Published in final edited form as:
Nat Biotechnol. 2002 April ; 20(4): 359–365. doi:10.1038/nbt0402-359.

Multiplexed protein profiling on microarrays by rolling-circle amplification

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Abstract

Fluorescent-sandwich immunoassays on microarrays hold appeal for proteomics studies, because equipment and antibodies are readily available, and assays are simple, scalable, and reproducible. The achievement of adequate sensitivity and specificity, however, requires a general method of immunoassay amplification. We describe coupling of isothermal rolling-circle amplification (RCA) to universal antibodies for this purpose. A total of 75 cytokines were measured simultaneously on glass arrays with signal amplification by RCA with high specificity, femtomolar sensitivity, 3 log quantitative range, and economy of sample consumption. A 51-feature RCA cytokine glass array was used to measure secretion from human dendritic cells (DCs) induced by lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α). As expected, LPS induced rapid secretion of inflammatory cytokines such as macrophage inflammatory protein (MIP)-1β, interleukin (IL)-8, and interferon-inducible protein (IP)-10. We found that eotaxin-2 and I-309 were induced by LPS; in addition, macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), soluble interleukin 6 receptor (sIL-6R), and soluble tumor necrosis factor receptor I (sTNF-RI) were induced by TNF-α treatment. Because microarrays can accommodat ~1,000 sandwich immunoassays of this type, a relatively small number of RCA microarrays seem to offer a tractable approach for proteomic surveys.

Several recent reports have established the feasibility of protein arrays for a variety of applications[1-7]. To meet the emerging needs of expression proteomics, however, such arrays must yield highly multiplexed, sensitive, quantitative, and reproducible measurements of protein levels. It is also desirable that assays on these arrays utilize small sample volumes and be compatible with hardware and software used by the DNA microarray industry. Microarrays of ordered immobilized capture antibodies and attendant sandwich immunoassays are a straightforward, near-term approach for highly parallel measurement of protein levels. Polyclonal or monoclonal antibodies for several thousand proteins are available, and are being supplemented with affinity probes generated by phage and ribosomal display, affibodies, and aptamers[8-11]. Indeed, recent studies have described sensitive[12,13] or highly multiplexed[14] arrays of ordered capture antibodies and sandwich immunoassays for measurement of protein levels. Concomitant achievement of adequate sensitivity and multiplexing for practical proteomics studies has not been demonstrated

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Competing interests statement
The authors declare competing financial interests: see the Nature Biotechnology *website (http://biotech.nature.com) for details.*

because unamplified immunoassays are insensitive, and conventional signal amplification procedures, such as enzyme-catalyzed chemiluminescence, have limited multiplexing capabilities because of signal diffusion.

RCA is a useful alternative for on-chip signal amplification[15-17].It permits sensitive and highly multiplexed assays on microarrays because RCA-amplified signals remain localized at the microarray spot (Fig. 1)[16,17]. When utilized on microarrays of printed proteins, RCA has been shown to allow detection of protein analytes with zeptomole sensitivity and broad dynamic range[16,18,19]. In the present study, we establish the utility of RCA for high-throughput analysis of protein expression on microarrays, providing assays that are highly sensitive, quantitative, and reproducible. We describe highly multiplexed, microarray immunoassays with four steps: sample application and protein capture by specific antibodies affixed to a microarray, binding of second antibodies to captured proteins, binding of a universal antibody to the second antibodies, and RCA signal amplification on the universal antibody. This approach was used to examine the time course of cytokine secretion by human DCs cultured with or without the maturation agents LPS or TNF-α (ref. [20]). This study confirmed the detection of a number of cytokines that have been previously reported to be induced in DCs, and also revealed the secretion of several proteins that had not been heretofore reported.

Results and discussion

Antibody microarrays

Microarrays were printed on thiolsilanecoated and crosslinker-activated glass slides divided by Teflon boundaries into 16 circular analysis sites of 0.5 cm diameter, or “subarrays”. This format minimized reagent consumption, segregated immunoassays into relatively small groups, and allowed different samples to be applied to each. Subarray spacing allowed automated processing by a liquid-handling robot with an eightpipette tip head. Cytokines representing both inflammatory and homeostatic groups were chosen for analysis (Table 1), because they represented low-abundance proteins whose absolute level was of biological significance[21-24]. Within each subarray, 256 “features” were printed at known locations. Approximately 150 of these represented 37–38 single monoclonal antibodies, each spotted in quadruplicate and each specific for a single cytokine. The remaining features represented internal calibrators (dilutions of cyanin 5 (Cy5)-labeled BSA) or controls for binding and signal amplification by the anti-biotin–DNA conjugate and RCA (biotin-labeled IgG). The latter features permitted standardization between subarrays and microarrays. Thus, each microarray slide allowed measurements of 75 human cytokines in eight samples. Coefficients of variation (CV), as measured by fluorescent intensity after incubation with Cy5-labeled goat anti-mouse antibody, were ~10% for quadruplicate features on the array, whereas CVs between different antibodies were ~25% (see Supplementary Figure 1 on the *Nature Biotechnology* website). Repeated testing of antibody microarrays stored dry at 4°C indicated that there was no loss in sensitivity for at least 1 month.

Immunoassays for each of the 75 cytokines were done with arrayed capture monoclonal antibodies and biotinylated, polyclonal, second antibodies. Because assays were performed simultaneously with a cocktail of 37–38 second antibodies applied to 37–38 capture antibodies on a subarray, antibodies were extensively evaluated for sensitivity, cross-reactivity, and nonspecific signals using purified cytokines, and approximately one half were replaced. Most remaining nonspecific signals were eliminated in the two groups of 37–38 immunoassays by iteratively switching between subarrays capture antibodies that gave nonspecific signals with particular polyclonal second antibodies. Residual cross-reactivity and nonspecific signals were minimized by optimization of washing and blocking conditions, antibody concentrations, and incubation times (see Supplementary Figure 2).

Sensitivity and specificity of cytokine detection by RCA

Amplification of immunoassay signals on microarrays was necessary because customary sensitivity using fluorescence detection without amplification was ~1 ng/ml; this sensitivity was insufficient for measurement of biologically significant cytokine perturbations (Fig. 2)[21-24]. Immunoassay sensitivity enhancement was achieved by covalent attachment of an oligonucleotide for RCA priming to an anti-biotin antibody with a stoichiometry of ~5:1. RCA was carried out with an 80-mer synthetic DNA circle and a DNA polymerase, yielding, as a single product, a long ribbon of single-stranded DNA after a 45 min reaction[15,25]. This product remained attached to the anti-biotin antibody and was detected by hybridization of complementary fluorescent oligonucleo-tides to tandem copies of circle sequence[16]. RCA was done at 37°C in an isotonic buffer of neutral pH. Nonspecific signals related to RCA were significantly less than those related to antibody cross-reactivity, and were minimized by appropriate washing and blocking conditions (see Supplementary Figure 3). RCA immunoassay sensitivity on microarrays, as measured by serial dilution of single, purified cytokines, was femtomolar, representing more than a 1,000-fold improvement (Fig. 2A): 45 (60%) of the 75 cytokine features had a sensitivity of ≤10 pg/ml, 22 (29%) had a sensitivity of ≤100 pg/ml, and 8 (11%) had a sensitivity of ≤1,000 pg/ml (Table 1). For 21 of the cytokines represented on the chip, no corresponding ELISA kit is commercially available. Importantly, the dynamic range and precision of RCA microarray immunoassays was similar to unamplified immunoassays (approximately 3 orders of magnitude dynamic range with an array scanner (Fig. 2) and precision of ~5% CV for assays carried out on four arrays, even at low cytokine concentrations (see Supplementary Figure 4)). Femtomolar sensitivity and 3 log dynamic range are adequate for measurement of most biologically relevant changes in cytokine secretion and, probably, for most biologically relevant changes in protein expression (Table 1)[26-28].

Specificity of RCA microarray immunoassays was examined in three ways. First, microarrays were incubated with relatively high concentrations of 2 cytokines (1 ng/ml macrophage chemoattractant protein (MCP)-1 or fibroblast growth factor (FGF)-7), followed by detection with 24 biotinylated antibodies and RCA. Signals were only observed at the appropriate features, and signal:noise ratio was >100:1 (see Supplementary Figure 5A). Second, serial dilutions of a mixture of 7 cytokines were applied to microarrays, followed by detection with 7 biotinylated antibodies and RCA. Dose-dependent signals were observed only at the appropriate features, and sensitivity for each was 1–10 pg/ml (Fig. 2B, C). Third, a mixture of 10 cytokines (100 pg/ml) was applied to microarrays, but detection was with biotinylated polyclonal antibodies to only 4, followed by RCA. Signals were observed only where both cytokine and specific polyclonal antibody were present (see Supplementary Figure 5B). Low, fixed, nonspecific signal was detected with a few cytokines. This nonspecific signal could be eliminated by adsorbing secondary antibodies on mouse IgG (data not shown), but this was unnecessary in practice.

Application of antibody arrays to Langerhans cell maturation

Dendritic cells are critical in both initiating and directing the immune response. DCs, which include multiple subsets[29], sample and process antigen and then, given the proper environmental cues, mature, migrate, and present these antigens to naive T-cell populations in draining lymph nodes. An earlier version of the microarray consisting of 51 different anti-cytokine antibodies was used to study factors secreted during maturation of Langerhans cells (LCs), a type of DC found in the epidermis of skin, to shed light on LC signals for helper T-cell (T_H) maturation. The identification of such cytokines is important, because LCs elaborate numerous cytokines that affect both the development of LCs and lymphocytes in the surrounding area[29,30]. Factors secreted by LCs at certain stages of differentiation induce naive helper T lymphocytes (T_{H0}) to differentiate into either T_{H1} or T_{H2} cells that, in turn,

stimulate cellular or humoral immune responses, respectively. Following antigen stimulation, the relative induction of T_{H1} and T_{H2} populations is believed to be an important determinant of immune response and immunopathology. Synchronized LC maturation can be induced *in vitro* by LPS or TNF-α (ref. [20]); LPS induces LC differentiation to maturity, associated with cytokine secretion sufficient to stimulate $T_{H0} \rightarrow T_{H1}$ transition, whereas TNF-α treatment drives LCs to an intermediate stage of differentiation, associated with cytokine secretion that stimulates $T_{H0} \rightarrow T_{H2}$ transition[20,30].

$CD34^+$ cells from granulocyte colony-stimulating factor (G-CSF)–treated patients were cultured without FCS (to allow precise measurement of secreted proteins) in medium containing a defined cocktail of cytokines for 7–8 days. Resultant immature LCs were isolated and recultured for 4 days in the presence of either LPS, TNF-α, or unsupplemented growth medium, as described[20]. Supernatant samples were collected at six time points after start of reculture, and the levels of 51 cytokines in each sample were measured simultaneously in duplicate on the protein chips. The fluorescence intensity of microarray features was averaged for each feature and sample, and the resulting time courses of cytokine secretion were determined (see Supplementary Figure 6). For selected cytokines, fluorescence intensities were converted to protein levels with standard curves generated from serial dilutions of purified analytes in unsupplemented growth medium (Fig. 3).

Protein chip analysis revealed that about one third of the cytokines represented on the 51-feature microarray increased in abundance at least fourfold during the 72 h culture (Fig. 4). LPS induced 16 cytokines by at least fourfold, whereas TNF-α induced 12. Only 3 cytokines were secreted by LCs cultured without addition of LPS or TNF-α; in no case was a cytokine secreted in untreated LCs but not in LPS- or TNF-α-treated cells. In contrast, 6 cytokines (IL-1β, IL-6, IL-8, IL-11, IL-12, and CNTF) were specifically induced by LPS, and 1 cytokine (ANG) was induced only by TNF-α (Fig. 4). A total of 9 cytokines (Eot-2, I-309, IP-10, MCP-1, RANTES, MIP-1β, IL-6sR, TARC, and MDC) were induced by both LPS and TNF-α, although the levels and time course of secretion induced by the treatments were usually significantly different (Fig. 3). It was clear, therefore, that multiplexed measurements of cytokine secretion on these chips were useful for characterizing LC activity during induced differentiation.

Validation of the cytokine microarray

We validated the results obtained with our protein chip platform by comparison with the results of previous studies and conventional formats (Table 2). MDC, for example, was induced sufficiently to allow reliable confirmation of the microarray data with a less sensitive ELISA assay. Results from both assays were concordant (Fig. 5), although the ELISA assay would have consumed 1,000-fold more sample to produce the data acquired with the microarray. MDC and a related chemokine, TARC, have previously been shown by RT-PCR to be induced in DCs by LPS (Table 2)[31]. Our results confirmed these findings but also extended them by demonstrating that MDC and TARC are induced by TNF-α with faster kinetics and a greater abundance than LPS (Fig. 3). Concordance with previous studies was also found with IL-8, IP-10, IL-12, MIP-1β, and RANTES, all of which were induced with LPS (Table 2, Fig. 3)[20,31,32]. Of note were IL-12p70, RANTES, and MIP-1β induction by LPS; previous studies have shown greater induction of these cytokines in monocyte-derived DCs than was observed with LCs in the current study. As has been shown for IL-12p70 (ref. [14]), this may reflect differences between monocyte-derived DCs and LCs, rather than immunoassay platform differences. These discrepancies notwithstanding, the observed patterns of cytokine expression were consistent with previous studies of LPS- and TNF-α-treated LCs, attesting to the validity of the cytokine microarray method.

Insights and future applications

The suggestion that quantitative-expression proteomics surveys are of greater biological relevance than RNA level measurement[33] seemed to be supported in the current study by the ability not only to measure changes in protein abundance but also to predict effects of these changes on biological activity. For example, IP-10, whose RNA is known to be induced in LPS-stimulated DCs (ref. [31]), was shown in the present study to be secreted at 0.5 ng/ml, which exceeds the 50% effective dose (ED_{50}) for induction of T_{H1} chemotaxis by IP-10 (refs [24,34,35]). Another example was IL-8, which induces T-cell chemotaxis and suppresses $T_{H0}\rightarrow T_{H2}$ differentiation with an ED_{50} of 0.1–0.5 ng/ml (ref. [36]), and which was present in LPS-treated LC supernatants at a concentration of 6 ng/ml (Fig. 3). In contrast was MIP-1β, which, while induced significantly by both LPS and TNF-α, achieved levels in supernatants (0.3 ng/ml) that were 10-fold below the ED_{50} for chemotaxis of T_{H1} cells (Fig. 3)[34]; this result may indicate that MIP-1β secretion by LPS-induced LCs is irrelevant for T_{H1} chemotaxis. These results should be interpreted carefully, however, because the actual biological activities of the LC supernatants were not measured directly. Furthermore, ED_{50} of cytokines measured in model assays in isolation may not accurately reflect biological activity, because multiple cytokines may act synergistically.

Potentially biologically relevant cytokine induction was also observed following TNF-α treatment. For example, MCP-1, which had not been previously reported to be secreted by DCs, was induced by TNF-α at 0.1 ng/ml, a concentration sufficient to induce $T_{H0}\rightarrow T_{H2}$ differentiation (Fig. 3)[37-39]. Similarly, induction of 20 ng/ml MDC and TARC by TNF-α should be sufficient to induce T_{H2} chemotaxis (Fig. 3)[40]. It was notable that RANTES, which was secreted by both LPS- and TNF-α-treated LCs in similar amounts (Fig. 3), is known to enhance both humoral- and cell-mediated immune responses[41,42].

RNA expression levels do not always correlate with protein levels[33,43]. In the present study, there were two examples of apparent discordance in abundance of RNA and protein: IL-8 protein was rapidly induced by LPS and then remained at high levels throughout the culture period (Fig. 3). This contrasted with a previous study in which IL-8 mRNA levels of LPS-treated monocytederived DCs peaked at 3 h after LPS treatment, followed by a decline to baseline level at 30 h (ref. [31]). The discordance between mRNA and protein levels at 30 h may reflect ongoing secretion of stored intracellular IL-8, or IL-8 longevity in the culture media. Striking discordance was observed with RANTES, wherein mRNA levels had been found to gradually increase with time[31], whereas the protein level in culture supernatants peaked 24 h after LPS introduction and then declined. Under these conditions there appeared to be no correlation between RANTES transcription and translation/exocytosis. It should be noted that the RNA results were obtained with different DC lineages and in different laboratories; nevertheless, our results clearly demonstrate the value of direct and detailed measurements of protein abundance in describing a biological system.

An exciting application of the cytokine protein chip is in comparison of the abundance of a particular protein with that of its cognate receptor on putative target cells. For example, RANTES levels peaked 24 h after LPS introduction and then declined. A previous report has shown that CCR1 and CCR5, receptors for RANTES, decrease in abundance on the surface of DCs within 2 h of LPS exposure[44], indicating the existence of an autocrine loop. Similarly, the RCA immunoassay was of sufficient sensitivity to enable the demonstration that 20 pg/ml eotaxin-2 was induced starting 48 h after LPS introduction, and increasing to 140 pg/ml at 72 h (Fig. 3). Of note is a recent report that after 48 h of LPS stimulation, DCs lose the capacity to promote $T_{H0}\rightarrow T_{H1}$ differentiation, and instead induce $T_{H0}\rightarrow T_{H2}$ differentiation[45]. Eotaxin-2 provides a possible molecular explanation for this change, in that it has been shown to act specifically on T_{H2} lymphocytes expressing its receptor, CCR3 (refs [24,46]). Finally, CCR4, the receptor for MDC and TARC, has been implicated as a

specific marker for T_{H2} lymphocytes[40,47-49]. Thus, the observation that TNF-α-induced LCs secrete significant amounts of MDC and TARC suggests a role for MDC and TARC in LC-induced $T_{H0} \rightarrow T_{H2}$ differentiation or recruitment.

Other intriguing cytokine–cytokine receptor findings were that two soluble receptors, sIL-6R and sTNF-RI, were specifically induced by TNF-α (Fig. 3). Induction of sIL-6R, but not IL-6, by TNF-α is of interest because IL-6 was induced by LPS, and sIL-6R is known to cause maturing DCs to differentiate to macrophages[50]. Specific induction of sTNF-RI by TNF-α was not an unexpected finding considering that the activity of TNF-α is known to be modulated by proteolytic shedding of the soluble extracellular domain of its receptor[51]. Finally, I-309 was secreted at relatively high levels by both LPS- and TNF-α-treated LCs (Fig. 3). This chemokine was recently implicated in recruiting a $CCR8^+$ T_H subtype characterized by the production of high levels of IL-10 and low amounts of interferon-γ and IL-4 (ref. [52]). Thus, I-309 also appears to be a novel product of stimulated LCs of potential biological importance.

In summary, the application of an RCA cytokine chip to the study of LC maturation illustrated several advantages over conventional assays. First, universal RCA signal amplification was shown to be compatible with protein arrays. Second, RCA-amplified protein arrays allowed, for the first time, >50 members of a family of proteins to be measured simultaneously without compromise of biologically relevant sensitivity. Because cytokines in cocktails can elicit biological effects that are different from those observed in isolation, global patterns of cytokine expression are more likely to yield biologically relevant and clinically useful information than assays of single cytokines. Third, multiplexed protein measurement was shown to be practicable in terms of sample economy and reproducibility, and to elicit biological information beyond that obtained with the most prevalent method for surveying gene activity, the measurement of RNA levels. RCA-amplified protein arrays represent a significant advance from ELISAs in terms of their requirement for sample, scalability to high throughput, and compatibility with statistical analysis methods developed for DNA microarrays. The cytokine chip should be useful in a variety of studies of basic immunology, infection, autoimmunity, immunodeficiency, and inflammation. Additional chips, featuring 50–100 RCA sandwich immunoassays, are planned that will focus on other collections of proteins that mediate signal transduction, apoptosis, and toxic drug responses. Finally, chip-based RCA signal amplification may also prove useful for chips that examine protein–protein or protein–drug interactions.

Experimental protocol

Conjugate synthesis

Mouse monoclonal anti-biotin IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was conjugated to a 5′-terminal amine-modified oligonucleotide, 5′-NH_2-AAA AAA AAA AAA AAA CAC AGC TGA GGA TAG GAC AT-3′, as described[16].

RCA microarray immunoassays

Glass slides coated with Teflon except for 16 circular areas or “subarrays” were functionalized with thiol silane and activated with *N*-(γ-maleimidobutyryloxy)succinimide ester (GMBS)[12]. Monoclonal antibodies (R&D Systems, Minneapolis, MN; PharMingen, San Diego, CA) were diluted to 0.5 mg/ml in PBS with 0.05 mg/ml BSA, and ~0.5 nl of each was spotted in quadruplicate onto the slides using a pin-tool type microarrayer (Genemachines, San Carlos, CA) at 80% humidity. Immediately before use, slides were blocked as described[16]. A 10 μl volume of sample containing either purified antigen or supernatant from cell cultures was applied to each subarray and incubated for 30 min. After

incubation, subarrays were washed twice with 30 μl PBS/0.5% Brj-35 with a 2 min interval between each wash. A mixture of biotinylated secondary antibodies (25 μl diluted to 0.1 μg/ml in PBS/0.5% Brj-35) was applied to each subarray, incubated for 30 min, and washed as described above.

The anti-biotin antibody conjugate was annealed for 30 min in PBS/0.5% Brj-35/2 mM EDTA at 37°C with an oligonucleotide (5′-CTC AGC TGT GTA ACA ACA TGA AGA TTG TAG GTC AGA ACT CAC CTG TTA GAA ACT GTG AAG ATC GCT TAT TAT GTC CTA TC-3′) that had been circularized as described[15]. A total of 25 μl was applied to each subarray and, after incubation for 30 min, microarrays were washed twice. The RCA reaction was carried out for 45 min at 37°C in a 25 μl volume containing T7 native DNA polymerase as described[16] in the presence of 0.05 μM detector probe 5′-Cy5-TGT CCT ATC CTC AGC TGG-Cy5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank K. Kukanskis and S. Cooley (MSI) for manufacturing the protein microarrays used in this study. We also thank Ron Lennox for suggesting the collaboration between Molecular Staging, Inc. and Cellular Genomics, Inc.

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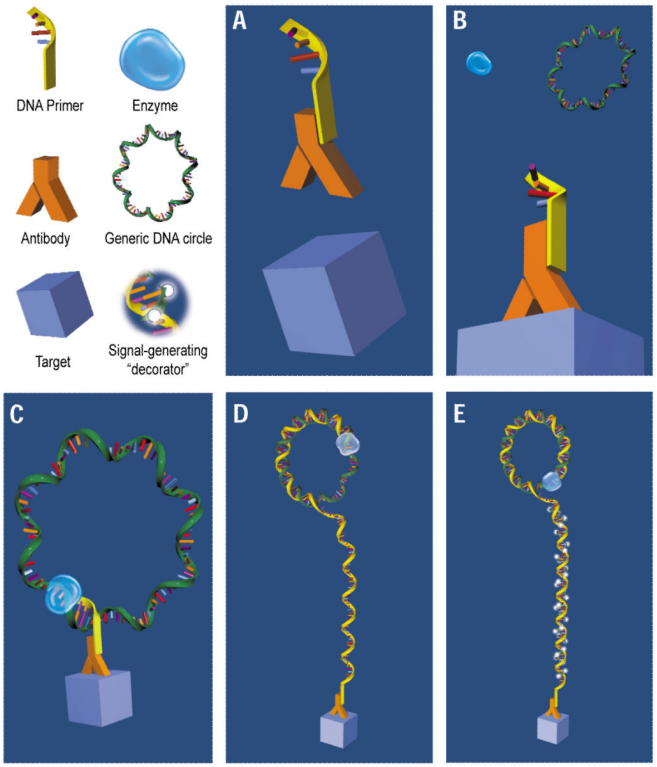

Figure 1.

Schematic representation of immunoassays with RCA signal amplification. (A) In the adaptation of RCA used for protein signal amplification, the 5′ end of an oligonucleotide primer is attached to an antibody. (B) The antibody–DNA conjugate binds to its specific target molecule; in the multiplexed microarray immunoassay, the targets are biotinylated secondary antibodies and the conjugate is an anti-biotin antibody. (C) A circular DNA molecule hybridizes to its complementary primer on the conjugate, and in the presence of DNA polymerase and nucleotides, rolling-circle replication occurs. (D) A long single DNA molecule that represents a concatamer of complements of the circle DNA sequence is generated that remains attached to the antibody. (E) This RCA product is detected by hybridization of multiple fluorescent, complementary oligonucleotide probes. RCA product fluorescence is measured with a conventional microarray scanning device. The amount of fluorescence at each spot is directly proportional to the amount of specific protein in the original sample.

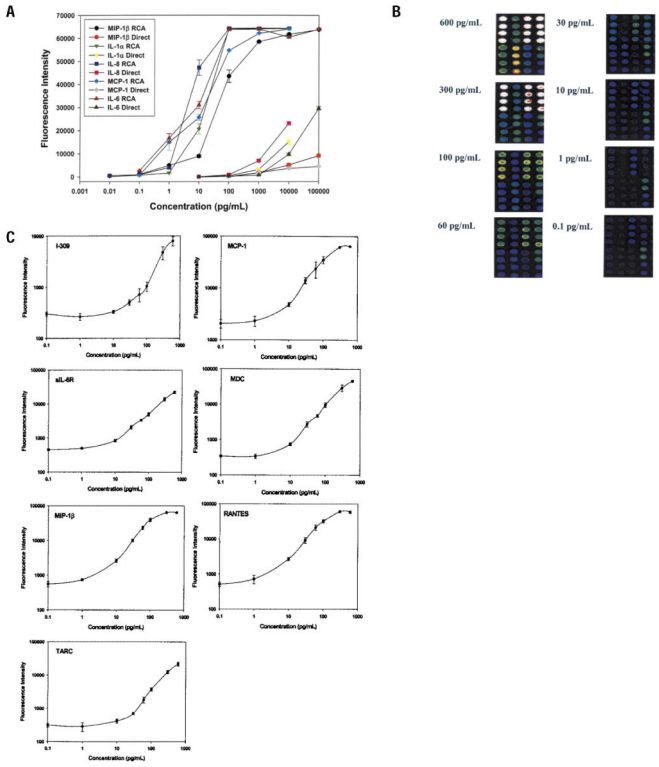

Figure 2.

Sensitivity of cytokine detection by RCA and direct detection. (A) Serial dilutions of individual cytokines were incubated on duplicate subarrays. On one set of subarrays, detection was carried out using RCA signal amplification. On the second set of subarrays, “direct” detection was performed using Cy5-labeled streptavidin. Fluorescence intensity of each spot was measured with a microarray scanner, and averages of the eight replicates of each antibody were plotted. (B) Seven cytokines were mixed, serially diluted, and incubated on subarrays containing monoclonal antibodies spotted in quadruplicate columns. Shown are fluorescence images of subarrays obtained with a microarray scanner. Top row of quadruplicate columns, left to right: MIP-1β, TARC, MCP-1, RANTES; bottom row of quadruplicate columns, left to right: sIL-6R, MDC, I-309, biotin-mIgG (positive control). (C) Indicated on each graph are mean fluorescence intensities and standard deviations derived from two subarrays, with four spots per subarray.

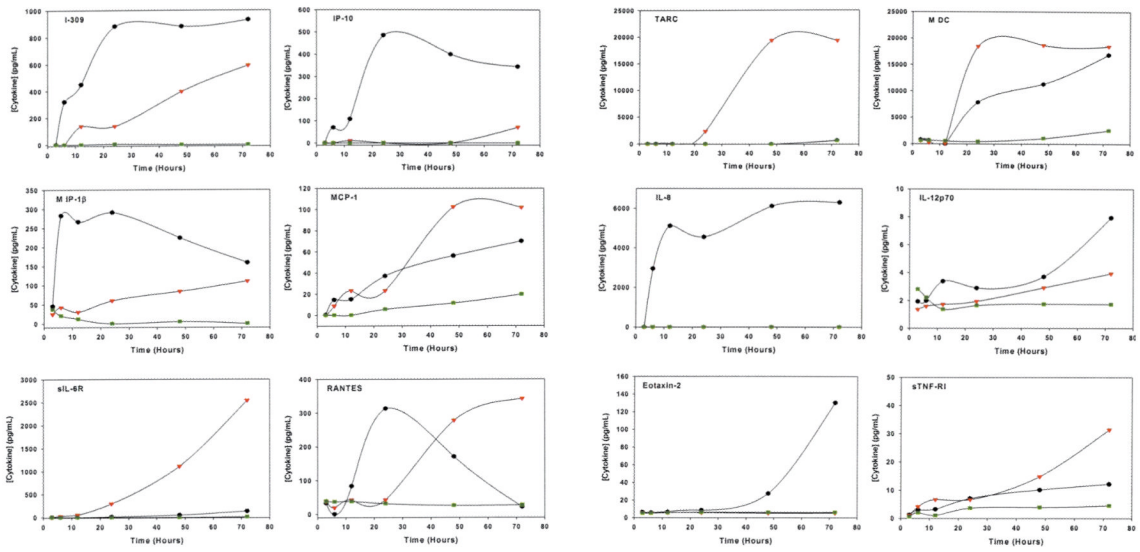

Figure 3.

Kinetics of cytokine production in maturing LCs on microarrays. Cytokine levels present in LC culture supernatants at six time points without induction or after LPS or TNF-α stimulation were determined by microarray immunoassay. Fluorescence intensities were converted to pg/ml using standard curves generated from mixtures of purified cytokines serially diluted in X-VIVO culture medium. The data for IL-8, MDC, TARC, and sIL-6R were generated from experiments using 1:20 dilutions of culture supernatants, and were corrected for this dilution factor. Black circles, LPS-treated; red triangles, TNF-α; green squares, uninduced.

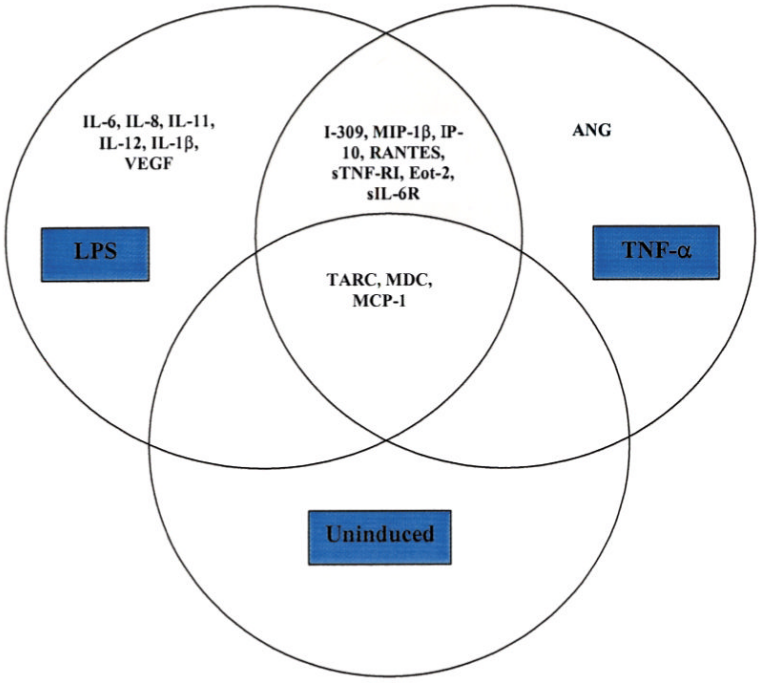

Figure 4.
Cytokines with at least fourfold increased abundance during 72 h culture with or without LPS or TNF-α.

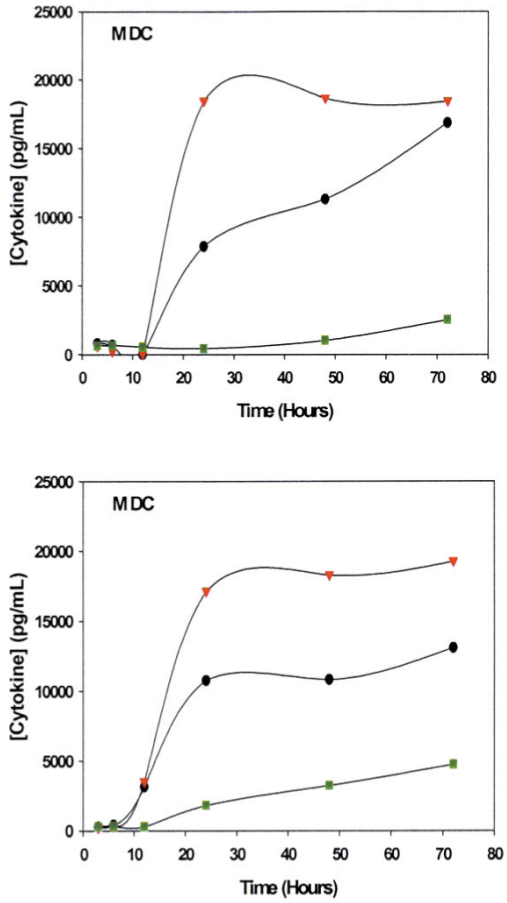

Figure 5.

Comparison of MDC measurement in supernatants by commercial ELISA and multiplexed RCA-amplified microarray immunoassay. MDC levels present in LC culture supernatants at six time points without induction or after LPS or TNF-α stimulation were determined by RCA microarray immunoassay (above) or commercial ELISA (below). Fluorescence intensities of the RCA microarray immunoassay were converted to pg/ml using standard curves generated from mixtures of purified cytokines. Black circles, LPS-treated; red triangles, TNF-α; green squares, uninduced.

Table 1
Performance characteristics of the 75-feature cytokine microarray

Cytokine	Biological activity (ED_{50}, pg/ml)	Mean serum levels (pg/ml)	ELISA sensitivity (pg/ml)	Array sensitivity (pg/ml)
ANG	NA[a]	3.6×10^6	6	5
AR	5,000–15,000	NA	NA	10
BDNF	3,000–10,000	28,000	20	10
BLC	5,000–20,000	NA	NA	10
CNTF	50,000–150,000	ND[b]	8	500
EGF	100–400	336	0.7	1
ENA-78	3,000–15,000	1,449	15	30
EOT	10,000–20,000	70	5	5
EOT-2	10,000–50,000	981	8.5	5
FAS	10,000–40,000	9,406	20	50
FGF-6	100–300	NA	NA	300
FGF-7	15,0000–25,000	ND	15	20
FGF-9	1,000–2000	NA	NA	100
FLT-3L	500–1,000	94	7	10
G-CSF	20–60	22	20	1,000
GDNF	1,000–3,000	NA	NA	10
GM-CSF	20–80	ND	3	3
GRO-α	1,000–4,000	93	10	10
HCC-4	$1.5–7.5 \times 10^4$	NA	NA	300
I-309	3,000–9,000	NA	NA	10
IFN-α	3.8×10^8 units/mg	NA	25	10
IFN-γ	800–1,500	ND	8	10
IL-1α	30–70	ND	1	1
IL-1β	13–20	5.4	1	1
IL-1rA	20,000–60,000	418	22	10
IL-1sRI	$5 \times 10^6–1 \times 10^8$	NA	NA	100
IL-2	250–500	ND	7	10
IL-2sRa	$5 \times 10^6–1 \times 10^8$	1,346	10	10
IL-4	50–200	ND	10	10
IL-5	100–200	ND	3	10
IL-6	200–800	1.62	0.7	1
IL-6sR	5,000–15,000	31,000	7	30
IL-7	200–500	4	1	1
IL-8	100–500	13.2	10	0.5
IL-10	500–1,000	ND	3.9	100
IL-12p40	50–200	77	15	30
IL-12p70	50–200	ND	5	10
IL-13	3,000–6,000	ND	32	20

Cytokine	Biological activity (ED_{50}, pg/ml)	Mean serum levels (pg/ml)	ELISA sensitivity (pg/ml)	Array sensitivity (pg/ml)
IL-15	500–2,000	ND	2	60
IL-16	100,000	171	13	500
IL-17	2,000–6,000	ND	15	60
IL-18	NA	126	12.5	20
IP-10	3,000–12,000	89	4.46	5
LIF	500	ND	8	100
MCP-1	5,000–20,000	370	5	1
MCP-2	30,000–120,000	NA	NA	5
MCP-3	20,000–80,000	NA	NA	10
M-CSF	500–1,500	670	9	5
MDC	3,000–9,000	1,089	62.5	5
MIF	50,000–100,000	NA	NA	100
MIG	$2–6 \times 10^6$	NA	NA	20
MIP-1α	2,000–10,000	ND	10	10
MIP-1β	10,000–30,000	80	11	5
MIP-1δ	2,000–8,000	NA	NA	50
MPIF-1	$2–6 \times 10^6$	536	17.5	20
MSP	10,000–30,000	NA	NA	50
NAP-2	$1–3 \times 10^6$	NA	NA	50
NT-3	10,000–30,000	NA	NA	10
NT-4	5,000–15,000	NA	NA	10
OSM	50–300	ND	6	10
PARC	$5 \times 10^6–2 \times 10^8$	NA	NA	1
PIGF	NA	ND	7	20
RANTES	10,000–20,000	49,137	8	3
SCF	2.500–5,000	984	9	5
sGP130	3,000–9,000	NA	NA	300
TARC	3,000–9,000	331	7	10
TGF-β1	15–20	48.6	7	10
TGF-β3	15–30	NA	NA	10
TNF-α	20–50	1.25	4.4	10
TNF-β	20–50	ND	16	10
sTNF-RI	45,000–90,000	1,198	3	6
sTNF-RII	NA	NA	100	50
TRAIL	50,000–150,000	NA	NA	1,000
uPAR	50,000–150,000	2,370	33	300
VEGF	5,000–10,000	220	9	30

ED_{50} information was obtained from product literature by R&D Systems.ELISA sensitivity information was obtained from product literature provided by R&D Systems for the Quantikine Immunoassay Kits.

[a] NA, not available

[b] ND, not detectable.

Table 2

Comparison of protein chip results with published reports

Treatment	Study	Cytokine	Assay	Result	Present study
LPS	14	IL-12	ELISA	20 pg/ml at 48 h	8 pg/ml at 72 h
	25	RANTES	ELISA	400 ng/ml at 24 h	300 pg/ml at 24 h
		MIP-1β	ELISA	5 μg/ml at 24 h	300 pg/ml at 24 h
		P-10	Northern	Upregulated at 3 h	500 pg/ml at 24 h
		IL-8	Northern	Upregulated at 3 h	5 ng/ml at 12 h
		TARC	RT-PCR	Upregulated at 30 h	2 ng/ml at 24 h
		MDC	RT-PCR	Upregulated at 30 h	20 ng/ml at 24 h
TNF-α	25	RANTES	ELISA	10 ng/ml at 24 h	80 pg/ml at 24 h
		MIP-1β	ELISA	1 μg/ml at 24 h	50 pg/ml at 24 h