Quantification of Cytokine Gene Expression Using an Economical Real-Time Polymerase Chain Reaction Method Based on SYBR® Green I

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Abstract

Assessment of cytokine expression has become crucial to understand host responses to infections as well as autoimmunity. Several approaches including Northern blot, RNase protection assay and enzyme-linked immunosorbent assay have been used for this purpose, but they are time consuming, labour intense, and relatively large quantity of the samples is usually required. Recently, a technique termed real-time reverse transcriptase-polymerase chain reaction (RT-PCR) has been developed to determine genetic expression with great sensitivity and specificity; however, specialized instrumentation and costly reagents are usually needed. We aimed at using low-cost reagents for real-time PCR. This was achieved by adapting a conventional RT-PCR protocol to the quantitative real-time format, by the addition of the SYBR® Green I reagent. We validated the approach by assessing the cytokine gene expression of murine splenocytes upon stimulation with phorbol 12-myristate 12-acetate (PMA)–ionomycin. The results using this technique were compared with those obtained with the well-established gene array method. We conclude that the use of the SYBR® Green I reagent during real-time RT-PCR provides a highly specific and sensitive method to quantify cytokine expression with accuracy and no post-PCR manipulation.

Introduction

Quantification of cytokine gene expression is essential for analysing immune responses; however, this goal requires highly sensitive techniques. While there are several methods for the quantification of nucleic acids, quantitative reverse transcription coupled to the polymerase chain reaction (RT-PCR) is becoming the technique of choice to detect low amounts of mRNA copies, because of the exponential increase of the template during thermal cycling [1, 2]. Moreover, the recent application of fluorescence techniques to the PCR, together with a suitable instrumentation capable of combining amplification, detection and quantification, has led to the development of real-time PCR methodologies [3], which are indeed revolutionizing the possibilities for nucleic acid quantification [4–6].

The instruments used for real-time PCR have a thermal cycler and a fluorescence detection system controlled by software and can monitor real-time product accumulation by measuring the increase in fluorescence during each cycle of the PCR to generate quantitative results. Currently, there are several reagents available to detect the amplified products with similar sensitivities. In general, they can be classified into two groups: fluorogenic probes and DNA-binding dyes.

SYBR® Green I dye is thought to bind in the minor groove of DNA; in its unbound state it has relatively low fluorescence but when bound to DNA it fluoresces brightly. As the amount of DNA in the PCR increases, the amount of fluorescence from the dye increases proportionally [7]. The incorporation of SYBR® Green I into real-time RT-PCR allows the detection of any double-strand DNA generated during PCR [7, 8]. This provides great flexibility because no target specific probes are required; however, both desired and undesired products will generate a signal. This problem can be overcome by

analysing the PCR product melting curve. The melting temperature (T_m) of an amplicon depends markedly on its size and nucleotide composition; hence, it is feasible to distinguish the fluorescence signal of the desired product from the signal(s) of undesirable products that melt at lower temperatures. This is the only post-PCR analysis that is necessary, making the technique both rapid and reliable [6].

High-throughput analysis of gene expression is now possible with the use of complementary DNA (cDNA) microarray technologies. The expression of a large number of genes can be monitored simultaneously and the expression profiles in different samples compared [9, 10]. Recent applications of this technique in immunology include the monitoring of changes in the expression caused, for instance, by inflammation [11], cytokines [12] and bacterial infections [13], or comparisons between cell populations [14]. However, low-intensity hybridization signals are difficult to interpret; besides, the high cost of the arrays severely limits their use in routine studies.

In this study, we assessed the cytokine expression profile of murine splenic mononuclear cells with or without phorbol 12-myristate 12-acetate (PMA)–ionomycin stimuli, using SYBR® Green I real-time RT-PCR. In parallel analysis, gene arrays were used to evaluate the results. We demonstrated that SYBR® Green I real-time RT-PCR is an economical, specific and sensitive method for cytokine expression profiling.

Materials and methods

Culture and stimulation of splenic mononuclear cells. Single-cell suspensions were prepared from spleens of 6–8-week-old-female BALB/*c* mice, and mononuclear cells were separated in a Histopaque-1119 gradient (Sigma-Aldrich, St Louis, MO, USA) by centrifugation at 800 *g* for 30 min. About 5×10^6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 5% bovine fetal serum at 37 °C in a humidified 5% CO_2 atmosphere. To compare the gene expression profiles, cells were incubated for 5 h in medium alone or with 1 μg/ml of each PMA and ionomycin (Sigma-Aldrich).

RNA isolation and DNase I treatment. Mononuclear cells were treated as described above and lysed in 1 ml of TRIZOL® reagent (Gibco BRL, Life Technologies, Grand Island, NY, USA). Total RNA was isolated in accordance to the manufacturer's instructions. To remove the chromosomal DNA, the samples were incubated with DNase I (0.5 U/10 μg of RNA, Gibco BRL) at 37 °C for 5 min in a total volume of 50 μl. DNase-treated RNA was re-extracted again with TRIZOL®. RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm in a SmartSpec 300 spectrophotometer (Bio-Rad, Hercules, CA, USA).

cDNA synthesis. Synthesis of single-strand cDNA was carried out, as described elsewhere [15]. Briefly, 1 μg of oligo-dT was annealed to 2 μg of RNA in a total volume of 10 μl at 70 °C for 10 min and chilled on ice. Then, 10 μl of reaction mix [1× first strand buffer, 10 mM dithiothreitol (DTT), 250 μM of dNTPs, 20 U RNAguard® (Amersham Pharmacia Biotech, Uppsala, Sweden) and 200 U M-MLV Reverse Transcriptase (Gibco BRL)] was added. The reaction was incubated at 25 °C for 5 min, at 37 °C for 60 min and at 70 °C for 10 min.

PCR primers. Specific cDNA sequences were obtained from the public GenBank sequence database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and primers were designed with the Oligo™ software of the DNA Star programme. In conventional RT-PCR, all primers generated only one amplification band visualized by agarose gel electrophoresis, demonstrating specificity (Table 1).

SYBR® Green I real-time PCR. . Real-time PCR was performed in a GeneAmp 5700® Sequence Detection

Table 1 Sequence of primers used in real-time reverse transcriptase-polymerase chain reaction (RT-PCR), amplicon sizes and their melting temperatures

Targets	Sequences*	Amplicon length (bp)	T_m (°C)†
β-actin	F: GTGGGCCGCTCTAGCCACCAA R: TCTTTGATGTCACGCACGATTTC	540	91
IFN-γ	F: GAAAGCCTAGAAAGTCTGAATAACT R: ATCAGCAGCGACTCCTTTTCCGCTT	388	88
TGF-β	F: GACCGCAACAACGCCATCTA R: GGCGTATCAGTGGGGGTCAG	236	88
IL-2	F: ATGTACAGCATGCAGCTCGCATC R: GGCTTGTTGAGATGATGCTTTGACA	502	90
IL-12 p40	F: CAGAAGCTAACCATCTCCTGGTTTG R: CCGGAGTAATTTGGTGCTCCACAC	396	89

IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; IL-2, interleukin-2.
*F and R stand for forward and reverse primers, respectively, in 5′→3′ orientation.
†The specific amplicon melting temperature (T_m) was determined by direct observation in its melting curve.

System SDS (Applied Biosystems, Foster City, CA, USA) using SYBR® Green I (10,000× concentration; Molecular probes, Eugene, OR, USA) as the detection format. Amplification was carried out in a total volume of 50 µl containing 0.5× SYBR® Green I, PCR buffer [50 mM KCl, 20 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2% glycerol and 0.2% dimethyl sulphoxide (DMSO)], 0.2 µM each primer, 0.2 mM dNTPs, 1.25 U AmpliTaq DNA polymerase (Applied Biosystems) and 5 µl of 1 : 5 diluted cDNA. The addition of SYBR® Green I (0.5× final concentration) to the reactions was the main modification to our procedures for standard PCR. The reactions were cycled 35 times under the following parameters: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and 85 °C for 5 s. At the end of the PCR, the temperature was increased from 60 to 95 °C at a rate of 2 °C/min, and the fluorescence was measured every 15 s to construct the melting curve. A nontemplate control (NTC) was run with every assay, and all determinations were performed at least in duplicates to achieve reproducibility.

PCR data analysis. At the end of the PCR, the GeneAmp 5700® SDS software saves the results, allowing instant manipulation or storage of the data for further analysis. Some initial cycles should be considered as a baseline (BL) or background in which no changes in fluorescence intensity occur, and the level above this, at which increments in fluorescence become detectable, is termed the threshold (Th). The software also determines the cycle number when a reaction reaches the Th. This value, termed the 'cycle threshold' (C_t), always appears during the exponential phase of the PCR and is inversely proportional to the initial number of template molecules in the sample (Fig. 1A). A BL from 1 to 5 and a Th of 0.7 were used for all determinations.

The software only determines the number of templates present in the reactions; if the amounts of RNA and cDNA used in the RT-PCR are considered, the number of specific molecules of mRNA per µg in the samples can be calculated. Results were expressed as absolute copy number per µg of RNA or as relative expression. The number of cytokines mRNAs calculated were normalized with the β-actin housekeeping gene then, the normalized values of stimulated cells were divided by those in control cells to obtain the relative expression.

Preparation of cDNA standards. External cDNA standards for each gene under study were constructed by cloning the corresponding RT-PCR product in the pCR®2.1 vector using 'The original TA cloning kit' (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The identity of the insert was confirmed by sequencing with the dideoxy chain termination method using the T7 Sequenase® version 2.0 DNA Sequencing kit (USB, Cleveland, OH, USA). The concentration (µg/µl) of the standard was determined by measuring the OD at 260 nm, and the copy number of plasmids per µl (CN) was calculated with the following formula: $CN = 9.1 \times 10^{11}$ (concentration of the plasmid/size of the plasmid in Kb).

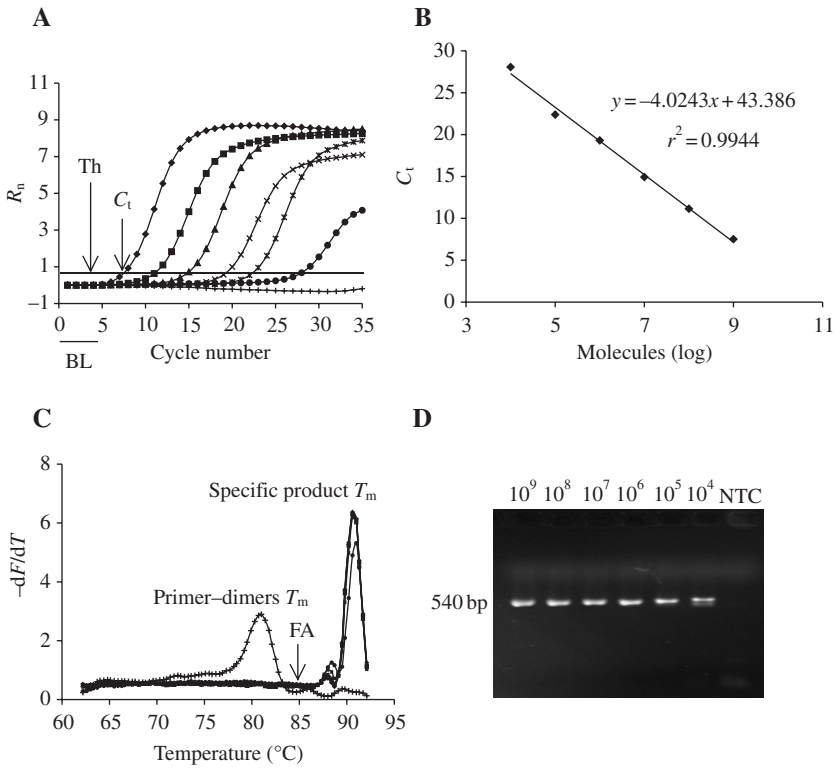

Figure 1 β-Actin standard curve. Plasmid serial 1 : 10 dilutions ranging from 1×10^9 to 1×10^4 β-actin molecules were amplified by real-time polymerase chain reaction (PCR) in the presence of 0.5× SYBR® Green I. (A) Amplification plot of 1×10^9 (♦), 10^8 (■), 10^7 (▲), 10^6 (×), 10^5 (*), 10^4 (●) and nontemplate control (+). (B) Standard curve. (C) Melting curve of PCR products. (D) Electrophoresis of PCR products in a 2% agarose gel. Th, threshold; C_t, cycle threshold; BL, baseline; R_n, normalized fluorescence intensity; T_m, melting temperature; $-dF/dT$, negative derivate of fluorescence; FA, temperature of fluorescence acquisition in each cycle; NTC, nontemplate control.

Preparation of ^{33}P-radiolabelled cDNA and array hybridization. Panorama® Mouse Cytokine Gene Arrays and cDNA labelling hybridization kits were purchased from Genosys (Sigma Genosys, The Woodlands, TX, USA). ^{33}P-radiolabelled cDNA probes for array hybridization were prepared following manufacturer's instructions. Briefly, 2 μg of total RNA in 11 μl was mixed with 4 μl of Mouse Cytokine labelling primers (Sigma Genosys), incubated at 90 °C for 2 min and cooled to 42 °C. This solution was added to a mix containing 1× reverse transcriptase buffer, 333 μM of each dATP, dGTP, dTTP and 1.67 μM dCTP, 20 μCi [α-^{33}P] dCTP (2000–3000 Ci mmol) (Amersham Pharmacia), 20 U of RNAguard® and 50 U of Arian Myeloblastic sis Virus (AMV) Reverse Transcriptase, in a total volume of 30 μl. After incubation at 42 °C for 2 h, the probes were purified by Sephadex® G-25 cDNA Spin Columns. The arrays were prehybridized for 4 h at 65 °C and hybridized to the denatured, labelled cDNA at 65 °C for 18 h. They were then washed twice under high stringency conditions (0.1× 150 mM sodium chloride/10 mM sodium phosphate/1 mM EDTA (SSPE)/1% SDS at 65 °C for 30 min) and finally subjected to autoradiography for 4 days using a BioMax MR X-ray film (Kodak, Rochester, NY, USA).

The autoradiograph was scanned to make a densitometric analysis of the arrays. The pixel intensity of each spot in both arrays was corrected for the background of the X-ray film, and the average signal (density value) of the pair of duplicate spots representing each gene was calculated. To correct the differences in probe labelling, the density values were normalized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene in both arrays; then values of stimulated cells were divided by their corresponding values in control cells to obtain the fold-induction or fold-reduction in genetic expression between samples.

Results

Absolute quantification with external standards

Real-time RT-PCR enables quantification in two ways: relative and absolute. The former is easier and inexpensive, but it only determines the expression level in comparison with a reference sample. In the latter, the exact copy number of a specific messenger in a sample is obtained and neither comparisons nor references are needed.

In order to perform absolute quantifications, external cDNA standards for β-actin, interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), interleukin-2 (IL-2) and IL-12 p40 were constructed, as described in *Materials and methods*. The linear range of the assay was determined by kinetic amplification of log serial plasmid dilutions from 1×10^9 to 1×10^4 molecules. In Fig. 1A, a typical amplification plot for β-actin dilutions is shown, demonstrating the quantitative nature of this technique. The GeneAmp 5700® SDS software plotted the log of molecules versus the C_t values in order to obtain a standard curve (Fig. 1B); a correlation of 0.994 was observed. Any unknown β-actin concentration sample can be calculated by simple interpolation using this curve.

The melting curve of the β-actin PCR products is shown in Fig. 1C. The specific β-actin amplicon T_m peak can be observed at 91 °C, which is clearly distinguishable from the lower T_m peak of primer–dimer artefacts at 82 °C in the NTC reaction. In order to demonstrate specificity, PCR products were electrophoresed in a 2% agarose gel (Fig. 1D); only the 540 bp β-actin-specific product was observed. The same steps were followed for each gene analysed (data not shown).

The general specificity in a PCR is provided by the set of primers and optimal reaction conditions, and it is understood solely as the amplification of the specific target. In real-time PCR, the detection of the fluorescence generated by the specific amplicon is also important because quantification is based on it. As the fluorescence acquisition (FA) during amplification is made at 85 °C in the last step of each cycle (above primer–dimer T_m, but just below the specific product T_m), only the specific PCR product signal is registered (Fig. 1C). This, in conjunction with the melting curve analysis, produces a highly specific and sensitive method, which can be applied to detect subtle differences in expression levels in at least five orders of magnitude.

Quantification of cytokine messengers by SYBR® Green I real-time RT-PCR

First, the integrity of RNA was confirmed in a denaturing agarose gel (Fig. 2A); the 28 S and 18 S rRNA bands visible in the control (C) and stimulated cells (S) were in the correct ratio (2 : 1), and no contaminating DNA or RNA degradation was observed. Afterwards, the RNA was reverse-transcribed and the real-time PCR was set-up together with a standard curve. To confirm reproducibility, all determinations were performed at least two times. In Fig. 2B, the β-actin and IFN-γ amplification plots are shown; β-actin C_t values were very similar between the control ($C_t = 24.22$) and stimulated samples ($C_t = 26.26$), and as expected, IFN-γ was only detected in stimulated cells ($C_t = 20.03$). At the end of the PCR, the instrument performed a melting curve. In Fig. 2C, the specific β-actin T_m peak (91 °C) was observed in control and stimulated cells. The specific IFN-γ peak (88 °C) was present in the stimulated cells and a primer–dimer peak (81 °C) was observed in control cells. PCR products were analysed by electrophoresis to demonstrate specificity (Fig. 2D); both control and stimulated cells expressed β-actin (540 bp), but the IFN-γ band (388 bp) was present only in stimulated cells, which confirms the results observed in the amplification (Fig. 2B) and melting plot.

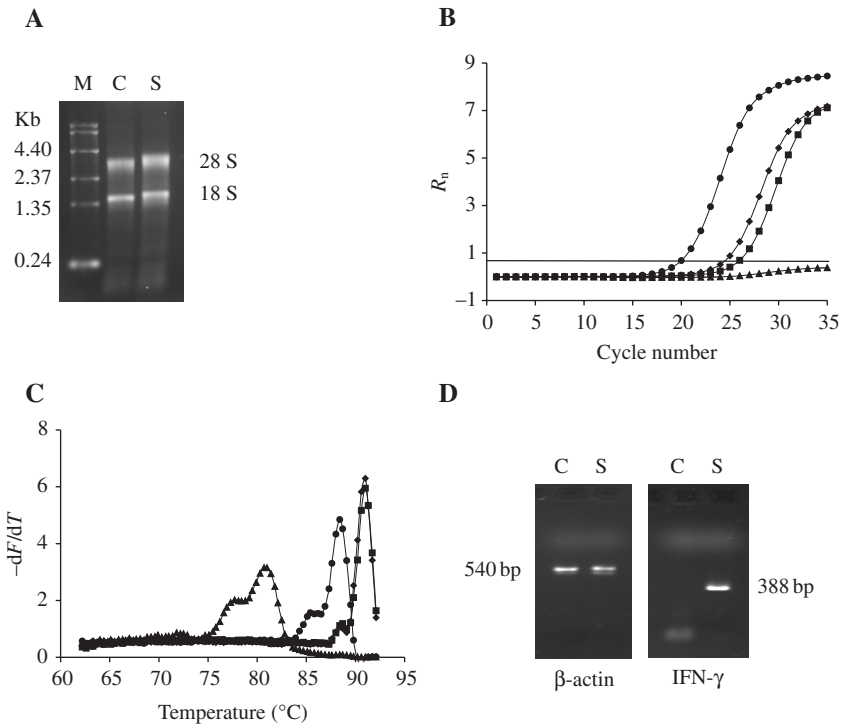

Figure 2 Determination of β-actin and interferon-γ (IFN-γ) mRNA copies. (A) Electrophoresis of 2 µg of total RNA in a denaturing 1% agarose formaldehyde gel; the 28 S and 18 S rRNA bands were in the correct 2 : 1 ratio. M, 0.24–9.5 Kb RNA ladder (Gibco BRL); C, control murine splenic cells; S, stimulated cells. (B) Amplification plot for β-actin (♦) and IFN-γ (▲) in control cells and for β-actin (■) and IFN-γ (●) in stimulated cells. Note that there is not a cycle threshold (C_t) value for IFN-γ in control cells (▲) because of the absence of the specific polymerase chain reaction (PCR) product. (C) Melting curve of β-actin and IFN-γ PCR products in control (♦, ▲) and stimulated cells (■, ●). (D) Electrophoresis of PCR products in a 2% agarose gel; the 540 bp band corresponds to β-actin and the 388 bp to IFN-γ amplified from control and stimulated cells.

The copy numbers of β-actin, IFN-γ, TGF-β, IL-2 and IL-12 p40 messengers were calculated in control and stimulated cells by the GeneAmp 5700® SDS software, interpolating the C_t of each sample in the corresponding standard curves. The results in Table 2 show an increase in the copy numbers for IFN-γ, TGF-β, IL-2 and IL-12 p40 in the stimulated cells.

Cytokine expression profiles using gene arrays

Panorama® Mouse Cytokine Gene Arrays consist of two charged nylon membranes containing PCR products spotted in duplicate. Each array contains 514 different mouse cytokine-related genes, eight housekeeping genes, three *Escherichia coli* genes and mouse genomic DNA.

The arrays were hybridized with ^{33}P-radiolabelled cDNA probes from unstimulated (Fig. 3A) and stimulated PMA–ionomycin mononuclear splenic cells (Fig. 3B). Table 3 summarizes genes that were either down- or upregulated. We found that IFN-γ, TGF-β and IL-2 were highly expressed in stimulated cells, confirming the result obtained by real-time RT-PCR. However, IL-12 p40 was not detected because of the low expression level of this interleukin in the cells. Other regulated genes, but not measured by real-time RT-PCR, were also included in Table 3. There was an upregulation of lipopolysaccharide-binding protein (LBP), macrophage inflammatory protein-1β (MIP-1β), lymphotactin (SCM-1) and IL-2 receptor alpha (IL-2 Ra) in stimulated cells.

Table 2 Quantification of cytokine messengers by SYBR® Green I real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene	Control	Stimulated
IFN-γ	ND*	18,516,897† (±376,474‡)
TGF-β	1,021,819 (±4328)	1,124,072† (±37,904‡)
IL-2	ND	6,417,552† (±13,429‡)
IL-12 p40	31,483 (±625)	309,012† (±5586‡)

IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; IL-2, interleukin-2.
*ND, not detectable (below the level of detection).
†Absolute copy number per µg of RNA in control and stimulated cells.
‡Standard deviation.

Discussion

Cytokines play a central role in modulating host immune responses to infections and other pathologies, and many techniques have been used to assess their presence in tissues or blood cell samples [16]. Some methods such as enzyme-linked immunosorbent assay (ELISA), ELISPOT and intracellular flow cytometry measure the protein concentration [17], while others such as Northern blot and RNase protection assay determine the gene expression levels; however, their relatively low sensitivity constitutes a limitation in cases where small quantity of the samples are only available. The application of conventional or semiquantitative RT-PCR has overcome these limitations [18]. Currently, real-time RT-PCR is the most suitable method for gene expression quantification [5, 19], but the use of specialized thermal cyclers and costly reagents has hampered its use.

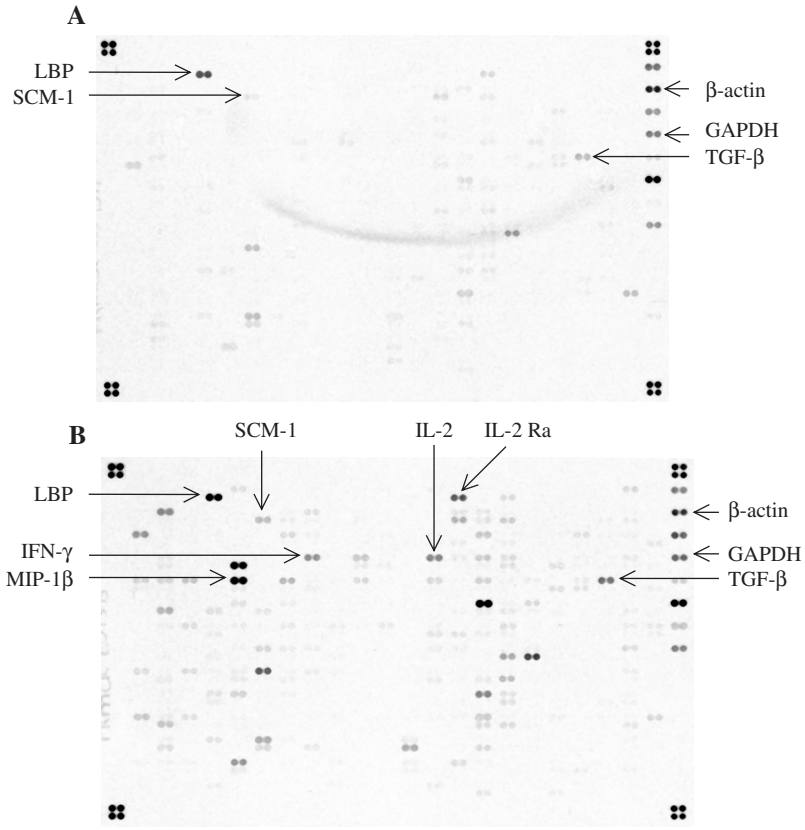

Figure 3 Gene array hybridizations. Membranes were hybridized with cDNA probes from control (A) or stimulated cells (B). There was an increased expression of interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), and interleukin-2 (IL-2) in stimulated cells. No change in expression occurs for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene between the samples. Other upregulated genes observed were lipopolysaccharide-binding protein (LBP), macrophage inflammatory protein-1β (MIP-1β), lymphotactin (SCM-1) and IL-2 receptor alpha (IL-2 Ra).

In order to reduce running costs, we adapted a conventional RT-PCR protocol to the quantitative real-time format by the addition of the SYBR® Green I dye. First, we determined the exact SYBR® Green I concentration to use in comparison with the commercial kit LightCycler DNA Master SYBR Green I from Roche Molecular Biochemicals (data not shown). This method allows any cytokine gene, which has been studied previously by conventional RT-PCR, to be investigated by real-time RT-PCR whilst retaining the same reagents and reaction conditions. The introduction of the final step at the end of each cycle allows measuring the specific amplicon fluorescence signal. When a new gene is being analysed, the specific T_m is readily obtained by melting the conventional PCR product in the presence of the dye.

Table 3 Densitometric analysis of the gene arrays

Gene	Control	Stimulated†
IFN-γ	ND*	3822
TGF-β	3332	4998
IL-2	ND	3724
IL-12 p40	ND	ND
LBP	6468	9800
MIP-1β	ND	9212
SCM-1	1404	3185
IL-2 Ra	ND	5439

IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; IL-2, interleukin-2; LBP, lipopolysaccharide-binding protein; MIP-1β, macrophage inflammatory protein-1β; SCM, lymphotactin; IL-2 Ra, IL-2 receptor alpha.
*ND, not detectable (below the level of detection).
†Density values of gene expression in control and stimulated cells.

In this study, the cytokine gene expression of murine splenocytes upon stimulation with PMA–ionomycin was assessed. We determined the absolute mRNA copies for β-actin, IFN-γ, TGF-β, IL-2 and IL-12 p40 using SYBR® Green I real-time RT-PCR. In a parallel assay, Panorama® Mouse Cytokine Gene Arrays were used to evaluate the results. We found that the relative cytokine expression levels obtained by real-time RT-PCR had similar tendencies to those obtained with the gene array method. A fold-induction for IFN-γ (49,810,453), IL-2 (17,263,214), TGF-β (2.97) and IL-12 p40 (26.4) was calculated by real-time RT-PCR in the stimulated cells. Using gene arrays, a fold-induction for IFN-γ (3517), IL-2 (3427) and TGF-β (1.3) was observed. IL-12 p40 was not detected because of its low expression level in the samples but was easily detected and quantified by our real-time RT-PCR method.

In conclusion, our adapting protocol using SYBR® Green I as the detection format and product verification

by melting curve analysis was confirmed to be a rapid, flexible, sensitive, specific and economical procedure for the study of cytokine gene expression.

Acknowledgments

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