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Research paper

Performance evaluation of cytometric bead assays for the measurement of lung cytokines in two rodent models ☆

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Abstract

There is a growing demand for a cost-effective, efficient, and high-throughput method for measuring cytokines. Currently, many studies are using flow cytometric bead-based multiplex assays in the measurement of cytokines. However, limited data are available regarding the performance of these cytometric bead assays versus enzyme-linked immunosorbent assay (ELISA) or correlation with mRNA expression using real time reverse transcriptase-polymerase chain reaction (RT-PCR). In one of our studies, cytometric bead array (CBA) was used to measure inflammatory cytokine protein levels in bronchoalveolar lavage (BAL) samples from mice exposed to welding fume, an inflammatory particulate. The results were then compared to whole lung mRNA levels of the same cytokines measured by real time RT-PCR in the same mouse model. It was found that the trends in cytokine profiles measured via CBA agreed with the whole lung mRNA results. In a separate experiment, we used a rat zymosan infectivity model to induce a pulmonary immunomodulatory response and determined cytokine concentrations in recovered BAL fluid by ELISA and two different types of cytometric bead-based assays, CBA and FlowCytomix (FC). The sample-to-sample correlation was good between ELISA and CBA with correlation coefficient R values of 0.76, 0.66, and 0.92 for rat IFN-γ, TNF-α, and IL-6, respectively. ELISA only correlated significantly with the FC assay for TNF-α with $R=0.43$. Patterns of cytokine response in our rat model also differed among the assays but overall, the ELISA and CBA yielded similar results. For a method-to-method comparison, we assayed supplied cytokine standards from ELISA kits using both ELISA and CBA to determine the R values and found it to be greater than 0.90 for all the cytokines tested. It was found that the ELISA was more sensitive in the low range of the standard curve while the bead assays were capable of detecting higher protein concentrations, which would allow for direct measurement of concentrated samples. There was a lack of agreement between the absolute protein values for the ELISA and flow cytometric bead-based assays; in most cases, the latter method tended to give higher protein concentrations than ELISA. In conclusion, direct comparisons between absolute protein values did not agree among the assays tested in this study, but patterns of

Abbreviations: CBA, cytometric bead array; RT-PCR, Reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assays; BAL, bronchoalveolar lavage; FC, FlowCytomix; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMA-SS WF, manual metal arc-stainless steel welding fume.

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cytokine response generally agreed between ELISA and CBA. In the case of the mouse CBA, a companion measurement is recommended if samples with low concentrations of an analyte are reported and extrapolated below sensitivity or zero.
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Keywords: Cytokines; ELISA; Flow cytometric bead assays; Multiplex immunoassay; RT-PCR

1. Introduction

Cytokine profiling can provide valuable mechanistic data for many inflammatory and immune disease states. A growing demand for a cost-effective, efficient, and high-throughput method of measuring cytokines exists. Currently, ELISA and RT-PCR are the most commonly accepted methods for the measurement of cytokines. Unfortunately, ELISA can only measure one analyte at a time, and real time RT-PCR does not detect native protein. In addition, a traditional ELISA for a single cytokine can take up to 4 h to complete; therefore, performing multiple ELISA assays for a number of different cytokines can be very expensive and time-consuming.

Currently, new methods are being used for the measurement of cytokines in biological samples, e.g., cytometric bead array (CBA; BD Biosciences, San Diego, CA) and FlowCytomix (FC; Bender MedSystems, Vienna, Austria). Multiplexing cytometric bead assays have been developed that allow for the simultaneous detection of multiple analytes. Fluorescently-labeled microbeads are coated with antibodies that specifically react with different analytes in a multiplex system. The microspheres can be differentiated by size and distinct fluorescence intensity using flow cytometry. Each microsphere provides a capture surface for a specific protein, similar to an individually coated well in an ELISA plate.

Flow cytometric multiplexed bead assays offer numerous advantages when compared with existing methods (Edwards et al., 2004; Diaz et al., 2006). For example, the user can simultaneously measure multiple analytes in a single sample volume and generate standard curves for all analytes from one standard mixture, thereby reducing analytical time, effort, and cost. Because of an increased surface area provided by the microbeads, the required sample volume is significantly less than the volume necessary for a conventional ELISA assay. The use of fluorescence, rather than colorimetric measurements, may provide more sensitivity and a broader range for cytokine quantitation. Flow cytometric bead assays allow for automated high-throughput screening, potentially improving reproducibility and reducing human error.

Despite the many potential advantages of flow cytometric bead-based assays, few comparative studies of these assays with other, more traditional, methods have been performed. The goal of the present study was to compare cytokine profiles obtained with two bead-based flow cytometric assays, real time RT-PCR, and ELISA in two rodent models. Previously established animal models of mouse lung inflammation (Solano-Lopez et al., 2006) and rat lung infection (Antonini et al., 2004; Young et al., 2006) were utilized to induce a lung inflammatory cytokine response which was subsequently measured by the aforementioned methods.

2. Materials and methods

2.1. Animals

All animal procedures were performed using protocols approved by the National Institute for Occupational Safety and Health Institutional Animal Care and Use Committee.

2.1.1. Mice

Male C57BL/6J and A/J mice, 3.5–4 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited, specific pathogen-free, environmentally controlled facility. Sentinel mice were free of endogenous viral pathogens, parasites, mycoplasmas, *Helicobacter*, and CAR Bacillus. Mice were separately housed in ventilated cages and provided HEPA-filtered air under a controlled light cycle (12 h light/12 h dark) at a standard room temperature (22–24 °C). Animals were acclimated to the animal facility for a minimum of one week and allowed access to a conventional diet (6% Irradiated NIH-31 Diet, Harlan Teklad, Madison, WI) and tap water *ad libitum*.

2.1.2. Rats

Specific-pathogen-free male Sprague–Dawley [H1a: (SD) CVF] rats approximately 7–8 weeks old at arrival (~225 g–250 g) were purchased from Hilltop Lab Animals (Scottdale, PA) and housed in an AAALAC-accredited, specific pathogen-free, environmentally

controlled facility. Sentinel rats were free of endogenous viral pathogens, parasites, mycoplasmas, *Helicobacter*, and CAR Bacillus. Rats were separately housed in ventilated cages and provided HEPA-filtered air under a controlled light cycle (12 h light/12 h dark) at a standard room temperature (22–24 °C). Animals were acclimated to the animal facility for a minimum of one week and allowed access to a conventional diet (6% Irradiated NIH-31 Diet, Harlan Teklad, Madison, WI) and tap water *ad libitum*.

2.2. Mouse: Welding fume inflammation study

2.2.1. Welding fume collection and characterization

Welding fume (WF) was collected during manual metal arc (MMA) welding using a stainless steel (SS) electrode (MMA-SS WF) and characterized as previously described (Antonini et al., 1999). Metal analysis indicated that the MMA-SS WF was composed of Fe, Cr, Mn, Ni, and trace metals at 41.1, 28.5, 16.7, 2.53, and 11 wt.%, respectively. In addition, the particle size of the fume was found to be of respirable size with a count mean diameter of <2.0 μm.

2.2.2. Welding fume exposure

MMA-SS WF was suspended in sterile Ca^{+2} and Mg^{+2}-free phosphate buffered saline (PBS) and sonicated for 1 min with a Sonifier 450 Cell Disruptor before each exposure (Branson Ultrasonics, Danbury, CT, USA). Mice were exposed four times, once every 3 days, to 85 μg (total dose equal to ~20 mg/kg or 340 μg) of MMA-SS WF by pharyngeal aspiration as previously described (Rao et al., 2003). The volume aspirated for each exposure was 25 μl and control mice were administered an equal volume of sterile PBS.

2.2.3. Mouse lung fluid recovery

Bronchoalveolar lavage (BAL) of the whole lung was performed 2 days after the fourth PBS or MMA-SS WF exposure. The mice were anesthetized with an overdose of Sleepaway (26% sodium pentobarbital, 7.8% isopropyl alcohol and 20.7% propylene glycol, Fort Dodge Animal Health, Fort Dodge, IA), then weighed. The mouse was exsanguinated via the vena cava and the trachea cannulated with a blunted 22 gauge needle. While continually massaging the thorax, 0.6 ml of cold sterile PBS was slowly instilled into the lung then withdrawn and placed into a 15 ml conical tube. This constituted the first fraction BAL fluid. The BAL fluid fractions for each animal were preserved on ice until four animals were sacrificed and then the samples were centrifuged (500 x*g*, 10 min, 4 °C). Aliquots of the first fraction acellular BAL supernatant were frozen at −80 °C for later cytokine analysis.

2.2.4. Mouse cytokines

Analysis of cytokines from the acellular BAL fluid was conducted using a mouse inflammation cytometric bead array kit (CBA; BD Biosciences, San Diego, CA) and was analyzed on a FACSCalibur flow cytometer. Standard curves were determined for each cytokine from a range of 20–5000 pg/ml. The lower limit of detection for the CBA, according to the manufacturer is 2.5–52.7 pg/ml, depending on the analyte. The following cytokines were measured: interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-12p70 (IL-12p70).

2.2.5. Real time RT-PCR

In a separate identical experiment, whole lungs were removed from PBS and MMA-SS WF-exposed mice then snap frozen in liquid nitrogen and stored at −80 °C for later RNA isolation. Using the TRIzol (Invitrogen, Carlsbad, CA) method, RNA was isolated from whole lung homogenates and then cleaned according to the manufacturer's instructions using an RNeasy Mini Kit (Qiagen, Valencia, CA). One μg of total RNA was reverse-transcribed using random hexamers (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen, Carlsbad, CA). Five μl of cDNA (in duplicates for each gene) was then used for gene expression determinations. Pre-designed Assays-on-Demand™ TaqMan® probes and primers (Applied Biosystems, Foster City, CA) were used for each gene of interest. The ribosomal subunit 18S was used as the housekeeping gene. Relative gene expression was calculated using the comparative threshold method ($2-\Delta\Delta Ct$) (Livak and Schmittgen, 2001).

2.3. Rat: Zymosan infectivity study

2.3.1. Zymosan treatment

Zymosan A was purchased from Sigma Chemical Company (St. Louis, MO). On day 0, rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% (w/v) solution of sodium methohexital (Brevital; Eli Lilly, Indianapolis, IN) and intratracheally instilled with zymosan at a dose of 2.5 mg/kg body weight or PBS as a vehicle control. The selection of the zymosan dose was based on a previous dose–response study that indicated 2.5 mg/kg body weight was the concentration

shown to induce a measurable inflammatory response in the lungs (Young et al., 2001).

2.3.2. Pulmonary bacterial inoculation

Listeria monocytogenes was cultured overnight in brain–heart infusion broth (Difco Laboratories, Detroit, MI) at 37 °C in a shaking incubator. Following incubation, the bacterial concentration was determined spectrophotometrically at an optical density of 600 nm. The sample was diluted to the concentration of 5×10^5 *L. monocytogenes* in 500 μl sterile PBS and was intratracheally instilled 3 days after zymosan instillation. The dose of *L. monocytogenes* used was found to give a uniform infection and did not result in mortality in untreated naïve Sprague–Dawley rats previously from our laboratory (Antonini et al., 2001).

2.3.3. Rat lung fluid recovery

At day 3 (before infection) and 6, 8 and 10 days (post-infection), the rats were anesthetized with an overdose of Sleepaway and then exsanguinated by severing the abdominal aorta. The left bronchus was clamped off, and BAL was performed on the right lungs of rats. The lungs were lavaged with 1 ml/100 g BW of PBS. This first BAL fluid fraction was centrifuged (500 x*g*, 10 min, 4 °C). Aliquots of the acellular BAL supernatant were frozen at −80 °C for later cytokine analysis.

2.3.4. Rat cytokines

The ELISA kit for the rat cytokines TNF-α, IL-6, and IFN-γ were purchased from Biosource (Camarillo, CA). The measurement of cytokines by ELISA was performed according to the manufacturer's instructions. Cytometric bead array (CBA) for rat IFN-γ, IL-6, and TNF-α were purchased from BD (San Diego, CA). A FlowCytomix kit (FC) which measure rat cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1α, IL-4, MCP-1, and TNF-α was purchased from Bender MedSystems (Burlingame, CA).

BAL fluid samples were analyzed for IFN-γ, IL-6, and TNF-α by using the CBA and FC multiplex cytometric bead assays and ELISA. Standard curves for each cytokine, from each kit, were generated by using the reference cytokine concentrations supplied by the manufacturers. Raw data of the FC bead assay were analyzed by FlowCytomixPro1.0 software. For CBA raw data, the quantification and analysis of rat BAL cytokines were done in Excel on raw data exported from the FACSCalibur flow cytometer. This approach was taken because the recommended software, Flow Cytometric Analysis Program (FACP), failed to recognize bead clusters that were close to each other for some analyses.

2.4. Statistical analysis

Statistical analysis was performed using SigmaStat v3.11 software (Systat Software, Inc.). All data are presented as means ± standard error of measurement (SEM). The significance was set at $p \leq 0.05$. Analyses for the RT-PCR data were done by Student's *t*-test. Pearson correlation analysis was used to calculate the correlation coefficient between ELISA, CBA and FC. A *t*-test was used to compare zymosan-treated group with saline group at each time point.

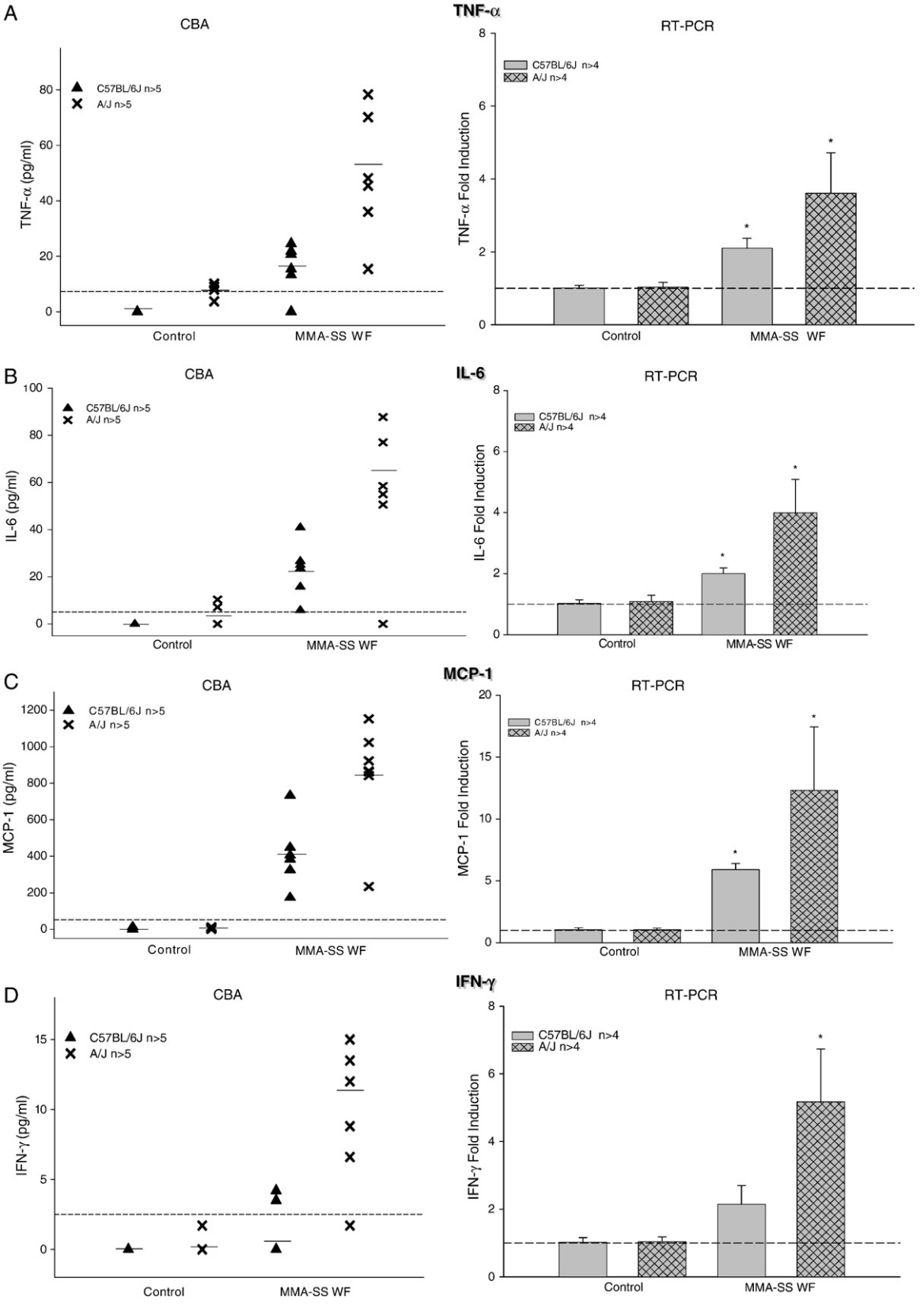
3. Results and discussion

3.1. Comparison of real time RT-PCR with cytometric bead assay for mouse inflammatory cytokine profiles

To confirm the results from the CBA assay, we compared the cytokine protein results with whole lung gene expression levels following pharyngeal aspiration of MMA-SS WF in mice at 2 days post-exposure. This was done because BAL protein concentrations occasionally were at or below the sensitivity level for a particular CBA cytokine which limited statistical analysis. Therefore, confirmation by another method was warranted. Unfortunately, ELISA could not be used to confirm the results from CBA due to limited (~400 μl) BAL sample so mRNA from whole lung, previously harvested in an identical experiment, were used for real time RT-PCR. RT-PCR was chosen as a confirmatory method in this study because inflammatory cytokine gene expression generally corresponds well with protein levels (Stemme et al., 2001).

Fig. 1A compares BAL TNF-α protein and whole lung gene expression for two mouse strains, C57BL/6J and A/J, following WF exposure. Fig. 1A, left panel shows that WF-induced TNF-α protein levels, measured by CBA, were greater in exposed A/J mice compared to

Fig. 1. Comparison between mouse TNF-α (A), IL-6 (B), MCP-1 (C), and IFN-γ (D) protein levels from CBA analysis and mRNA levels from whole lung homogenates in C57BL/6J and A/J mice at 2 days after exposure to MMA-SS WF. The dotted line represents the assay sensitivity and mean lines (—) is shown for each group. Due to limitations in assay sensitivity, statistical analysis could not be performed on CBA data. Values are means ± SEM ($n = 4$–5 per group). The asterisk (*) indicates a significant increase ($p < 0.05$) versus the corresponding control level.


A
CBA
C57BL/6J n>5
A/J n>5
TNF-α (pg/ml)
Control
MMA-SS WF
TNF-α
RT-PCR
C57BL/6J n>4
A/J n>4
TNF-α Fold Induction
Control
MMA-SS WF
B
CBA
C57BL/6J n>5
A/J n>5
IL-6 (pg/ml)
Control
MMA-SS WF
IL-6
RT-PCR
C57BL/6J n>4
A/J n>4
IL-6 Fold Induction
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MMA-SS WF
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RT-PCR
C57BL/6J n>4
A/J n>4
IFN-γ Fold Induction
Control
MMA-SS WF

Table 1
Method-to-method correlation between ELISA and CBA

Cytokines	Correlation coefficient	CBA values relative to ELISA value
Rat IL-4	0.99	$y=4.03x+7.02$
Rat TNF-α	0.99	$y=1.80x-1.38$
Rat INF-γ	1.00	$y=11.7x+178$
Rat IL-6	1.00	$y=0.92x-4.74$
Rat IL-10	0.93	$y=0.76x-1.41$
Mouse TNF-α	0.98	$y=0.69x+3.78$
Mouse MCP-1	1.00	$y=2.47x+22.78$

Table 2
Sample-to-sample correlation between ELISA and CBA or FC

Cytokines/vender	CBA	FC
Rat IFN-γ	$R=0.76$	$R=0.10$
	$p<0.001$	$p=0.38$
Rat TNF-α	$R=0.66$	$R=0.43$
	$p<0.001$	$p<0.001$
Rat IL-6	$R=0.92$	NA
	$p<0.001$	

Note. NA = not available.

the C57BL/6J mice. A similar profile was observed with TNF-α mRNA levels (Fig. 1A, right panel). Taken together, these data suggest that changes in TNF-α protein, detected by CBA, were confirmed by whole lung gene expression findings using real time RT-PCR in this model.

Fig. 1B, C, and D also demonstrate similar trends for protein levels and gene expression profiles for IL-6, MCP-1 and IFN-γ, respectively. IL-6, MCP-1, and IFN-γ BAL protein levels were higher in MMA-SS WF-exposed A/J mice compared to the C57BL/6J strain (Fig. 1B, C, and D, left panel). Gene expression also

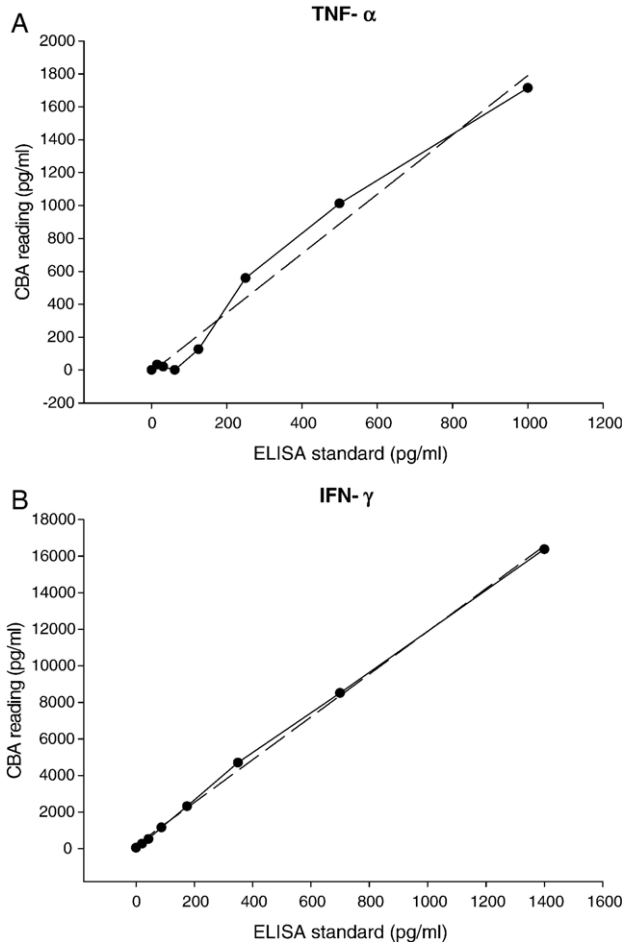

Fig. 2. Typical standard curves comparing rat TNF-α (A) and IFN-γ (B) values as determined by CBA and ELISA. The standard solutions for TNF-α and IFN-γ from ELISA kit were used for both CBA and ELISA measurements. The dashed line represents linear relationship. The correlation coefficients for TNF-α and IFN-γ when comparing CBA and ELISA measurements were 0.99 and 1.00, respectively. However, TNF-α and IFN-γ concentrations as measured by CBA were approximately 1.8 and 11.7-fold higher than ELISA values, respectively.

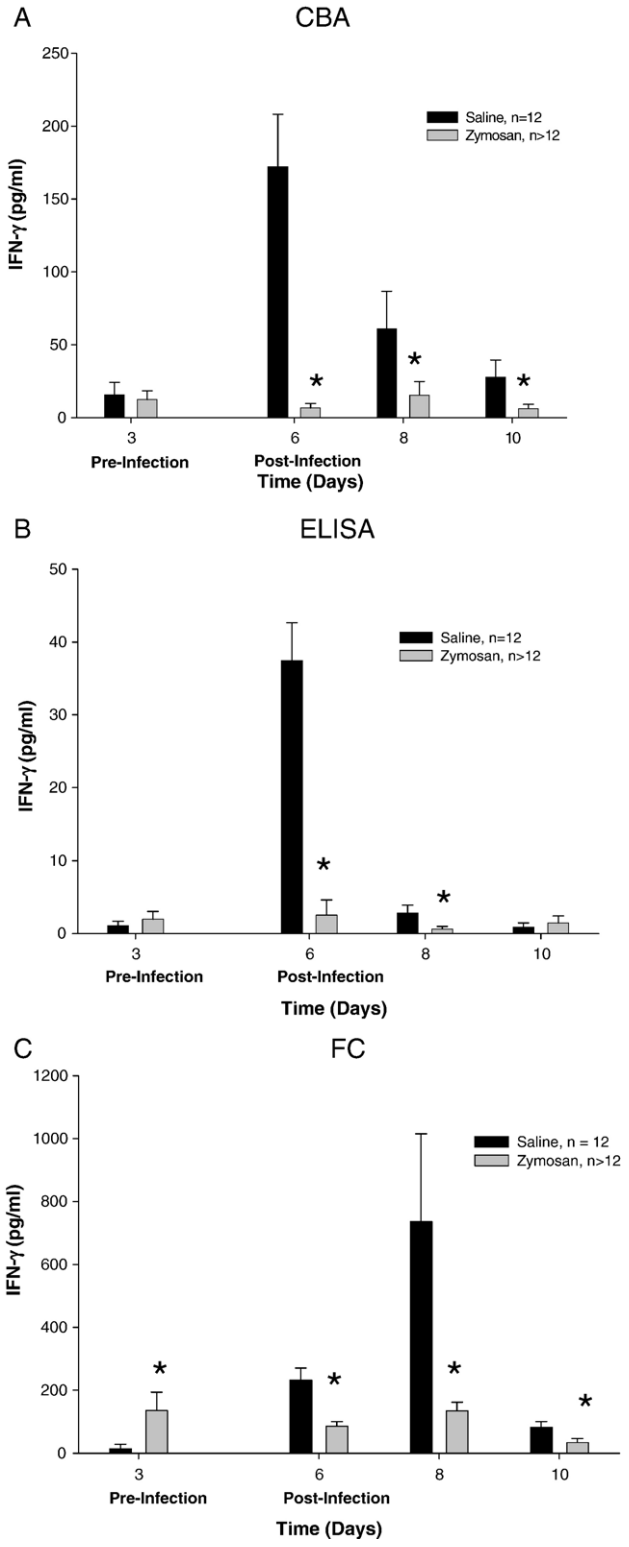

Fig. 3. Comparison of IFN-γ values as determined by CBA (A), ELISA (B), and FC (C) for BAL fluid samples collected from *Listeria* infected zymosan-treated rats. Note the difference in scale on the *y*-axis in all panels. The asterisk (*) indicates a significant difference ($p < 0.05$) versus the saline control level.

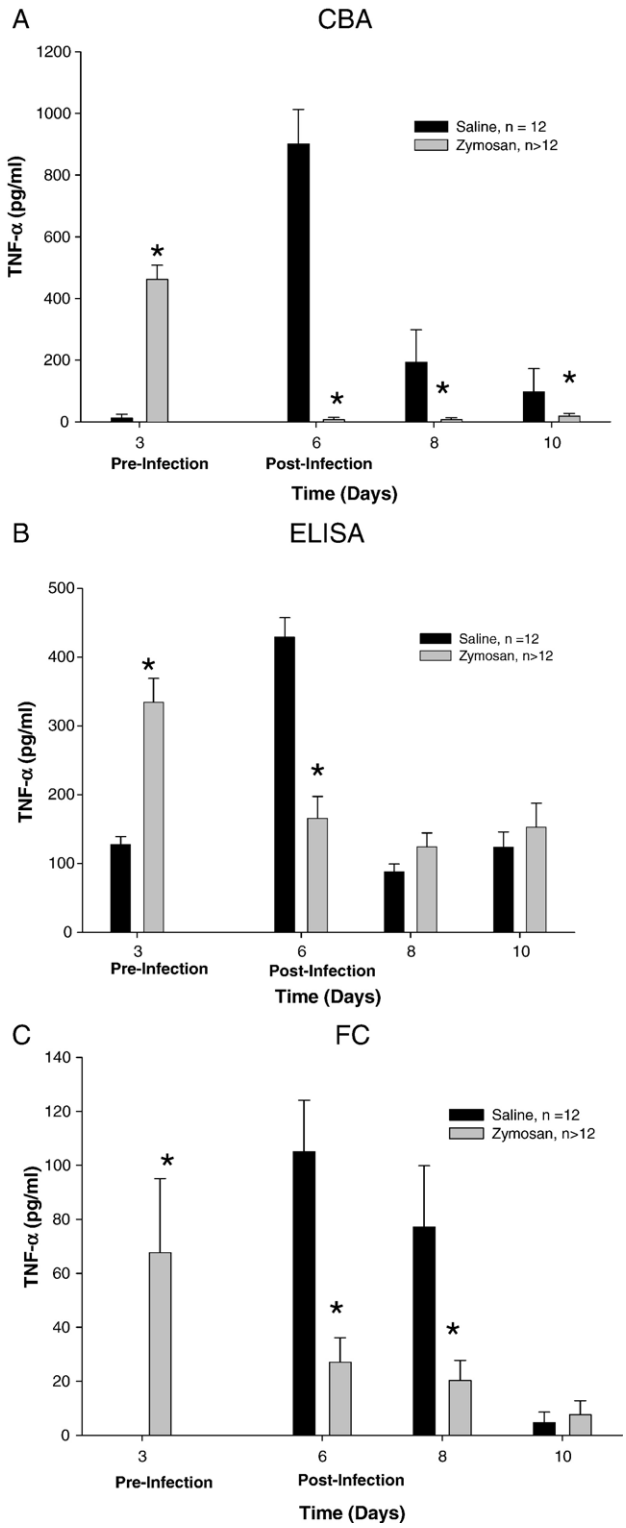

Fig. 4. Comparison of TNF-α values as determined by CBA (A), ELISA (B), and FC (Fig. 4C) for BAL fluid samples collected from *Listeria* infected zymosan-treated rats. Note the difference in scale on the *y*-axis in all panels. The asterisk (*) indicates a significant difference ($p < 0.05$) versus the control level.

revealed at least a 2-fold induction difference for these cytokines between the two mouse strains (Fig. 1B, C, and D, right panel). Therefore, a good correlation was found between mouse inflammatory cytokine BAL protein and whole lung gene expression. It was noted that protein levels of IL-10 and IL-12p70 were unaltered by exposure and in turn eliminated from further confirmation by RT-PCR (data not shown).

3.2. Comparison of cytometric bead assays and ELISA for rat cytokine measurement

3.2.1. Method-to-method comparison

In order to determine the absolute value differences between a flow cytometric bead assay and ELISA, ELISA kit standards were measured via the CBA assay. Table 1 shows the correlation coefficients and the corresponding linear relationships between the CBA and ELISA readings on five rat and two mouse cytokines. CBA correlated well with the ELISA results with a typical correlation coefficient greater than 0.90 (Table 1). Fig. 2 demonstrates a typical linear relationship observed between the CBA and ELISA for rat TNF-α and IFN-γ with a correlation coefficient of 0.99 and 1.00, respectively. However, CBA values for TNF-α and IFN-γ were higher than ELISA by approximately 1.8 and 11-fold, respectively (Table 1). Among the cytokines tested, rat IL-10 and mouse TNF-α values were lower than those obtained by ELISA. The values obtained for rat IL-6 showed the best agreement between the two assays (Table 1). Similarly, a report from Karlsson et al. (2003) also shows that values from cytokine flow cytometry assays were consistently higher than those from an ELISPOT (enzyme-linked immunospot).

3.2.2. Sample-to-sample comparison

BAL fluid samples from zymosan-exposed rats were assayed using the CBA, FC, and ELISA and the results were compared. Table 2 shows the correlation coefficients between the ELISA and CBA or FC assays for rat IFN-γ, TNF-α, and IL-6. IFN-γ levels as measured by CBA had a correlation coefficient of 0.79 compared with ELISA ($p<0.001$), whereas a correlation coefficient of 0.10 was observed for the FC assay versus with ELISA. A p value of 0.38 (>0.05) was obtained, indicating no statistically significant correlation between the FC method and ELISA when measuring rat IFN-γ. The correlation coefficient for rat TNF-α was 0.66 ($p<0.001$) for the CBA, whereas the FC assay had a lower, but significant, correlation ($R=0.43$) versus ELISA. Again, a highly significant correlation ($R=0.92$, $p<0.001$) was found for IL-6 between the CBA and ELISA.

Table 3
Manufacturer specified sensitivity for ELISA, CBA, and FC

Cytokines	ELISA (pg/ml)	CBA (pg/ml)	FC (pg/ml)
Rat IFN-γ	<13	26.5	10.6
Rat TNF-α	<4	27.2	5.1
Rat IL-4	2.0	3.1	1.6
Rat IL-6	<8	60.3	NA
Rat IL-10	<5	127.0	NA

Note. NA = not available.

Fig. 3 illustrates the comparison of rat IFN-γ levels in BAL fluid as measured by CBA, FC, or ELISA. Fig. 3A and B show that the IFN-γ response post-infection, as determined by CBA, was similar to that measured by ELISA however, the absolute values for IFN-γ at each time point were higher with the CBA. In contrast, the IFN-γ response post-infection, as determined by the FC assay, was somewhat different when compared to the ELISA (Fig. 3C). In addition, the absolute values at each time point were higher in comparison to those obtained from ELISA or CBA (Fig. 3). Overall, data presented in Fig. 3 and Table 2 suggest that the two cytometric bead assays correlated differently when compared to ELISA but the CBA and ELISA yielded similar IFN-γ profiles in this study.

Fig. 4 shows the pattern for BAL fluid TNF-α pre- and post-infection in rats for the different assays. Results using the CBA (Fig. 4A) and ELISA (Fig. 4B) were similar in pattern, in most cases, at the different time points. Again, the cytokine response pattern for TNF-α as measured by the FC method (Fig. 4C) was different when compared to the ELISA. In addition, the absolute values for TNF-α were quite different in some instances when measured by the FC method compared to either ELISA or CBA.

3.2.3. Sensitivity of assays and standard curve range

A comparison of the reported assay sensitivity from the different methods is listed in Table 3. ELISA provides the highest sensitivity among the different assays. On the other hand, the CBA and FC methods offer a broader standard curve range versus ELISA. For example, the standard curve range for rat IFN-γ for ELISA, CBA, and FC are 21.8–1400, 20–20,000, and 13.7–10,000 pg/ml, respectively.

4. Conclusions

The CBA mouse inflammation kit performed well and may be of practical value for studies with low sample volumes. However, this kit may require confirmation by another method if samples with low concentrations of an

analyte are reported as extrapolated below sensitivity or zero. In the rat, the cytokine response profiles, but not the absolute values or fold change differences, were similar between ELISA and CBA in BAL samples. It was also found that CBA performed better than the FC when compared to ELISA. If sample sensitivity is an issue, ELISA would be preferred, but CBA offers advantages such as simultaneous analysis of multiple analytes and cost-efficiency.

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