

# Multiplexing immunoassays for cytokine detection in the serum of patients with rheumatoid arthritis: lack of sensitivity and interference by rheumatoid factor

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## Abstract

### Objectives

*Accurately measuring cytokines in clinical material remains an important challenge in the development of biomarkers. Enzyme-linked immunoabsorbent assays (ELISAs) are considered 'gold standard'; however, their use is limited by the relatively large sample volume required for multiple analyte testing. Several alternatives (including membrane or bead-ELISA) have been developed particularly to enable multiplexing. Concerns were raised regarding their use in rheumatology due to interference by heterophilic antibodies, notably rheumatoid factor (RF). In this report, we compared several multiplex assays using serum from rheumatoid arthritis (RA) patients with respect to the presence of residual RF following attempted removal employing commonly used procedures.*

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### Methods

*Healthy control and RF-positive/negative RA sera were used to compare 4 multiplex assays with ELISA: bead-based 'Luminex' immunoassay, cytometric bead assays (CBAs), membrane-based and Mosaic™ ELISAs. Sera were tested following Ig blockade (mixed species serum) or removal (using PEG6000 or sepharose-L).*

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### Results

*Ig removal was only partially efficient and residual RF was detected in most sera. RF had no impact on cytokine measurement by ELISA. In single and multiplex Luminex, cytokine levels associated with false positive results correlated directly with RF titres. Following Ig-blockade/removal, these relationships remained suggesting false positivity was still associated with the presence of residual RF. Conversely, detection of cytokines in multiplex membrane-based or Mosaic- ELISA were not affected by the presence of RF; however, levels of cytokines readily detected by ELISA were often below the detection threshold of these assays. CBA assays were also low on sensitivity but unaffected by RF.*

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### Conclusion

*False positivity, due to the presence of heterophilic antibodies, mainly affected Luminex assays. Other assays however remained limited in their sensitivity. Multiplexing of cytokine measurement remains a challenge, particularly in rheumatological pathologies, until assays of adequate sensitivity are developed. ELISA remains the gold standard.*

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### Key words

cytokine, rheumatoid factor, ELISA, immunobead assays

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## Introduction

Enzyme-linked immunoabsorbent assays (ELISAs) have long been considered the 'gold standard' for detecting antibodies and antigens in biological fluids or cell lysates. They are easy to use and yield highly reproducible results. However, their limitations lie in the relatively large volume of sample required for testing, typically 50–200 µl of body fluid/cell lysate, and the time required for their implementation (3 hours to 1 day). It has been of interest to many in the fields of both medical diagnostics and research that an alternative methodology is found to enable faster processing and, most importantly, the possibility of 'multiplexing' analysis of many targets simultaneously from the same volume, including hormones, cytokines, antibodies and bacterial antigens. Several alternatives have been developed, including the bead-based immunoassay (hereafter called the Luminex assay), which has been proposed as a high throughput multiplex replacement for ELISA. This is a multiplex platform that can easily be used to include up to 96 targets. In addition a number of membrane-based ELISAs and Mosaic™ ELISAs have recently been released as well as cytometric bead assays (CBAs). These multiplex technologies represent an attractive alternative to ELISA, enabling multiple testing from a small volume of serum or body fluid.

Recently, we raised questions related to the sensitivity of not only multiplex solid-phase assays, such as that used with the Luminex technology, but also to the sensitivity of some ELISAs in their measurement of cytokines, specifically interleukin (IL)-7 in RA serum and synovial fluid samples (1–4). Our review of IL-7 measurements in 17 publications (1) reported that high IL-7 levels in RA samples (and other tested cytokines), were only observed in the papers using Luminex (singleplex or multiplex with wide variation) whereas all data in healthy controls were similar to matched data obtained by 3 different ELISAs (with the exception of a fourth one). RF, present in the serum of approximately 75% of RA patients, has been reported to hinder the accurate measurement of cytokines by immuno-assay (5–9). Two

multiplex cytokine profile studies using Luminex showed 20 fold (~50 pg/ml) to 100-fold (~300 pg/ml) higher serum IL-7 levels (10, 11) compared to levels of IL-7 (6.9±2.0 pg/ml) established using ELISA (12). In both of these Luminex studies, every cytokine/chemokine/growth factor analysed was increased compared to controls.

It has been demonstrated that heterophilic immunoglobulins, can interfere with the Luminex technology producing false positives (6, 9, 13, 14). Heterophilic antibodies encompass antibodies with low specificity, directed against multiple poorly antigenic immunogens. The occurrence of false-positive results in two-site immuno-assays (15–18) results from heterophilic antibodies non-specifically bridging the detection and capture antibodies in these assays (19, 20). These antibodies also reportedly interfere with nephelometric and turbidimetric assays such as those used for CRP quantification (21). Interference was reduced by treatment with polyethylene glycol (which is reported to reduce IgM by 80%) (5, 6, 22) or immunoglobulin saturation (23). Rheumatoid factor (RF) is an autoantibody directed against the Fc portion of IgG and is found in 75% of patients presenting with RA as well as other diseases such as Sjögren's syndrome, infective endocarditis, systemic sclerosis and systemic lupus erythematosus (SLE) (24). RF was shown to exhibit some of the properties of heterophilic antibodies cross reacting with several antigens (25).

This study, therefore, compared the output of sandwich ELISA, mosaic ELISA and membrane-based ELISA, with bead assays using both Luminex and CBA methods. This work was carried out to examine the possibility of multiplex cytokine detection taking in consideration interferences due to the presence of naturally occurring RF in RA serum samples, and tested known methods of immunoglobulin removal prior to measurements being performed.

## Methods

### *Patients and healthy controls*

Serum samples were obtained from healthy controls and RA patients with reported presence or absence of RF.

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*Competing interests none declared.*

Ethical approval was obtained from the Leeds East Research Ethics Committee, and informed consent was obtained from each participant. RA was diagnosed using the ACR criteria of 1987. Sera were stored at  $-20^{\circ}\text{C}$  prior to use. Healthy control individuals were recruited from local blood donors ( $n=8$ ).

#### Immunoglobulin removal

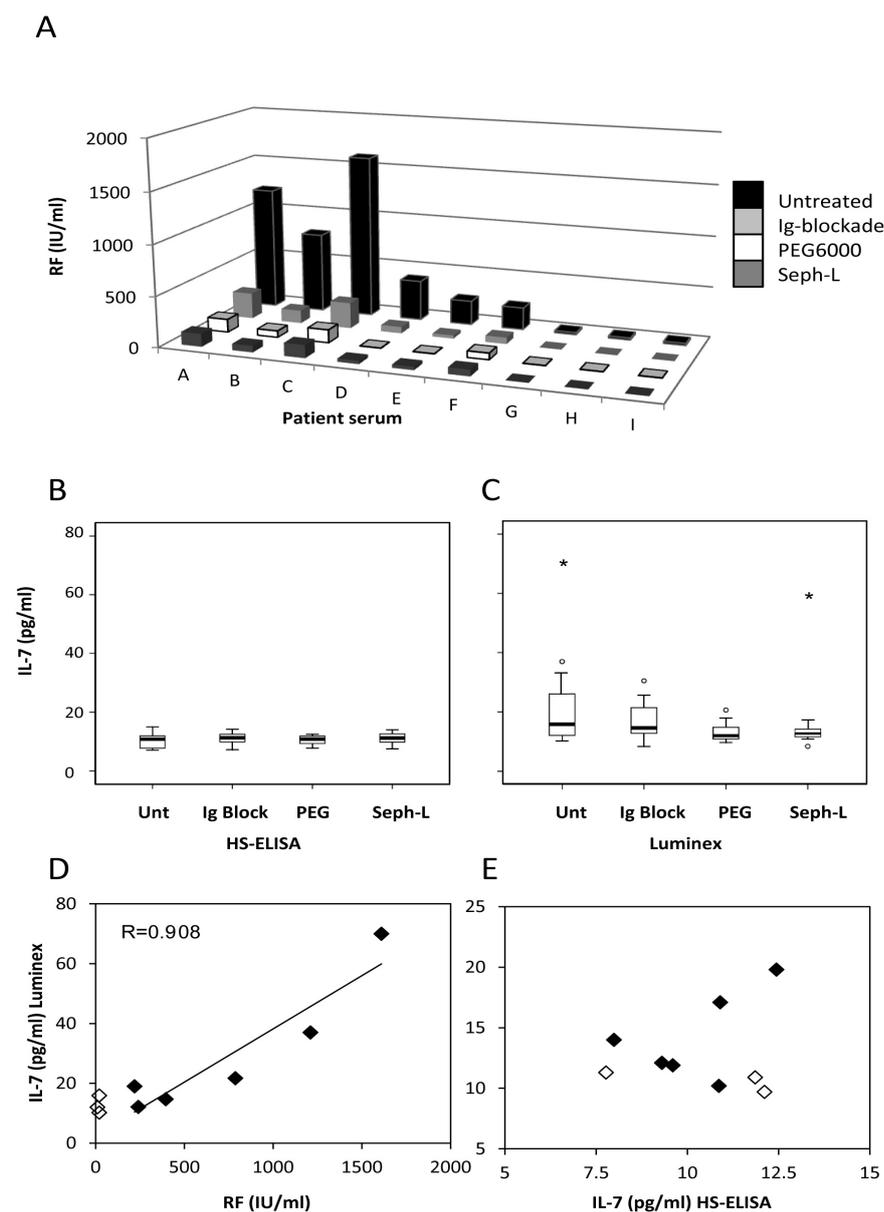
To remove the potential influence of/interference by RF in the detection of cytokines in serum samples, blocking of immunoglobulin (Ig) and Ig removal were compared. Samples underwent each of the following treatments: 1) untreated, volumes were corrected with PBS; 2) addition of Ig blocking mix (final concentration 20% normal mouse serum, 10% goat serum, 10% rabbit serum, 10% PBS) and incubated at  $21^{\circ}\text{C}$  for 30 minutes (5); 3) Ig removal by addition of 0.1% v/v 30% PEG6000 (Fluka) in 0.1 M phosphate pH 7.4 (Sigma); samples were then incubated for 30 minutes at  $4^{\circ}\text{C}$  and centrifuged at 700 g for 45 minutes at  $4^{\circ}\text{C}$  (6). Sera were then treated by addition of blocking mix as in treatment '2'. 4) IgG removal by application to Nab sepharose-L columns (Pierce/Thermo) with 10 minute incubation ( $21^{\circ}\text{C}$ ) and collection of the first elution by centrifugation followed by addition of blocking mix as in treatment '2'.

#### RF determination

Prior to, and following the above treatments, the semi-quantitative SERODIA-RA particle agglutination test for detection of rheumatoid factor (Mast Diagnostics) was used to determine initial and residual IgG RF in the serum samples. This was carried out in accordance with the manufacturer's instructions and scored after 18 hours.

#### ELISAs

ALL ELISAs were Quantikine ELISA kits (R&D Systems, UK). High sensitivity (HS) kits were used when available and all were carried out in duplicate according to the manufacturer's instructions (20–150  $\mu\text{l}$  serum/analyte). The sensitivities were: IL-7 (HS), 0.1 pg/ml; IL-2, 7 pg/ml; IFN $\gamma$ , 8 pg/ml; TNF- $\alpha$ , 1.6

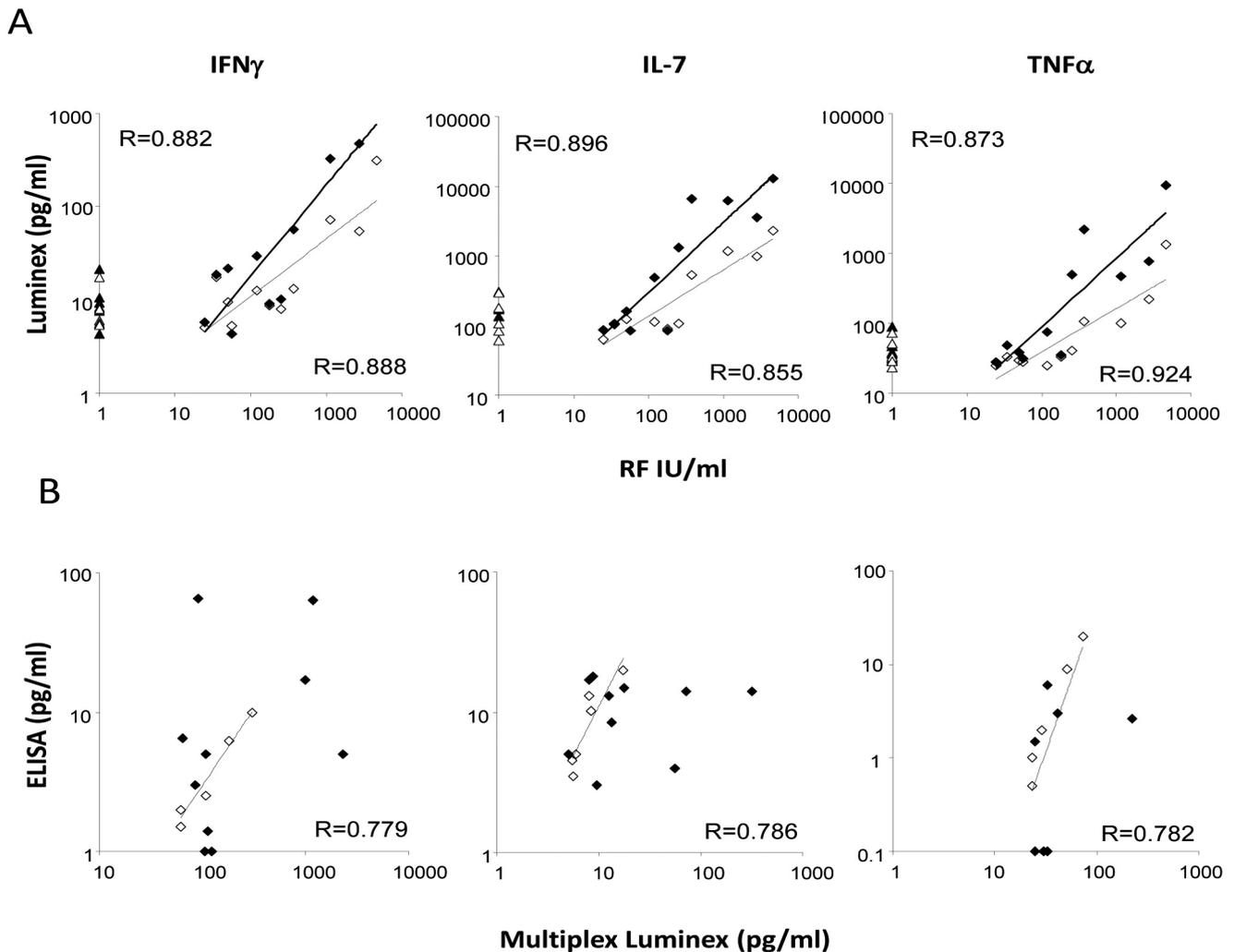


**Fig. 1.** (A) RF levels remain detectable following different methods of Ig blocking and RF removal (sepharose-L + Ig blockade and PEG6000 + Ig blockade) compared to untreated serum samples from RA patients. (B) IL-7 levels measured by HS-ELISA and (C) singleplex Luminex in the same RA patient sera, stars indicate outliers and circle extremes of the IL-7 distributions. (D) Correlation between RF titre and the level of IL-7 detected by singleplex Luminex ( $\rho=0.908$ , RF-negative samples in open diamonds and RF-positive samples in black diamonds), (E) Absence of relationship between IL-7 detected by HS-ELISA and singleplex Luminex after RF removal (by PEG6000/Ig blockade) in the same RA patient sera (RF-negative samples in open diamonds and RF-positive samples in black diamonds).

pg/ml; TNF- $\alpha$  (HS), 0.191 pg/ml; IL-6 (HS), 0.039 pg/ml and TGF- $\beta$ 1, 4.61 pg/ml; the ranges were 0.25–16 pg/ml (IL-7), 31.2–2000 pg/ml (IL-2), 15.6–1000 pg/ml (IFN $\gamma$ ), 15.6–1000 pg/ml (TNF- $\alpha$ ), 0.5–32 pg/ml (TNF- $\alpha$  (HS)), 0.156–10 pg/ml (IL-6) and 31.2–2000 pg/ml (TGF- $\beta$ 1). Assays were read at 490/450 nm and corrected at 630/570 nm on an OpsysMR microplate reader (Dynex Technologies Ltd, UK).

#### Luminex

This assay is based on the principal of a sandwich immunoassay, using 5 micron beads as the solid phase, with the capture antibodies bound to these beads. Incubation of the sera with the beads, was followed by washing, detector antibody incubation and streptavidin conjugated fluorescent proteins were then added, yielding a measurable immune complex. The assays were carried out



**Fig. 2.** (A) Levels of RF from 16 serum samples from RA patients measured by multiplex Luminex (IFN $\gamma$ , IL-7 and TNF- $\alpha$ ) before (closed symbols, rho values top left) and after (open symbols, rho values bottom right) RF pre-absorption with PEG6000/Ig blockade. Cytokine levels detected in RF-positive samples showed false positivity in direct relationship with RF titres in sera before and also after RF removal. (B) Following RF removal (PEG6000/Ig blockade), the Luminex measured values still remain unrelated to ELISA derived values in RF-positive samples (closed samples), whereas for RF-negative samples (open symbols) relationships could be observed between methods (rho values on plot, R).

according to the recommended protocol using 50  $\mu$ l of serum. The individual properties of the cytokine specific beads are then measured on the Luminex 100 platform. Single plex – IL-7 and 25-plex; sensitivity in pg/ml) IL-1B (15), IL-1R $\alpha$  (30), IL-2 (6), IL-2R (30), IL-4 (5), IL-5 (3), IL-6 (3), IL-7 (10), IL-8 (3), IL-10 (5), IL-12 P40/70 (15), IL-13 (10), IL-15 (10), IL-17 (10), IFN $\alpha$  (15), IFN $\gamma$  (5), TNF- $\alpha$  (10), IP-10 (5), MCP-1 (10), MIG (4), MIP-1 $\alpha$  (10), MIP-1B (10), RANTES (15), eotaxin (5) and GM-CSF (15), assays were supplied by Biosource (UK).

#### Membrane-based ELISA

A Proteome Profiler<sup>TM</sup> Array for Human Cytokine Array (Panel A, R&D) was

tested according to the manufacturer's instructions. Briefly, 500  $\mu$ l serum was mixed with a cocktail of biotinylated detection antibodies and incubated with a membrane spotted with capture antibodies. Unbound material was washed off and bound cytokines measured by chemiluminescence, as instructed, where light emitted is proportional to cytokine bound.

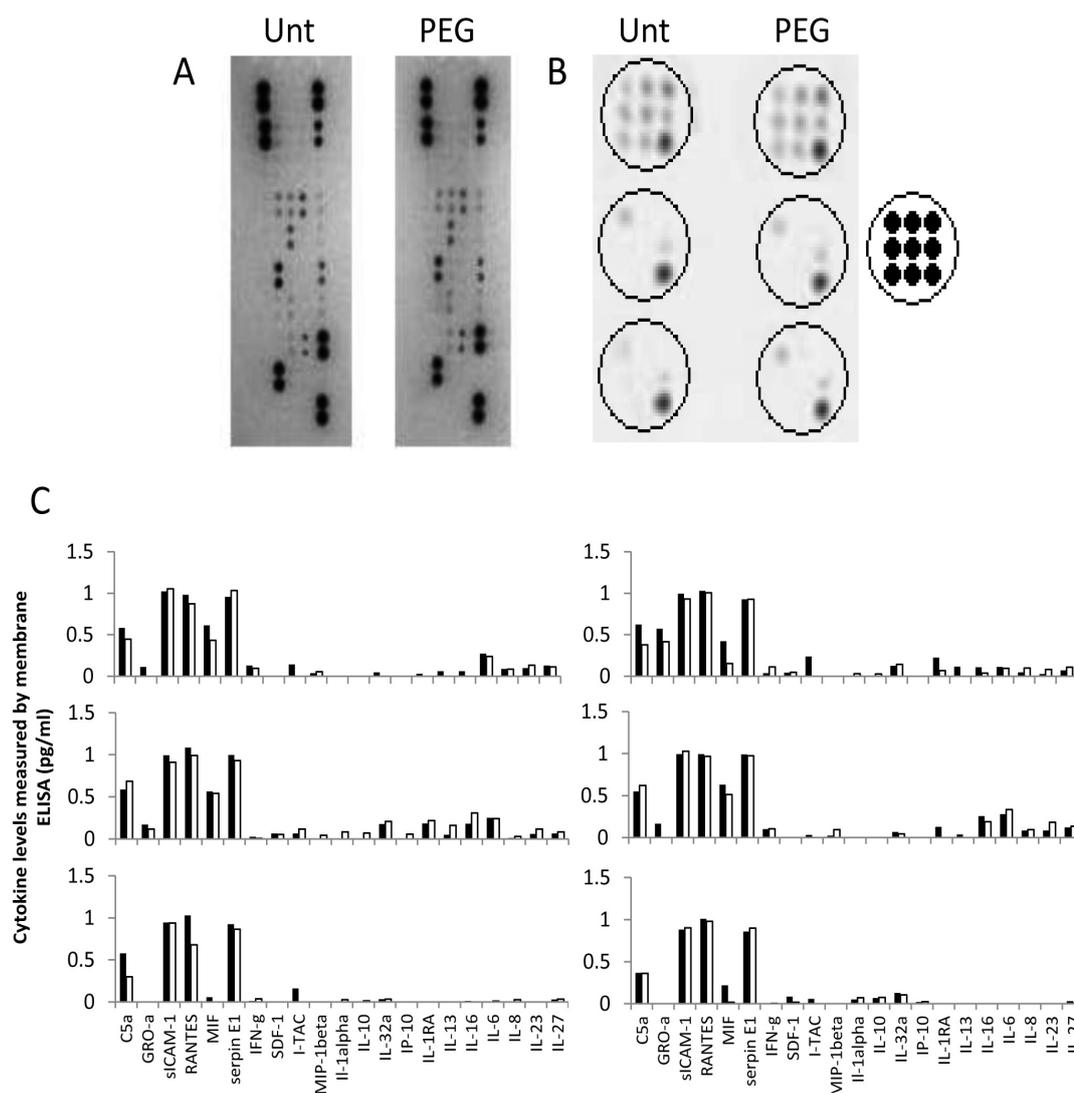
#### Mosaic ELISA

Mosaic<sup>TM</sup> ELISA Human Cytokine Panel 1 (R&D) follows the same principle as traditional sandwich ELISA, but with eight individual capture antibodies spotted into each well. The cytokines were measured by chemiluminescent detection (according to

the protocol) with signal proportional to cytokine. Using a digital camera and analytical software, pixel intensity on the images yields the cytokine level. For these experiments 60  $\mu$ l of serum were used. Cytokines tested in this panel were (sensitivity, range in pg/ml); CD40L (2.23, 1.43–5.31), IFN- $\gamma$  (1.38, 0.77–2.08), IL-1 $\alpha$  (0.59, 0.33–0.74), IL-1 $\beta$  (0.20, 0.11–0.31), IL-6 (0.41, 0.21–0.76), IL-8 (0.59, 0.26–1.24), IL-17 (0.17, 0.06–0.35) and TNF- $\alpha$  (1.65, 0.76–2.13)

#### Cytometric Bead Array (CBA)

The cytometric bead assay (CBA) was performed using a Becton Dickinson Human Enhanced Sensitivity Master Buffer Kit (BD, UK), according to the



**Fig. 3.** (A) Representative example ( $n=6$ ) of membrane-based ELISA for a RF-positive serum sample (1150 IU/ml) from a RA patient before (Unt) and after PEG6000/Ig blockade treatment (PEG).

(B) representative mosaic ELISA results from the highest concentration of the dose scale (top panel) and 2 RA patients (middle panel) - RF positive 1150 IU/ml) and lower panel RF-negative)

(C) (Left) Cytokine levels measured by membrane ELISA (black- before and white- after PEG6000/ Ig blockade) in six RA patients (top two RF-negative, bottom four RF-positive). Several cytokines (including TNF- $\alpha$ , IL-1 $\beta$ , IL-12p70, IL-2, IL-4, IL-5, IL-17, MCP-1, MIP-1 $\alpha$ , Strem-1) were not detected by this methods and are not displayed on these plots.

recommended protocol. This involved capture of cytokines onto antibody coated beads. To detect cytokines, beads have a distinct size and fluorescence for each analyte, enabling their identification using a second detection antibody and flow cytometry to measure the amount of cytokine bound ("theoretical sensitivities" according to manufacturer in pg/ml); IL-2 (11.2), IL-6 (1.6), IL-7 (0.5), IL-10 (0.13), IL-12 (0.6), IL-17A (0.3), IFN $\gamma$  (1.8), TNF- $\alpha$  (0.7), MIP-1 $\alpha$  (0.2) and RANTES (0.002), range of standard curve 10-2500 pg/ml for all). Here 50  $\mu$ l of sera were tested and analysis was performed on a BD LSRII flowcytometer.

#### Statistics

Spearman's rank correlation coefficient rho (abbreviated R on figures) was used

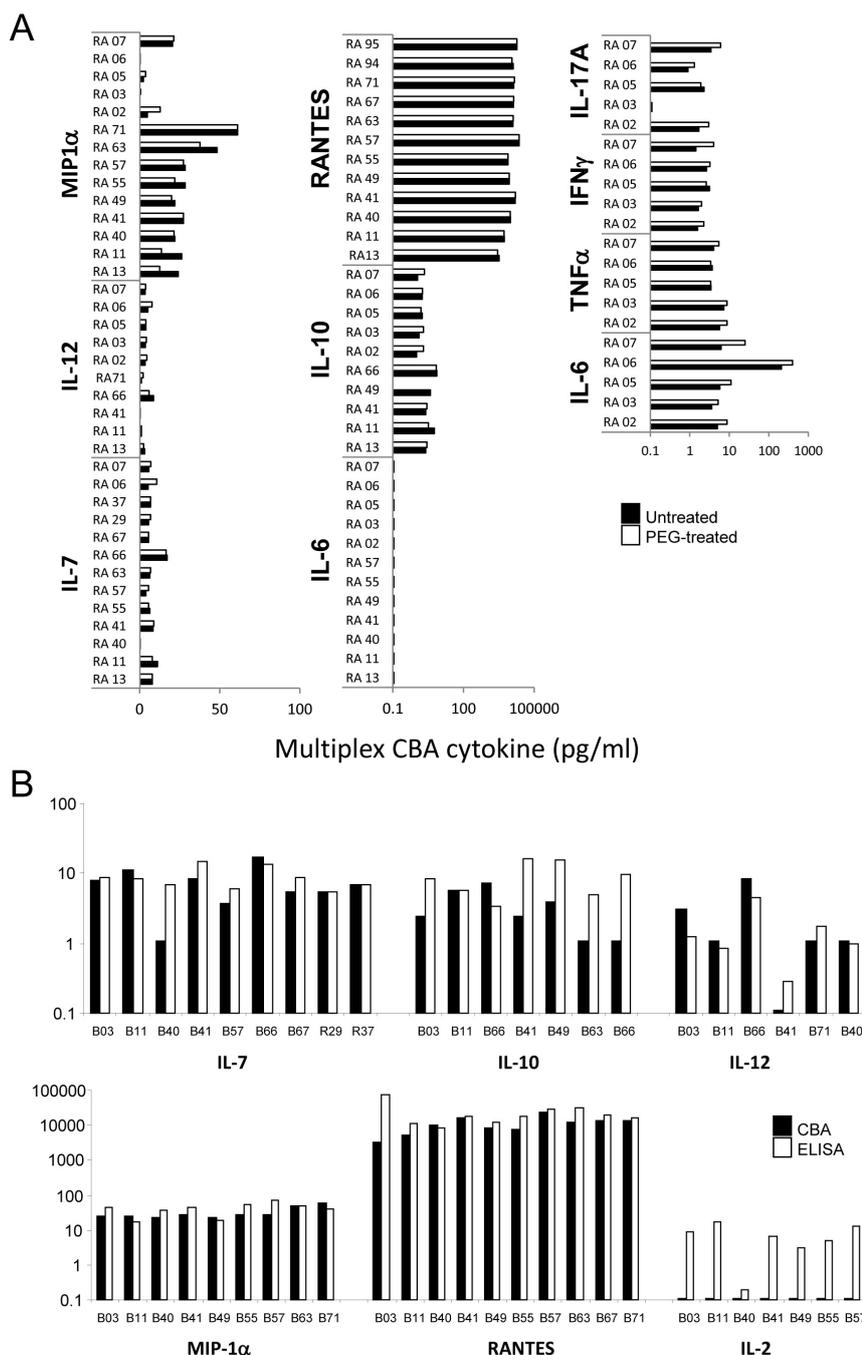
to correlate two variables and only reported when significant. SPSS software version 13 was used.

#### Results

##### Rheumatoid factor removal

In the initial part of this study, two previously described methods of RF removal were tested (5, 6). These RF removal techniques were evaluated using an IL-7 ELISA on serum samples from 9 RA patients in clinical remission to minimise disease effect (CRP<10 mg/l and DAS<2.6) but with variable levels of IL-7 as previously reported (ranging from ~2.5-25 pg/ml (12)), 6 RF-positive (range 219-1610 IU/ml, and 3 RF-negative) predetermined by nephelometry (data obtained from clinical notes). The level of RF remaining in the patients' serum was then meas-

ured using a laboratory method of RF determination (Serodia RA). The untreated serum was also re-measured using this technique in order to ensure suitable comparisons. Results from the untreated sera showed high correlation with titres reported in patients' notes ( $\rho=0.898$ ,  $p<0.0001$  data not shown), showing that the routine NHS and laboratory tests were highly comparable. Residual RF was analysed in samples post different treatments. Ig blocking alone using serum mix (Ig blockade, Fig. 1A) already had a blocking effect on detectable RF levels. The sepharose-L and PEG6000 RF-removal methods further reduced RF levels and were equally efficient at removing relatively low quantities of RF (serum D, E, F). At higher levels of RF (>100 IU/ml, serum A, B and C) PEG6000 was more



**Fig. 4.** (A) Cytokine levels detected in RA patient sera by CBA for 10 cytokines before and after PEG6000/ Ig blockade. (B) Samples with available ELISA data (white bars) were compared with CBA data (Black).

efficient than sepharose-L; however, both methods were only capable of reducing RF levels, but not completely removing it.

**Sandwich ELISA**

Two IL-7 ELISAs were compared; low sensitivity (LS-, range 6–200 pg/ml, Diacclone) and high sensitivity (HS-, range 0.1–20 pg/ml, R&D). Discrepancies

between LS data (median 6.88, range 1.88–47.10) and expected data were observed as previously reviewed (1). In contrast the HS-IL-7 ELISA produced data similar to that expected range (11–12, 26–27) (median 14.33, range 10.33–23.95). We found no relationship between the two sets of ELISA results (n=20, correlation rho=0.004). To evaluate the impact of RF on the sandwich

ELISA, the two RF removal methods and direct Ig blocking were used with the HS-IL-7 ELISA. The mean levels of IL-7 following Ig blocking before and after RF removal were similar in each case with a small increase (>2 pg/ml) compared to untreated samples (Fig. 1B). Coefficients of Variation (CV) were calculated for each method and compared to the results obtained in untreated samples; CVs were similar in range (<18%) for all 3 categories of treatment. This confirmed that the presence of RF did not generate false positive results and has limited effect on both the efficiency and/or accuracy of the ELISA. The small discrepancy with untreated samples could be attributed to the concentration effect of immuno-complex removal, as indicated by the manufacturer.

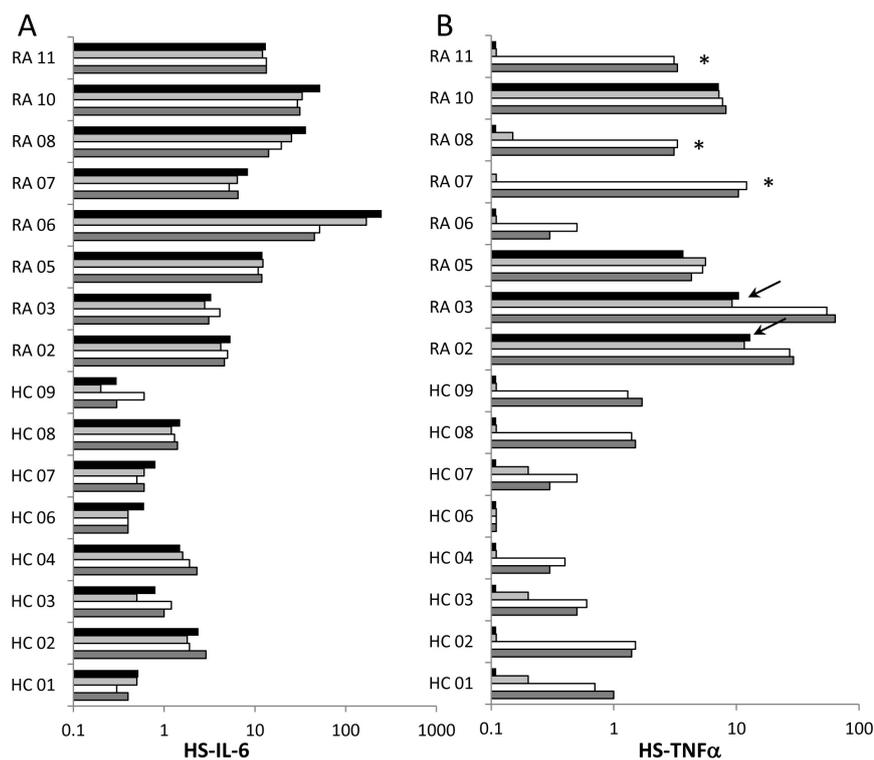
**Luminex: IL-7 single-plex**

Serum from 10 healthy controls was tested using normal-range single-plex IL-7 beads and compared with HS-ELISA IL-7 data. A strong correlation was observed between the single-plex and the HS-ELISA (rho=0.7950, data not shown).

Next, RA patient sera were tested using HS-ELISA (Fig. 1B) and single-plex IL-7 beads (Fig. 1C). Values obtained in untreated samples by single-plex ranged from 10.2–70.8 pg/ml. Direct Ig blocking as well as RF removal reduced the measurements of IL-7 (CV up to ~26% in RF-negative samples and >70% in RF-positive) confirming the occurrence of false positive. Furthermore, a direct relationship between IL-7 and RF titres was observed (Fig. 1D, for all samples n=9, rho=0.908 and for only RF-positive samples n=6, rho=0.972). RF removal (using PEG6000/Ig blockade) was unable to restore a direct relationship with results obtained by HS-ELISA (Fig. 1E) despite the final range of values obtained (9.7–19.8 pg/ml) being close to those of the HS-ELISA (7.0–15 pg/ml).

**Luminex: 25 multi-plex**

Sixteen RA sera were tested using a 25-plex immunoassay before and after RF removal (using PEG6000/Ig blockade). The cytokines tested in-



**Fig. 5.** Cytokine levels detected in healthy control (n=8) and RA patient (n=8) sera samples tested by CBA/ELISA before (black/white) and after (light grey/dark grey) PEG6000/Ig blockade. Arrows indicate samples where the ELISAs detect higher levels than the CBA. Asterisks indicate loss of sensitivity by the CBA compared to the ELISA.

cluded IL-2, IL-7, IL-10, IL-12, IFN $\gamma$ , RANTES and TNF- $\alpha$ , (full list in the methods). RF range was <20–4096 IU/ml. As seen with the IL-7 singleplex, all cytokines exhibited false positive levels that were directly correlated with RF titres ( $\rho > 0.750$ ). Example data presented in Figure 2A, for IFN $\gamma$   $\rho = 0.882$ , IL-7  $\rho = 0.896$  and TNF- $\alpha$   $\rho = 0.873$  (n=11 RF-positive samples closed diamonds n=6 RF-negative samples closed triangles).

As with the pilot study, the efficiency of RF removal remained variable, most notably for samples with titres >100 IU/ml. Following RF removal, the level of cytokine detected decreased with CV up to ~22% in RF-negative and up to 96% in RF-positive samples. However, detected levels remained directly related to RF titres (Fig. 2A, open diamonds for RF-positive and open triangle for RF-negative samples, IFN $\gamma$   $\rho = 0.888$ , IL-7  $\rho = 0.855$  and TNF- $\alpha$   $\rho = 0.924$ ) suggesting that residual RF interference was still generating false positive.

Finally, comparison between Luminex

data after RF removal and ELISA data (Fig. 2B) suggested absence of correlation in RF-positive samples (closed symbols) but a relationship in RF-negative samples despite the low number of sera tested (n=5, open symbols IFN $\gamma$   $\rho = 0.779$ , IL-7  $\rho = 0.786$  and TNF- $\alpha$   $\rho = 0.782$ ). Furthermore, the cytokine levels, detected by Luminex, matched quite closely those elucidated using ELISA however, only in RF-negative samples (Fig. 2B).

#### Multiplex ELISA:

##### Membrane based and Mosaic ELISA

The ability to multiplex solid phase ELISA appears very appealing. Systems were developed using either membrane-based dot-blot (Fig. 3A) or plastic-based dot-ELISA (Mosaic, Fig. 3B). Both systems were tested using RA serum samples, untreated and after RF removal using PEG6000/Ig blockade. The detection of 20 different molecules was little affected by the presence of RF (Fig. 3C, CV <25%); however, several cytokines of major interest were not detected (including TNF- $\alpha$ , IL-1B,

IL-12p70, IL-2, IL-4, IL-5, IL-17A, MCP-1, MIP-1 $\alpha$ , data not displayed in Fig. 3C) but known to be present as they were detected by direct sandwich ELISA in these same samples (data not shown). Similarly, Mosaic ELISA provided results indicating no interference of RF but low sensitivity, thus not allowing cytokine detection in serum samples (Fig. 3B).

#### Cytometric bead assay

A 10-plex (IL-2, IL-6, IL-7, IL-10, IL-12, IL-17A, IFN $\gamma$ , TNF- $\alpha$ , MIP-1 $\alpha$  and RANTES) cytometric bead assay (CBA), was used to test RA serum cytokines for which some had matched ELISA data. In untreated sera, cytokines (with the exception of RANTES) were detected in some, but not all samples, however, IL-2 was not detected at all (Fig. 4A, black bars). Cytokine levels were not greatly affected by RF removal (white bars, CV remained <27%) suggesting lack of interference and no false positivity.

Cytokine levels detected by CBA (Fig. 4B, black bars) were then compared to those detected by ELISA (white bars). For most samples levels of IL-7, IL-12, MIP-1 $\alpha$  and RANTES were similar, but for IL-10, several samples showed lower levels detected by CBA suggesting issues with the sensitivity of the assays. IL-2 detected by ELISA was not detected by CBA.

More recently high sensitivity CBA beads have become available for a few cytokines with further new releases in the pipeline. We therefore tested two cytokines in healthy control (HC) and RA samples; TNF- $\alpha$  and IL-6 using both HS-CBA and HS-ELISA assays (Fig. 5). Results showed quite good correlation between ELISA and CBA data (irrespective of RF removal) for IL-6, notably in HC where levels were quite low compared to RA patients. In contrast, it was apparent that CBA remained limited in its detection efficiency for TNF- $\alpha$ , not only in samples with low levels (below 1 pg/ml) as detected by ELISA, but also in some RA samples with quite high levels (indicated by stars). Furthermore, levels detected by the CBA were often less than by ELISA (indicated by arrows).

## Discussion

Cytokines play a central role in the normal processes of inflammation and repair in the body. Several cytokine targets have been successfully used as biotherapeutic agents for malignant, inflammatory and infectious disease; with blockade of proinflammatory cytokines well-established as a therapeutic tool for autoimmune diseases, such as RA and SLE. Several anti-cytokine agents are available with more emerging; despite demonstrating significant success, heterogeneity of response is evident. The challenge in such diseases is to identify predictors of response to enable a more effective personalised management. The accurate measurement of cytokines is likely to be a critical component of this. In this study, we sought to investigate the interference by residual IgG RF on the measurement of cytokines in the serum of patients with RA using several multiplex platforms. The PEG6000 and sepharose-L methods act by physical removal of the immuno-complexes and the serum only/Ig blockade method causes the formation of immune-complexes (5), which are then removed by centrifugation. In our hands, whilst the sepharose-L method was efficient at removing relatively low RF quantities and the PEG6000 method slightly more efficient, neither of these methods (in addition to Ig blockade) was completely effective and residual RF still generated false positive results, hence the cytokine data post RF removal remained related to RF titre in the samples. In solid phase assays (membrane-based ELISA or Mosaic ELISA), RF did not seem to interfere with detection, nor did this occur in the CBA. The sensitivity was however not sufficiently robust in these assays, even when using improved high sensitivity CBA beads.

Several studies have reported cytokine levels in RA patients using solid phase multiplexing technology. These included Luminex (5, 7-9), RayBiotech (a glass chip/chemilluminescence platform) or SearchLight (multiplex sandwich ELISA) (8), and take account of interference by heterophilic antibodies by using Heteroblock (a commercially available blocking reagent optimised for two-site immuno-assays), protein L

spharose, column affinity absorption or Ig-blockade. These showed reduction of signal in RF-positive sera but not in negative samples. One study considered the titres of RF and concluded that only high-titre RF samples (>100 IU/ml) showed false positive results (8). These data are confirmed in a more detailed manner by our evidence of direct relationships between RF titres and the generation of false positive values. Another study correlated the presence of high levels of cytokines (using Luminex following protein L-sepharose RF removal) with the presence of anti-citrullinated peptide antibodies (ACPA); another autoantibody associated with RA (9). Seropositivity for ACPA is often associated with the presence of RF in RA (~90% overlap). Our data therefore suggest an alternative explanation: the lack of effective RF removal (sepharose-L or Heteroblock) may still allow for the generation of false positive data and may bring into question the relationship between cytokines and the presence of ACPA.

In conclusion, our results demonstrate that RF removal remains inefficient in samples with titre >100 IU/ml hence in most RA patients. Furthermore, multiplex Luminex bead assays are particularly affected by interference with heterophilic antibodies such as RF, whilst the other solid-phase assays tested appear to be less affected by the presence or absence of RF. Multiplexing cytokine measurement will therefore remain a challenge until highly reliable assays are developed, potentially utilising an improved version of CBA (high sensitivity). In the meantime, sandwich ELISAs offer a reliable alternative, albeit with the known constraint of required sample volume limiting the number of analytes that can be simultaneously profiled.

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