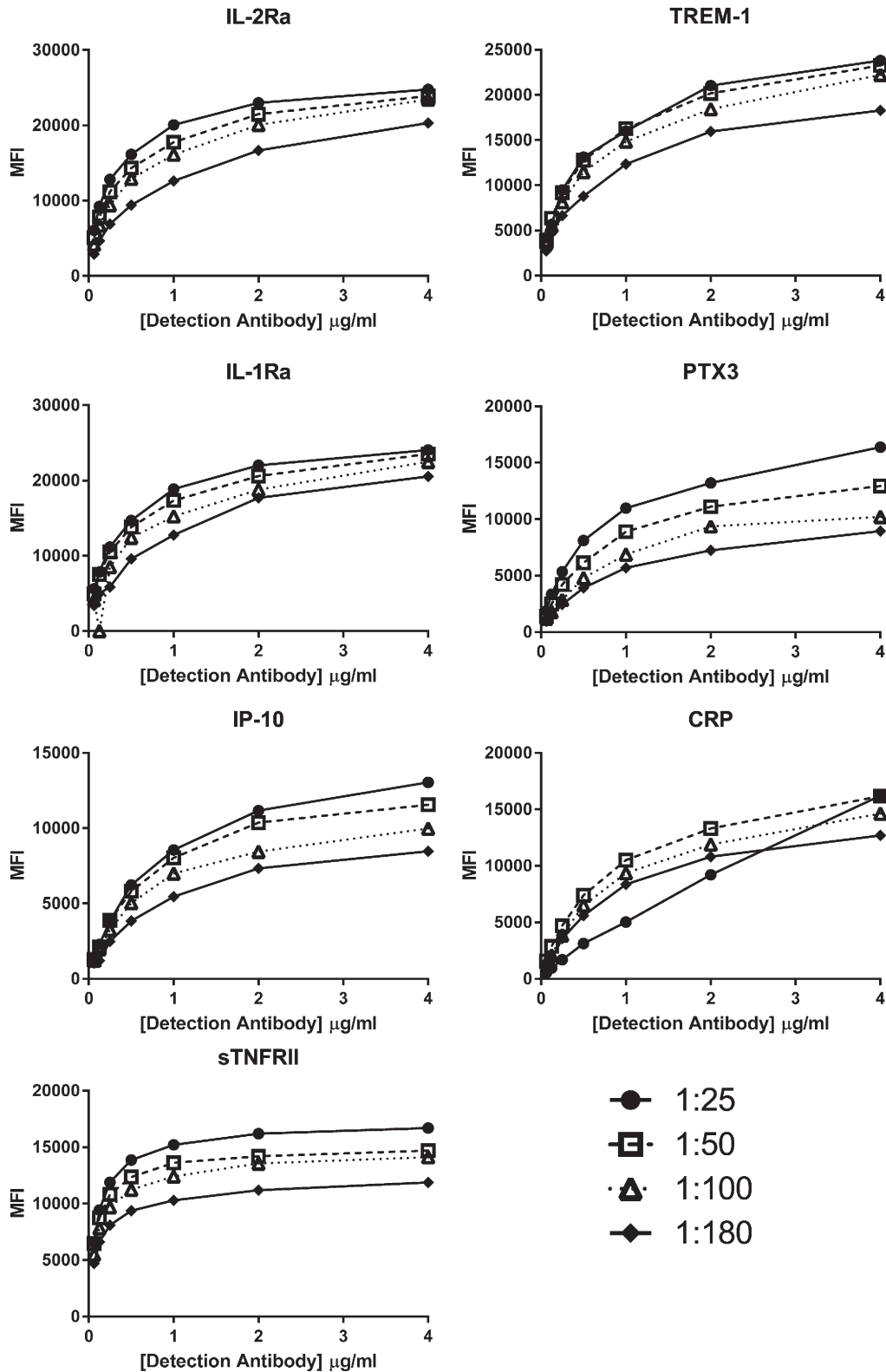

Fig. 2. Optimal capture antibody coating of Protein G-coupled beads. To determine optimal coating amounts for the proposed array, different Protein G-coupled bead regions were each incubated with varying dilutions (ranging from 0.8 to 14.4 µg/ml; refer to Table 1) of 7 different capture antibodies. These were detected by varying concentrations of PE-conjugated species-specific antibodies in singleplex xMAP® assays. Optimal capture antibody coating generates a curve that saturates above 10,000 MFI. A 1:50 dilution (ranging from 1.44 to 7.2 µg per 2.5×10^5 beads) was deemed to be suitable for all capture antibodies investigated.

incubation. Activated beads were washed twice using 150 μl PBS, and then coupled with 1 to 125 μg Protein G in 500 μl PBS for 2 h. Coupled beads were washed with 500 μl PBS then blocked in 250 μl PBS-TBN# for 30 min. Beads were washed and stored in PBS-BN# at 4 °C in the dark. The bead concentration was determined using an Invitrogen Countess® Automated Cell Counter. All incubations were carried out in the dark at room temperature with continuous mixing. Washing steps involved 1 min recovery of beads using a magnetic separator.
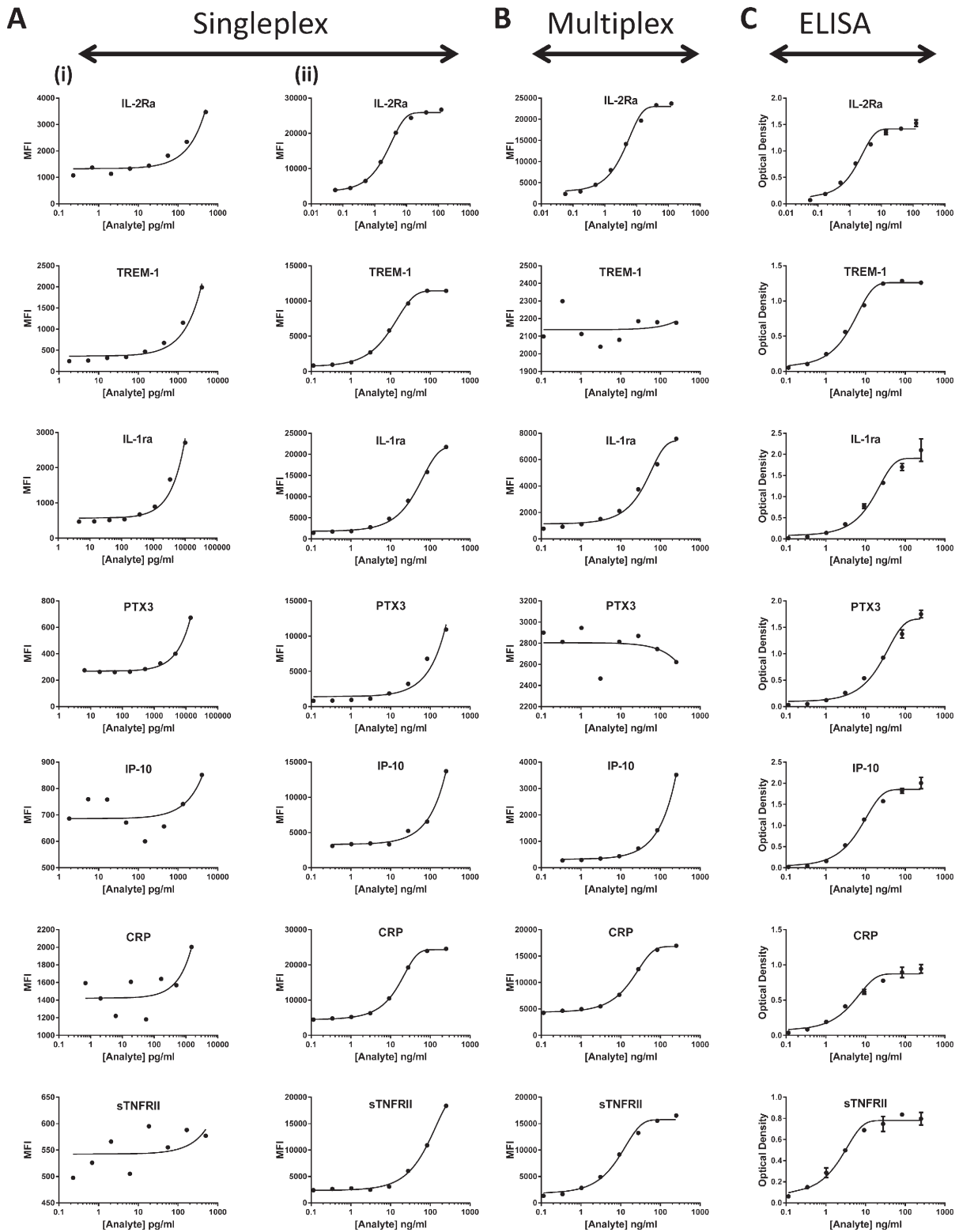

Fig. 3. Comparison of antibody pair performance in xMAP® vs ELISA. Standard curves generated for all 7 analytes using the xMAP® technology (A, B). In singleplex assays (A [i]), top standard concentrations ranging from 0.5 to 14 ng/ml recommended by the manufacturer for ELISA were utilised (refer to Table 1). Low MFIs and curve shapes indicated that the dilution series were too low for xMAP® analysis. Consequently, the top standard concentration was increased to 125 or 250 ng/ml for multiplex assays (A [ii and B). These revised 8-point 3-fold dilution series were used to test all 7 antibody pairs by ELISA (C).

2.3. Coupling confirmation and stability

Two-fold serial dilutions of biotinylated rabbit anti-Protein G antibody were mixed with 5000 beads per well of a 96-well plate. The final antibody concentration, in a total volume of 50 μl PBS-BN#, ranged from 0.0625 to 4 μg/ml. As a negative control, beads mixed with PBS-BN# were also included. The plate was incubated for 30 min, and beads were washed thrice in 100 μl PBS-BN#. Then 50 μl streptavidin-PE (2 μg/ml in PBS-BN#) was added to beads and incubated for 10 min. Beads were washed thrice then resuspended in 125 μl PBS-BN#. After a 30 s incubation at 1100 rpm on a plate shaker, 50 to 75 μl beads from each well was analysed on a Luminex® analyser instrument, according to the system manual. In general, coupling should yield at least a MFI of 10,000 (at standard PMT settings on Luminex® 200™ and FLEXMAP 3D® instruments or on a MAGPIX® instrument) at saturation for optimal use in immunoassays. The process was repeated in determining the stability of the Protein G-coupled beads after storage at 4 °C for over a year. All incubations were carried out at room temperature in the dark on a plate shaker at ~800 rpm, unless otherwise stated. Washing steps involved 1 min recovery of beads using a magnetic separator.

2.4. Coating Protein G-coupled beads with capture antibody

For the chosen bead region per analyte, 2.5×10^5 Protein G-coupled beads were incubated with varying dilutions (1:25 to 1:180) of capture antibody in 500 μl PBS for 2 h at room temperature, or overnight at 4 °C. (Refer to Table 1 for stock concentrations of the capture antibodies). Beads were washed twice and blocked with 500 μl PBS-BSA for 1 h. (Any unoccupied IgG-binding domains of the coupled Protein G were occupied by immunoglobulins within the BSA). Following two washes, beads were resuspended in 500 μl PBS-BSA. Once coated with capture antibody, the beads could be stored in the dark at 4 °C for a maximum of 4 days. To determine the optimal dilution of capture antibody to be used, 5000 antibody-coated beads were labelled with two-fold serial dilutions of PE-conjugated species-specific secondary antibody (0.0625 to 4 μg/ml in PBS-BSA) per well of a 96-well plate for 1 h. After two washes in PBS-BSA, beads were resuspended in 100 μl PBS-BSA, mixed for 5 min to prevent aggregation, then analysed on a Luminex® analyser instrument. A capture antibody dilution that saturated (or was on the way to saturation) between 10,000 and 20,000 MFI was chosen. All incubations were carried out at room temperature in the dark with agitation, unless otherwise stated. Washing steps involved 1 min recovery of beads using a magnetic separator.

2.5. xMAP® sandwich immunoassays

Per well, 25 μl sample was mixed with 25 μl antibody-coated beads; 5000 beads per analyte was used. The plate was incubated for 2 h. Beads were washed twice then incubated with 50 μl biotinylated detection antibody (1:500 dilution; Table 1) for 2 h. After two washes, beads were incubated with 50 μl streptavidin-PE (5 μg/ml) for 30 min. Beads
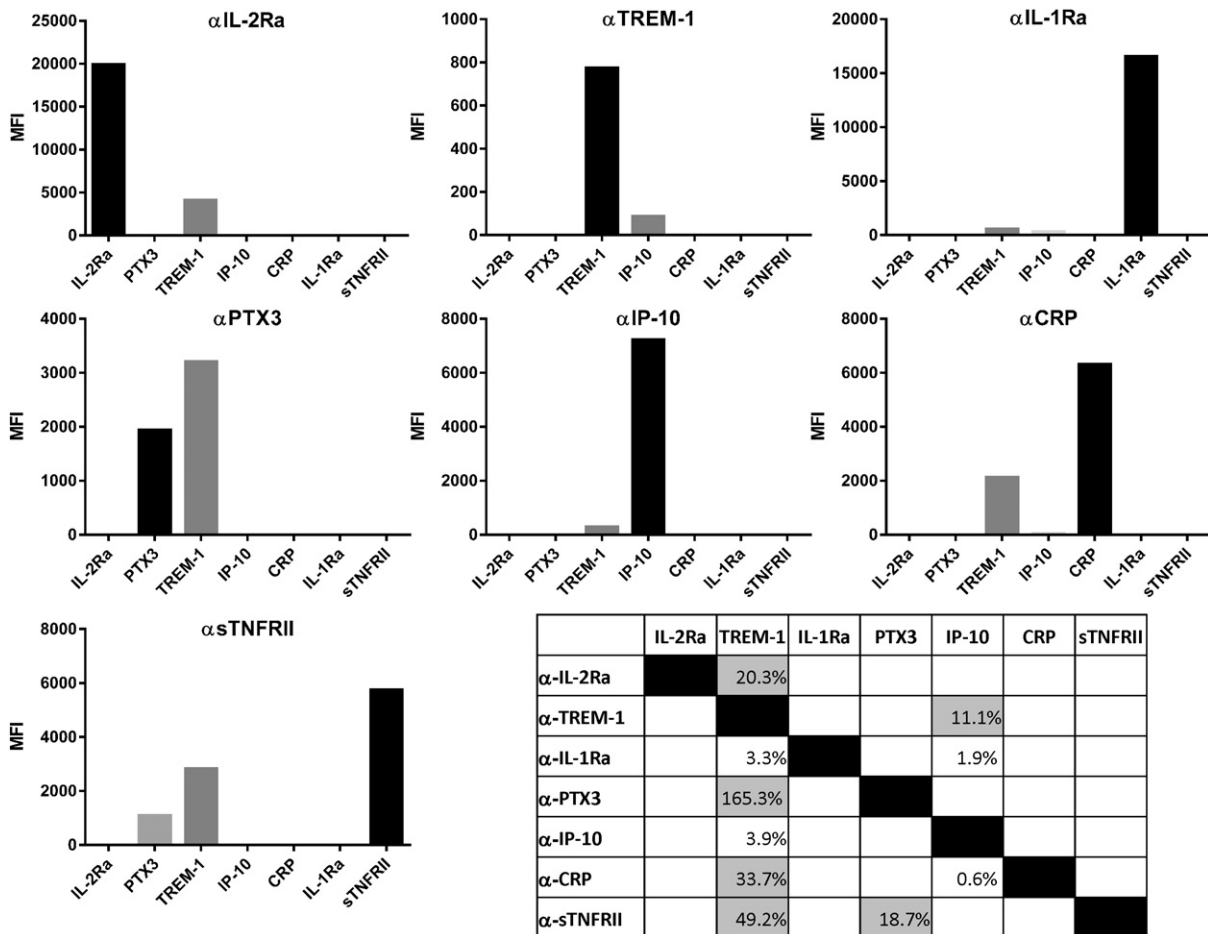

Fig. 4. Cross-reactivity of antibody pairs in multiplex sandwich immunoassays. xMAP® assays were carried out using a cocktail of antibody-coated beads. Each analyte (at the top standard concentration of 125 or 250 ng/ml) was individually tested against the bead cocktail. Black bars in the graph (and black regions in the table) denote specific antibody recognition. Grey bars in the graph (and percentage values in the table) denote cross-reactivity. Cross-reactivity is determined to be an off-target reactivity that was ≥5% of the MFI observed for the cognate ligand, and is represented as grey regions in the table.

were washed twice, resuspended in 100 μl PBS-BSA, agitated for 5 min to prevent aggregation then analysed on an analyser instrument. The MFI of PE was determined for each bead region in each well.

Four-parameter logistic curves were generated using GraphPad Prism. All incubations were carried out in clear-bottom, black 96-well plates (Bio-Rad) at room temperature in the dark on a plate shaker. PBS-BSA
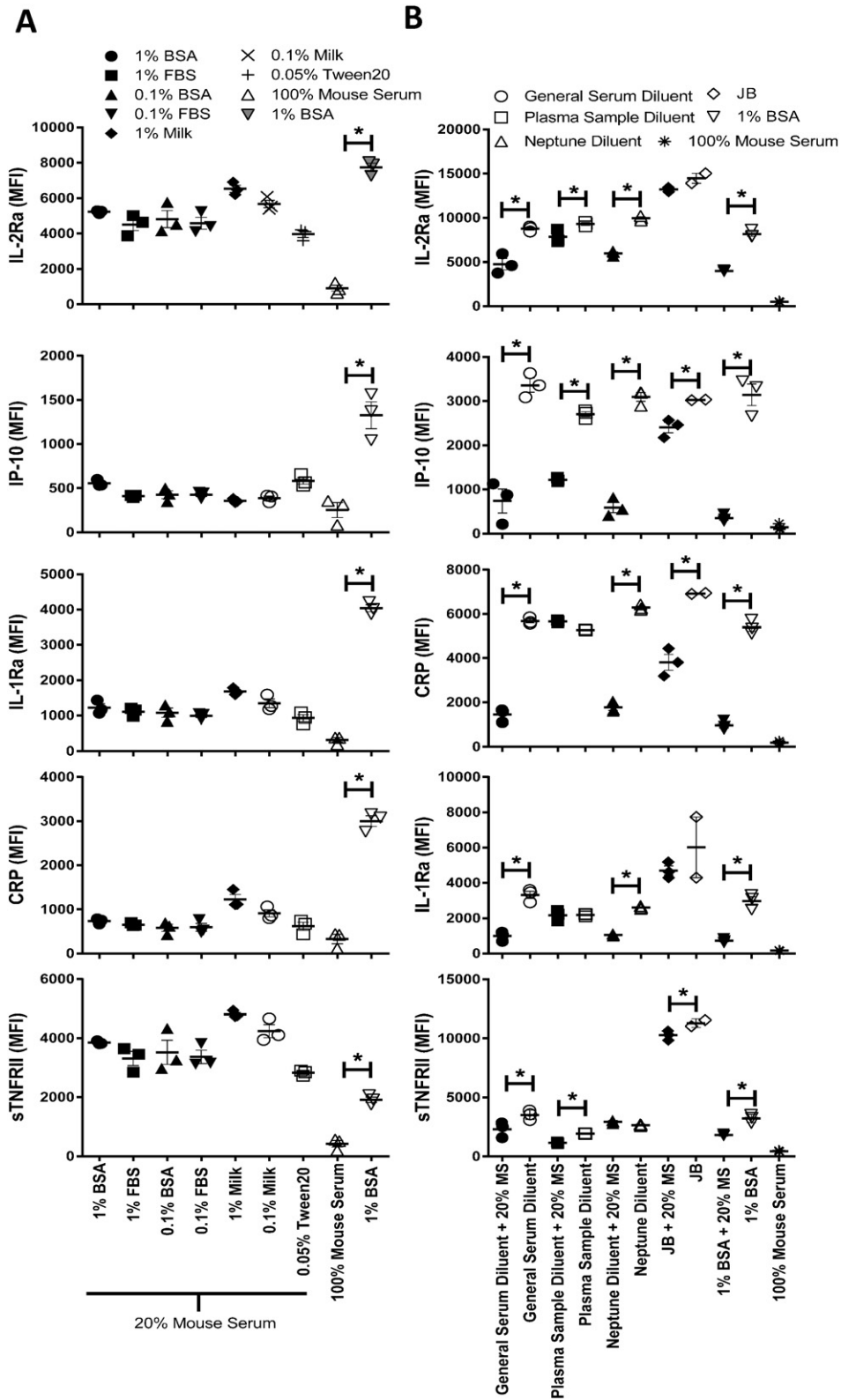

Fig. 5. J Buffer reduces serum matrix effects. Multiplex xMAP® assays were carried out to test PBS containing a variety of common blockers (A) or alternative (commercial or in house) buffers (B) as possible serum sample diluent. Where appropriate, 20% normal mouse serum (MS) was also included in the diluent buffers. Each analyte was spiked in the various diluents or 100% normal mouse serum at the top standard concentration of 125 or 250 ng/ml. One way anova with Dunnett's multiple comparison tests were used to determine significance (*P < 0.05).
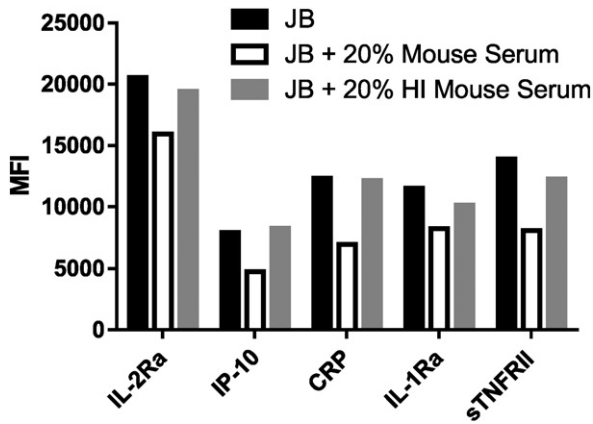
Fig. 6. Heat inactivation reduces serum matrix effects. Analytes in J Buffer (JB) spiked with 20% normal mouse serum were tested against a cocktail of antibody-coated beads using the xMAP® technology. Control or heat inactivated (HI) normal mouse serum was utilised.

served as wash buffer and reagent diluent. Washing steps involved 1 min recovery using a magnetic separator.

The samples used were commercial protein standards diluted in PBS-BSA or a variety of different buffers (Section 2.1). Where appropriate, normal mouse serum heat-inactivated at 56 °C for 30 min was used. Experiments generating standard curves involved an eight point standard curve using three-fold serial dilutions in PBS-BSA. Initial assays utilised the top standard concentrations recommended for the R&D systems DuoSet ELISA kits (Table 1). Subsequently, a 250 to 0.1143 ng/ml dilution series was carried out for all analytes except IL-2Ra, which was diluted from 125 to 0.0571 ng/ml. Multiplex assays involved pre-mixing an equal number of each antibody-coated bead region in PBS-BSA. In addition, detection antibodies were pre-mixed in PBS-BSA to achieve a final dilution of 1:500 each (Table 1); 50 μl of this antibody cocktail was incubated with each well of beads. To determine whether antibody pairs cross-reacted with non-cognate ligands in multiplex assays, pre-mixed antibody-coated beads were incubated with each analyte in different wells of a 96-well plate. The top standard concentration (125 or 250 ng/ml) was used for all analytes. PE signal was generated by incubation with the detection antibody cocktail

then streptavidin-PE. Cross-reactivity was determined to be off-target reactivity that was ≥5% of the MFI observed for the cognate ligand.

2.6. Sandwich ELISA

ELISAs were carried out using R&D Systems DuoSet kits according to the manufacturer's protocol, except for one deviation. Briefly, wells of a 96-well plate were coated with capture antibody (1:180 dilution; Table 1) overnight at room temperature. After three washes, wells were blocked in PBS-BSA for a minimum of 1 h. This was followed by three washes, then incubation with sample or standard for 2 h at room temperature. In deviation from the manufacturer's protocol, the top standard concentration for the eight-point three-fold serial dilution was 125 ng/ml for IL-2Ra and 250 ng/ml for the remaining analytes. Wells were washed then incubated with biotinylated detection antibody (1:180 dilution; Table 1) for 2 h at room temperature. The wells were washed then incubated with Streptavidin-HRP for 20 min at room temperature. After a final wash, wells were incubated with TMB substrate solution for 1 to 2 min at room temperature. The reaction was stopped by the addition of stop solution. Optical density of each well was measured using a microplate reader. Four-parameter logistic standard curves were generated using GraphPad Prism.

2.7. Mice

6 to 8 week old female CB6F1 mice (Harlan Ltd., Stamford Bridge, UK) were kept in specific-pathogen-free conditions in accordance with the United Kingdom's Home Office guidelines. All work was approved by the Animal Welfare and Ethical Review Board (AWERB) at Imperial College London. Mice were immunised intramuscularly with non-adjuvanted or MF59-adjuvanted trivalent subunit vaccine (Novartis Vaccines, Italy) at one-tenth of the human dose (~1.5 μg haemagglutinin) in a total of 100 μl (i.e. 50 μl per thigh muscle). Tail bleeds were taken at 0, 4, 6, 12, 24, 48, 72 and 144 h post immunisation. At each time point, blood draws were taken from 5 of 20 mice; different mice were used at each time point to refine animal usage. The blood samples were allowed to clot for 30 min on ice and sera were extracted by spinning at 13,680 ×g for 10 min. Sera samples were then analysed using the custom pentaplex xMAP® array.

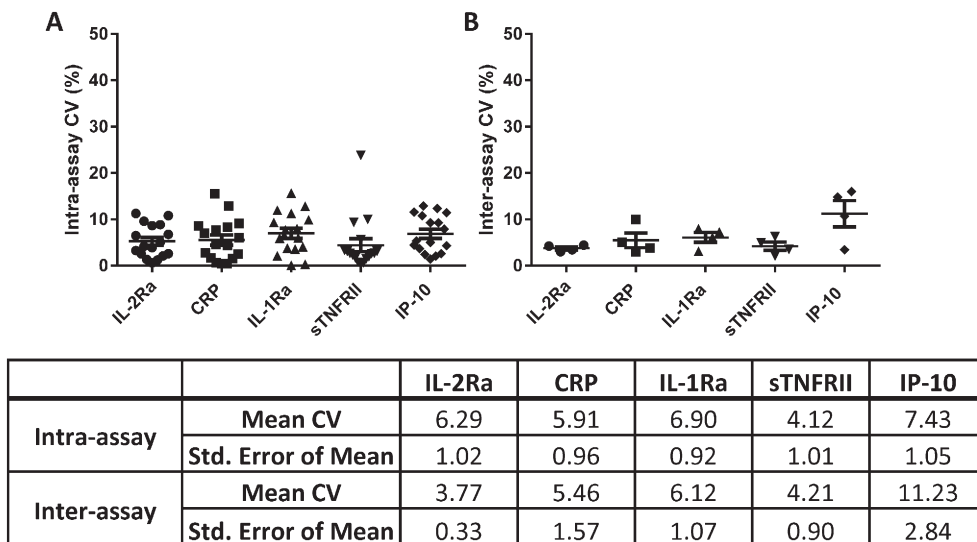|		IL-2Ra	CRP	IL-1Ra	sTNFRII	IP-10
Intra-assay	Mean CV	6.29	5.91	6.90	4.12	7.43
	Std. Error of Mean	1.02	0.96	0.92	1.01	1.05
Inter-assay	Mean CV	3.77	5.46	6.12	4.21	11.23
	Std. Error of Mean	0.33	1.57	1.07	0.90	2.84

Fig. 7. Reproducibility and reliability of the custom pentaplex assay. Intra-assay (A: 3 experiments) and inter-assay (B: 7 experiments) variations were calculated and plotted on a graph showing mean CV ± standard error of mean. The mean and standard error values are displayed in the table.

2.8. Statistical analysis

The coefficient of variation (CV) was calculated by dividing the standard deviation of a population by its mean then multiplying by 100 to give a percentage. Intra-assay variation within a plate was determined by assaying triplicates of 17 samples. Inter-assay variation (i.e. assay-to-assay variation) was assessed by testing triplicates of 4 samples in seven separate assay runs, run on seven different days. Mean and Standard Error of the Mean (SEM) for the CVs were calculated using GraphPad Prism. Statistical significance was determined via one way Anova with Dunnett's multiple comparisons test and calculated using GraphPad Prism.

3. Results

3.1. Protein G-coupled MagPlex® microspheres are stable for >1 year

In general, bead-based sandwich immunoassays involve conjugating capture antibodies to the solid surface via a carbodiimide reaction. Since amine groups in antibodies are cross-linked to carboxylic acids on the beads, the orientation of the capture antibody is unknown. Protein G is a streptococcal cell wall protein with two IgG-binding domains (Bjorck and Kronvall, 1984; Reis et al., 1984), as well as sites for albumin and cell surface binding (Akerstrom et al., 1987; Bjorck et al., 1987). Conjugating Protein G to beads generates a reagent that ensures optimal orientation of capture antibodies in xMAP® immunoassays, and allows the development of multiple types of assays with the same set of beads. To determine optimal coupling conditions, MagPlex® Microspheres (which are 6.5 μm magnetic carboxylated beads) were conjugated to various amounts of Protein G with the aid of EDC and Sulfo-NHS. Recombinant Protein G lacking the albumin and cell surface binding domains was utilised in order to reduce non-specific binding. Optimal coupling was achieved with as little as 1 μg per 1.25×10^6 beads, as demonstrated by a saturating MFI of ~25,000 when labelling with biotinylated anti-Protein G antibody and Streptavidin-PE (Fig. 1a). 17 months after bead conjugation, a reduction in MFI was observed at the lower anti-Protein G antibody concentrations, (Fig. 1b). Nevertheless, a saturating MFI of ~25,000 was achieved at the highest antibody concentrations. Altogether, the data indicate that the Protein G present on the beads over a year post coupling was still optimal for the intended purpose.

3.2. Use of Protein G-coupled beads in multiplex sandwich immunoassays

A multiplex bead array to measure IL-2Ra, TREM-1, IL-1Ra, PTX3, IP-10, CRP and sTNFRII in mouse sera was required. As a result, seven bead regions were coupled with Protein G. To determine the optimal amount of each capture antibody, Protein G coupled beads were coated with various antibody dilutions (resulting in concentrations ranging from 0.8 to 14.4 μg/ml) then detected by PE-conjugated species-specific secondary antibody. For all analytes except PTX3 and IP-10, curves saturating at a MFI >10,000 were generated for the highest antibody dilution of 1:180 (Fig. 2). To achieve the same criteria, a 1:50 dilution was necessary for the PTX3 and IP-10 capture antibodies (Fig. 2). A 1:50 dilution, which equates to concentrations ranging from 2.88 to 14.4 μg/ml (Table 1), was chosen for coating Protein G-coupled beads. Consequently, the chosen coating amount for each capture antibody per 2.5×10^5 beads (in 500 μl antibody solution) was as follows: (i) 1.44 μg for IL-2Ra, TREM-1 and IL-1Ra, (ii) 3.6 μg for IP-10, CRP and sTNFRII, (iii) 7.2 μg for PTX3. Once coated, beads were stable for use within 4 days of storage at 4 °C in the dark.

Antibody-coated beads were first tested for use in singleplex sandwich immunoassays. Standard curves could be generated for IL-2Ra, TREM-1, IL-1Ra and PTX3, but not for IP-10, CRP and sTNFRII (Fig. 3a[i]). However, the data indicated that the eight-point three-fold
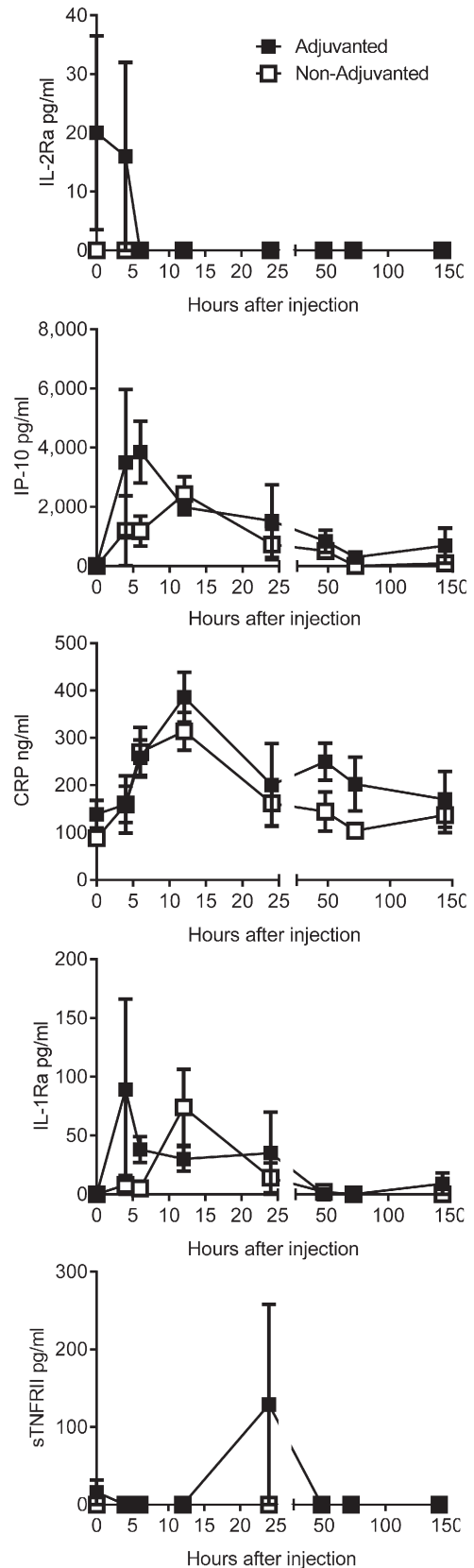

Fig. 8. Evaluation of serum biomarker responses to adjuvanted and non-adjuvanted influenza vaccine using custom pentaplex array. Mice were immunised intramuscularly with non-adjuvanted or MF59-adjuvanted trivalent subunit vaccine in a total of 100 μl. Tail bleeds were taken at 0, 4, 6, 12, 24, 48, 72 and 144 h post immunisation. Sera from each time point were analysed using pentaplex xMAP® array for IL-2Ra, IP-10, CRP, IL-1Ra and sTNFRII. Data points represent the mean of n = 5 mice ± SD.

dilution series recommended by the manufacturer for ELISA (Table 1) involved concentrations at the bottom of the standard curve. Consequently, higher top standard concentrations were chosen: 125 ng/ml for IL-2Ra and 250 ng/ml for the remaining analytes (Fig. 3a[ii]). All antibody-coated bead regions were pre-mixed then used in multiplex sandwich immunoassays. Good standard curves with a wide dynamic range covering ~3 log10 were generated for all analytes except TREM-1 and PTX3 (Fig. 3b). Use of these antibody pairs and top standard concentrations (i.e. 125 or 250 ng/ml) in ELISA generated good standard curves for all analytes (Fig. 3c).

3.3. Anti-TREM-1 and anti-PTX3 antibodies exhibit off-target reactivity

The inability to generate good standard curves for TREM-1 and PTX3 in the multiplex xMAP® assay indicated that the anti-TREM-1 and anti-PTX3 antibodies were cross-reacting with non-cognate ligands. No cross-reactivity was observed in ELISAs; the antibody pairs only bound their specific ligands (data not shown). To investigate antibody cross-reactivity in the multiplex assay, pre-mixed beads were tested against each analyte individually at the top standard concentration. Cross-reactivity was determined to be an off-target reactivity that was ≥5% of the MFI observed for the cognate ligand. All antibody-coated beads bound to TREM-1 to varying degrees, with the greatest effect seen with anti-PTX3 beads (Fig. 4). The increase in MFI observed for the anti-IL-1Ra and anti-IP-10 antibody pairs were just below the aforementioned cross-reactivity criteria. Cross-reactivity was also observed between anti-TREM-1 and IP-10, as well as anti-sTNFRII and

Fig. 9. Flow chart of multiplex array development using Protein G-coupled beads. A brief overview of the steps that are required and need to be optimised in order to develop custom xMAP® arrays.

PTX3. Consequently, TREM-1 and PTX3 detection were removed from the array. The resulting custom pentaplex array generated was for measurement of murine IL-2Ra, IP-10, CRP, IL-1Ra and sTNFRII.

3.4. Optimal sample preparation for custom pentaplex xMAP® assay

The custom bead array was for the measurement of five analytes in small volumes of mouse sera. Serum is a complex mix of proteins that may non-specifically bind to microspheres – known as matrix effects (Elshal and McCoy, 2006). These matrix effects can interfere with the performance of a xMAP® assay and may be observed as poor bead recovery, instrumentation clogging, low signals, or variable results. This can be overcome by sample dilution using an appropriate buffer. Typically, serum is diluted at least 1:5 to reduce non-specific reactivity responses (de Jager et al., 2005). In order to investigate serum matrix effects as well as identify a suitable sample diluent buffer, the pentaplex array was tested against protein standards diluted in a variety of buffers in the presence or absence of normal mouse serum. Where appropriate, 20% normal mouse serum was used to mimic the matrix that would be encountered in diluted ex vivo experimental samples.

Detection of cognate ligands by all antibody-coated beads (except anti-sTNFRII beads) was significantly impaired ($P < 0.0001$) in the presence of 20% normal mouse serum, compared to detection in PBS only containing 1% BSA (Fig. 5a). Use of the following blockers failed to alleviate the matrix-mediated interference: BSA (0.1–1%), FBS (0.1–1%), milk (0.1–1%) and 0.05% Tween 20 (Fig. 5a). Serum matrix effects were then investigated in three commercial diluent buffers as well as an in-house recipe (J Buffer). J Buffer is PBS containing 20 mM Tris–HCl, 0.05% Tween-20, 1% Goat Serum and 1% Sheep Serum. For all antibody-coated beads (except anti-CRP), commercial diluent buffers containing 20% normal mouse serum interfered with the detection of cognate ligands (Fig. 5b). Only J Buffer consistently exhibited limited interference from serum matrix effects. To enhance the effectiveness of J Buffer, the effect of serum heat inactivation was investigated. Heat inactivation significantly increased ($P < 0.05$) detection of all analytes (except IL-1Ra) in serum-containing J Buffer (Fig. 6); indicating that the cause of interference was a heat labile molecule (such as complement). IL-1Ra detection was notably increased but this was not statistically significant.

3.5. Inter- and intra-assay variation

The coefficient of variation (CV) is a statistical measure of the dispersion or variability of a population. In this instance, it was used to measure the precision and reproducibility of the custom pentaplex sandwich immunoassay. The intra-assay CV (Fig. 7a) specifically measures the accuracy of replicates within one assay, while the inter-assay CV (Fig. 7b) measures the reproducibility of the assay on multiple occasions. The CVs for all five analytes were low; intra-assay variation ranged from 4.12 to 7.43% whilst the inter-assay variation was 3.77 to 11.23%. According to the FDA Bioanalytical Method Validation guidelines, intra-assay CV of <10% and inter-assay CV of <20% are usually acceptable.

3.6. Analyses of sera from vaccinated mice using the pentaplex array

The pentaplex xMAP® array described here was specifically developed to analyse systemic biomarker changes in response to influenza vaccination in CB6F1 mice, using very small amounts of serum (20 μl). Mice were immunised with non-adjuvanted or MF59-adjuvanted trivalent subunit influenza vaccine. Of the five analytes investigated, three were detected in mouse sera; with IP-10, CRP and IL-1Ra being up-regulated post immunisation (Fig. 8). These data are consistent with previously published reports that show an increase in IP-10 and IL-1Ra expression in response to MF59 (Caproni et al., 2012; Mosca et al., 2008; Seubert et al., 2008), as well as increased CRP and IP-10
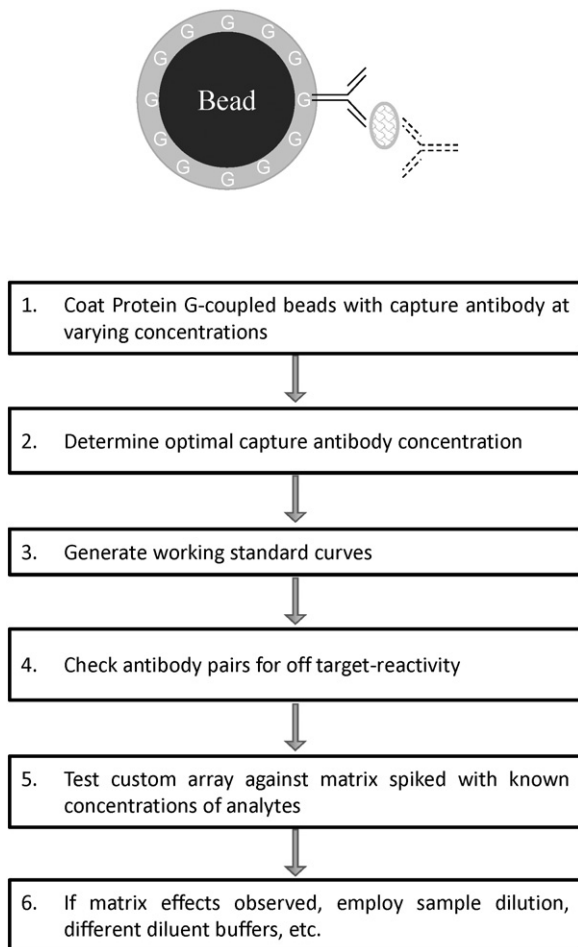
expression in response to influenza vaccination (Christian et al., 2011; Nakaya et al., 2011; Obermoser et al., 2013). IL-2Ra and sTNFRII remained at baseline levels in the serum, with no difference observed between the adjuvanted and non-adjuvanted animals in response to intramuscular immunisation.

4. Discussion

A plethora of commercial bead array formats are available, but the development of custom arrays is still necessary to support research activities. This is of particular importance for scientists utilising experimental animal models for which research reagents are rare; research activities centred around the ferret model is a notable example. In this study, we demonstrate one approach to develop custom xMAP® arrays using Protein G-coupled beads (summarised in Fig. 9). Use of Protein G means that (i) only one coupling procedure is necessary and (ii) capture antibodies are orientated in the correct manner. In addition, Protein G-coupled beads offer flexibility and robustness that make it possible to easily expand an array, and to do so within a wide time frame (>1 year) due to the stability of this reagent. Optimal Protein G coupling, capture antibody coating, sample preparation and diluent for the simultaneous analysis of five analytes in mouse serum samples using the xMAP® technology are demonstrated here. The analytes of interest are potential biomarkers of vaccine safety being investigated by the IMI-JU project BioVacSafe: soluble cytokine receptors sTNFRII and IL-2Ra, pattern recognition receptor CRP, antagonist IL-1Ra and chemokine IP-10. We demonstrate that the custom pentaplex array can be used to measure these mediators in the serum of immunised mice.

The xMAP® technology has many advantages over ELISA (de Jager and Rijkers, 2006; Houser, 2012); one of which is the requirement for significantly reduced sample volume. Typically, 25 μl of sample (or standard) is required per well of a 96-well plate, whilst 50–100 μl/well is used for each ELISA needed. Indeed, the pentaplex bead-based sandwich immunoassay described here requires 25 μl/well of sample/standard compared with a total of 500 μl for five ELISAs. Since a 1:5 dilution of mouse serum was required for the pentaplex array, this is equates to measuring 5 analytes using just 5 μl of serum in one well. This is of particular importance because this array was developed for the analysis of sera from neonatal, as well as adult and aged mice. ELISA also consumes relatively large volumes of reagents in comparison to the xMAP® technology. Typically, 100 μl/well of diluted detection antibody and streptavidin is needed per ELISA, whilst the xMAP® technology requires a total of 50 μl/well of each reagent regardless of the number of analytes to be investigated. Typically, 100 μl/well of diluted capture antibody is used in ELISA. For the custom pentaplex xMAP® array described here, only 12.5 μl/well of diluted capture antibody was needed to coat each Protein G-coupled bead region utilised. This means that xMAP® not only utilises significantly less reagent volumes, the total reagent amount required is also significantly reduced, especially with the use of Protein G-coupled beads. Consequently, there are significant cost and sample savings associated with using xMAP® instead of ELISA, particularly with regards to antibodies. Simultaneous analyte measurement also greatly reduces the amount of time and labour required to analyse large quantities of samples, thereby resulting in higher throughput. In addition, multiplex assays ensure that all analytes of interest are measured under the same conditions (i.e., same operator, identical incubation times and temperatures, etc.), which is of particular importance if a scientific question relies on a combination of biomarkers or the relationships between them (Lewczuk, 2012). Furthermore, bead-based assays can detect raw imprecision of repeated (duplicate) measurements (Lewczuk, 2012), and also offer improved imprecision because multiple, independent measurements (typically 50–100) are carried out within each bead region (Ellington et al., 2010).

It cannot be assumed that reliable singleplex assays can automatically be converted into a reliable multiplex array. Rather, this transition requires the development of optimal bead coupling procedures for each capture antigen required, as well as optimisation of the assay. Factors that can interfere with the development of multiplex assays include antibody cross-reactivity and assay diluents (de Jager and Rijkers, 2006; Ellington et al., 2010). It is well reported that antibody cross-reactivity to non-target proteins is ubiquitous and widespread. For xMAP®, the use of antibody/bead mixtures and agitation means that cross-reactivity against (i) analytes, (ii) capture antibodies, (iii) detection antibodies can occur. The references cited here, as well as the Luminex® cookbook (http://info.luminexcorp.com/xmap-cookbook-2nd-edition-free-download), provide helpful guidance and protocols on how to detect and avoid cross-reactivity when developing custom multiplex assays. The soluble pattern recognition receptor PTX3 and immunoglobulin-like receptor TREM-1 were eliminated from the panel of interest in this study due to antibody cross-reactivity issues detected by xMAP® but not by ELISA. This indicates that the presentation of antigenic epitopes on microspheres is different from that in ELISA, as has been previously reported (Khan et al., 2006). Expansion of the array to a heptaplex might be possible if screening of alternative PTX3 and TREM-1 antibody pairs (i.e. from different hybridoma clones) identifies reagents with minimal cross-reactivity. Further differences between ELISA and xMAP® used in this study were highlighted by the slightly decreased sensitivity of xMAP® analysis for IP-10 compared to ELISA; this scenario is not unusual. Khan et al. suggest that the availability and accessibility of the antigenic epitopes may be limiting due to the smaller surface area of xMAP® microspheres, whilst the concentration of antigenic molecules may not be limiting due to the larger available surface areas in ELISA microtitre plate wells (Khan et al., 2006).

In conclusion, the multiplex sandwich immunoassay described here is a rapid, simple, specific, reproducible and adaptable method for the analysis of small volumes of serum samples.

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