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Methodology article

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Abstract

Background: Cytokine mRNA quantification is widely used to investigate cytokine profiles, particularly in small samples. Real-time polymerase chain reaction is currently the most reliable method of quantifying low-level transcripts such as cytokine and cytokine receptor mRNAs. This accurate technique allows the quantification of a larger pattern of cytokines than quantification at the protein level, which is limited to a smaller number of proteins.

Results: Although fluorogenic probes are considered more sensitive than fluorescent dyes, we have developed SYBR Green real-time RT-PCR protocols to assay pro-inflammatory cytokines (IL1a, IL1b and IL6, TNFa), cytokine receptors (IL1-r1, IL1-r2, IL6-r, TNF-r2) and related molecules (IL1-RA, SOCS3) mRNA in rats. This method enables normalisation against several housekeeping genes (beta-actin, GAPDH, CypA, HPRT) dependent on the specific experimental treatments and tissues using either standard curve, or comparative C_T quantification method. PCR efficiency and sensitivity allow the assessment of; i) basal mRNA levels in many tissues and even decreases in mRNA levels, ii) mRNA levels from very small samples.

Conclusion: Real-time RT-PCR is currently the best way to investigate cytokine networks. The investigations should be completed by the analysis of genes regulated by cytokines or involved in cytokine signalling, providing indirect information on cytokine protein expression.

Background

Cytokines are regulatory proteins, which play a key role in inflammatory responses either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in numerous cell types. Knowledge of the local cytokine pattern is essential to elucidate the immune and pathological pathways involved in many inflammatory responses such as infectious diseases, autoimmune reactions, etc. However, cytokine protein detection, via techniques such as ELISA only allows the measurement of

a limited number of cytokines from a single sample. In addition, tissue samples are often too small to enable their quantification at the protein level. Until now, this point has been a critical one. Processing of rat samples with ELISA techniques is also impaired by the lack of sensitivity of currently commercialized ELISA kits. Fortunately, the development of quantitative reverse transcription polymerase chain reactions (RT-PCR) provides a highly sensitive tool. Thus, quantification of mRNA is widely used to investigate the cytokine profiles although mRNA is only an estimate of cytokine profiles at the protein level. This drawback can however be partially bypassed by studying the expression of genes regulated by cytokines and involved in cytokine signalling.

Though a variety of methods are used to measure mRNA expression, RT-PCR is the most sensitive thanks to the exponential amplification process. Development of real-time monitoring of the PCR has led to a large improvement in the reproducibility and rapidity of quantitative RT-PCR. Real-time PCR works equally well with a fluorescent dye (e.g., SYBR Green) as it does with fluorogenic sequence-specific probes (TaqMan™, molecular beacons, scorpions and hybridisation probes) and is currently the most accurate and sensitive method for quantifying the mRNA expression of cytokines, which are often expressed at very low levels [1].

Recent works have described real-time PCR quantification of Interleukin 1 alpha (IL1a) [2,3], Interleukin 1 beta (IL1b) [3-5], Interleukin 6 (IL6) [3] and Tumour Necrosis Factor alpha (TNFa) [2,5-7] in rat samples, essentially using fluorogenic probes. However, contrary to human and mouse species [8,9], no study has been carried out so far reporting real-time PCR quantification of an extended panel of pro-inflammatory cytokines and related molecules in the rat species.

Although fluorogenic probes are considered to be more sensitive than fluorescent dyes [10], we have developed a homogenous and reproducible SYBR Green RT-PCR assays which allow measurement of the basal expression of a wide panel of inflammatory cytokines as well as their receptors in many rat organs.

Results

Primer design and control of primer specificity

Except IL1b primers previously described [4], primers were specially designed for this study. Primer design and optimisation concerning dimerization, self-priming and melting temperature were carried out using MacVector software (Accelrys, San Diego, USA). The default parameters of the program were applied, except for the following; i) product size 75–120 bp, ii) percent G+C 47–53, iii) bonds primer versus primer (any) 4 and iv) bonds primer versus primer (GC) 3. Primers with G-C stretches are avoided. If possible primer sets with identical size and G-C content were chosen (Tables 1, 2). The short amplicon length did not always allow designing intron-spanning primers. Primer sets amplifying genomic DNA are pointed out in Tables 1, 2. Thus, intron-specific primers or RT-minus controls were used to ascertain the absence of

Table 1: Primer sequences used for cytokine and cytokine receptor real-time PCR assays

Gene name		5'-3' primer sequence	Position cDNA-gene[a]	Accession Number[b]
IL1a	FW	AAGACAAGCCTGTGTTGCTGAAGG	663–747	D00403
	RW	TCCCAGAAGAAAATGAGGTCGGTC	Int. span.	NW_047658
IL1b	FW	CACCTCTCAAGCAGAGCACAG	793–871	M98820
	RW	GGGTTCCATGGTGAAGTCAAC	Exon	NW_047658
IL1RA	FW	AAGACCTTCTACCTGAGGAACAACC	139–255	M63101
	RW	GCCCAAGAACACATTCCGAAAGTC	Int. span.	NW_047651
IL1R1	FW	GTTTTTGGAACACCCTTCAGCC	1209–1313	M95578
	RW	ACGAAGCAGATGAACGGATAGC	Exon	NW_047814.1
IL1R2	FW	CATTCAGACACCTCCAGCAGTTC	328–443	Z22812
	RW	ACCCAGAGCGTATCATCCTTCAC	Exon	NW_047814.1
IL6	FW	TCCTACCCCAACTTCCAATGCTC	532–610	E02522
	RW	TTGGATGGTCTTGGTCCTTAGCC	Exon	M26745
IL6R	FW	AAGCAGGTCCAGCCACAATGTAG	696–812	NM_017020
	RW	CCAACTGACTTTGAGCCAACGAG	Int. span.	NW_047626.1
TNFa	FW	AAATGGGCTCCCTCTCATCAGTTC	195–305	X66539
	RW	TCTGCTTGGTGGTTTGCTACGAC	Int. span.	D00475
TNF-r2	FW	TGCAACAAGACTTCAGACACCGTG	142–224	AF420214
	RW	AGGCATGTATGCAGATGGTTCCAG	Exon	NW_047727.1

Note. FW forward primer; RW reverse primer; IL, interleukin; IL-r, interleukin receptor; TNFα, Tumor necrosis factor α; TNF-r2, Tumor necrosis factor receptor 2. [a] Position of amplification product within cDNA sequence (upper line) and within genomic sequences (lower line). Exon; both primers bound on the same exon, Int. span.: primers bound on different exons, , [b] Genbank accession number of cDNA (upper line) and genomic sequences or contigs (lower line), available at http://www.ncbi.nlm.nih.gov/.

Table 2: Primer sequences used for house-keeping genes and cytokine related molecules real-time PCR assays

Gene name		5'-3' primer sequence	Position cDNA-gene[a]	Accession Number[b]
βactin	FW	AAGTCCCTCACCCTCCCAAAAG	3474–3570	J00691
	RW	AAGCAATGCTGTCACCTTCCC	Exon	V01217
CypA	FW	TATCTGCACTGCCAAGACTGAGTG	381–507	M19533
	RW	CTTCTTGCTGGTCTTGCCATTCC	Int. span.	NW_047430
GAPDH	FW	GTATTGGGCGCCTGGTCACC*	73–274	AB017801
	RW	CGCTCCTGGAAGATGGTGATGG	Exon	NW_047696.1
HPRT	FW	CTCATGGACTGATTATGGACAGGAC	211–333	S79292
	RW	GCAGGTCAGCAAAGAACTTATAGCC	Int. span.	NW_047696.1
SOCS3	FW	CCTCCAGCATCTTTGTCGGAAGAC	581–679	AF075383
	RW	TACTGGTCCAGGAACTCCCGAATG	Exon	AJ249240
C/EBPβ	FW	TGGACAAGCTGAGCGACGAG	656–760	NM_024125
	RW	TGTGCTGCGTCTCCAGGTTG	Exon‡	NW_047660.1
cFos	FW	TTCACCCTGCCTCTTCTCAATGAC	849–931	X06769
	RW	GCCTTCAGCTCCATGTTGCTAATG	Int. span.	NW_047762
cJun	FW	CAATGGGCACATCACCACTACAC	604–725	X17163
	RW	TCTGGCTATGCAGTTCAGCTAGG	Exon‡	NW_047717.1
COX-2	FW	TGTATGCTACCATCTGGCTTCGG	981–1074	S67722
	RW	GTTTGGAACAGTCGCTCGTCATC	Exon	NW_047397.1
I-KB	FW	TGAGTACCTGGACTTGCAGAACG	334–409	AF246634
	RW	TGTAGATGCCTCTCCAAGGATGG	Int. span.	NW_047557.1
NOS-2*	FW	CATTGGAAGTGAAGCGTTTCG	2103–2197	L12562
	RW	CAGCTGGGCTGTACAAACCTT	Int. span.	AABR03074184

Note. FW forward primer; RW reverse primer; CypA, cyclophilin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphorybosyl transferase; SOCS3, Suppressor of cytokine signalling 3; C/EBPβ (NF-IL6 rodent homologue); COX-2, cyclooxygenase 2; I-KB, KappaB inhibitors; NOS-2, nitric oxide synthase 2. [a] Position of amplification product within cDNA sequence (upper line) and within genomic sequences (lower line). Exon; both primers bound on the same exon, Int. span.: primers bound on different exons, , [b] Genbank accession number of cDNA (upper line) and genomic sequences or contigs (lower line), available at http://www.ncbi.nlm.nih.gov/. *primers are stemmed from mouse sequence. ‡ gene without intron.

genomic DNA, as described in "Methods".

PCR were carried out both from RT-products and from specific recombinant DNAs overlapping PCR products. Specificities of the PCR amplification are always analysed with melting curve analysis. Melting peaks obtained either from RT-product or from specific recombinant DNA are identical. The melting temperatures of PCR products are shown in Table 3. In addition, products were controlled with high-resolution gel electrophoresis. To sum up, all PCR amplifications lead to a single and specific product.

Linearity and efficiency of PCR amplification

The accuracy of mRNA quantification depends on the linearity and efficiency of PCR amplification. Both parameters were assessed using standard curves generated by increasing amounts of cDNA. Relationship between the threshold cycle (C_T) and the logarithm of the cDNA concentration were studied according to i), the correlation coefficient and ii), the slopes calculated by LightCycler Software 3 (Roche Applied Science, Mannheim, Germany).

Correlation coefficients (r) confirm the linear relationship between the threshold cycle (C_T) and the logarithm of the cDNA concentration. Standard curves, using five points, diluted over a 100-fold range, always led to a high linearity ($r \geq 0.99$) as observed with all primer sets (data not shown and Figure 1, Panel A and B).

The PCR efficiency (*Ex*) was calculated using the equation $Ex = (10^{-1/slope})-1$. A slope value of -3.32 implies a PCR efficiency of 1 (100%). The higher slope values mean that the PCR efficiency is less than 1. As PCR amplification depends on template preparation, i.e. RNA extraction and cDNA synthesis [11] the assay efficiency was checked on cDNA obtained with different RNA extraction methods and in different rat organs. As shown in Figure 1 (panel A), for TNFa amplification, there was no difference in PCR efficiency despite different template preparations (phenol and anion exchange resin RNA extraction). Similarly, there is no difference in PCR efficiency among different organs. Figure 2 presents the average slope for cytokine mRNAs of 14 independent assays ($Ex = 0.965 \pm 0.085$) in two rat brain structures (hypothalamus, hippocampus) and five rat organs (ileum, liver, lung, spleen, skin) using

Table 3: Detailed conditions used for real-time PCR assays.

Gene name	[primer][a] (μM)	[Mg^{++}][b] (mM)	Annealing conditions[c] (°C…s)	Product TM[d] (°C)	Amplicon Length[e] (bp)
βactin	0.3	3.5	58°C…4s	83.5	97
CypA	0.4	4	58°C…5s	85.2	127
GAPDH	0.4	3	58°C…4s	86.9	202
HPRT	0.4	4	60°C…5s	84.7	123
IL1a	0.4	4	65°C…5s	81.5	85
IL1b	0.4	3.5	58°C…5s	82.0	79
IL1RA	0.4	3	65°C…5s	80.7	117
IL1R1	0.4	3	64°C…5s	80.0	105
IL1R2	0.4	4	64°C…5s	84.5	116
IL6	0.4	3	65°C…5s	80.9	79
IL6R	0.4	5	60°C…5s	84.3	117
TNFa	0.4	3	58°C…4s	84.7	111
TNFR2	0.4	4	60°C…5s	84.9*	83
SOCS3	0.4	4	65°C…5s	84.7	99
C/EBPβ	0.4	4	59°C…5s	90.2	90.2
cFos	0.4	4	60°C…5s	84.6	84.6
cJun	0.4	4	62°C…5s	89.2	89.2
COX-2	0.4	4	60°C…4s	83.8	83.8
NOS-2	0.4	4	58°C…5s	83.8	83.8

Abbreviations: see Tables 1, 2.[a] final concentration of oligonucleotide primers (μM), [b] final concentration of Mg^{2+} (mM), [c] Annealing temperature (°C) and duration (s), [d] melting temperature of specific PCR product, [e] Amplicon length in base pairs. *TNF-r2: a second melting peak close to 88°C can coexist depending on template. The two peaks correspond to a single amplification product as controlled using high-resolution gel electrophoresis and PCR with recombinant and genomic DNA as templates.

phenol RNA extraction. When rat hypothalamus was used as a template (anion exchange resin RNA extraction) PCR amplification efficiencies were respectively 0.987 ± 0.041, 0.960 ± 0.031 and 0.959 ± 0.028 for cytokines (mean of 6 genes), cytokine receptors (mean of 5 genes) and housekeeping gene CypA (mean of 3 independent assays). Furthermore, only low intra-assay variations were observed as shown with SOCS3 mRNA quantification over a 1000-fold range of measurement (Figure 1, panel B).

In conclusion, the linearity and efficiency of amplification of PCR assays among different templates allowed an accurate quantification of different target genes. Moreover, there were slight differences in amplification efficiencies of PCR assays, which allow the use of the comparative C_T (ΔC_T) quantification method (cf. Quantification).

Average crossing point of cytokines PCR assays in various organs in control rats

The sensitivity of the SYBR Green PCR assay was tested through its ability to measure basal levels of target mRNAs in control rats. Average C_T of cytokines and SOCS3 PCR assays in control rats enable mRNA quantification in the PCR exponential amplification phase in all tested structures and organs (Figure 3. Panel A). Cytokine receptor C_Ts are similar to those of cytokines as shown in control rat hypothalamus samples (Figure 3, Panel B). Thus, the sensitivity of these assays enables the measurement of cytokines mRNA using in vitro LPS or lectin-stimulated blood samples (data not shown).

Normalization

A reliable quantitative RT-PCR method needs taking into account corrections for experimental variations in different samples, i.e. different amounts of cDNA and minor differences in PCR efficiency. Inter-sample differences in amplification efficiency are not a pitfall in real-time PCR as opposed to end-point quantification [1]. Indeed in real-time PCR, quantification is based on C_T values, which are measured in the early stage of the exponential phase of the reaction, although variations in input RNA or in reverse transcription efficiency must be corrected. Normalization to a housekeeping gene is currently the best method of avoiding these discrepancies [1]. We generally use cyclophilin A (CypA) as the reference gene owing to its stability in many physiological and pathophysiological conditions. However, the reliability of the results depends on the choice of the most relevant housekeeping gene according to the specific experimental treatments and organs. So beside cyclophilin A [12], primer sets, able to quantify the most common housekeeping genes (beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphorybosyl

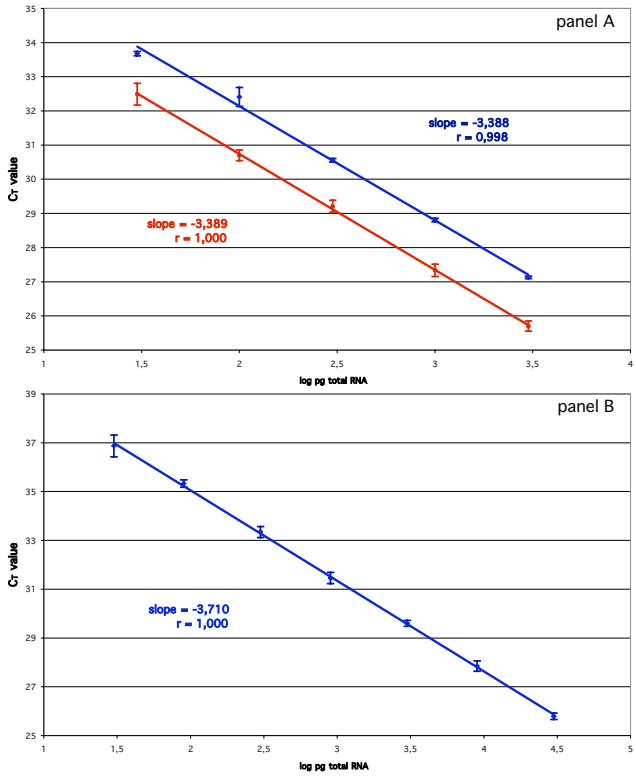

Figure 1
Representative standard curves for TNFa and SOCS3 mRNAs using the LightCycler device. RNA was isolated using spleen cell cultures stimulated with LPS. The primer sets are listed in Tables 1, 2 and amplification conditions are described in "methods". Slopes and statistical value are assessed using LightCycler 3 software. Data are means ± SEM. A) Variation of standard curve for TNFa mRNA quantification according to mRNA extraction: (blue diamond) anion exchange resin RNA extraction (RNeasy mini, QIAGEN), (red diamond) phenol extraction [26]. Each value is the average of four (phenol) or three (RNeasy) independent mRNA quantifications. Only a weak difference is observed in PCR efficiencies between the templates (-3.398 and -3.399). The C_T variation is probably due to the difference in mRNA purity between both extraction methods. B) Intra-assay variation for SOCS3 mRNA quantification. Each value is the mean of three repetitions in the same experiment.

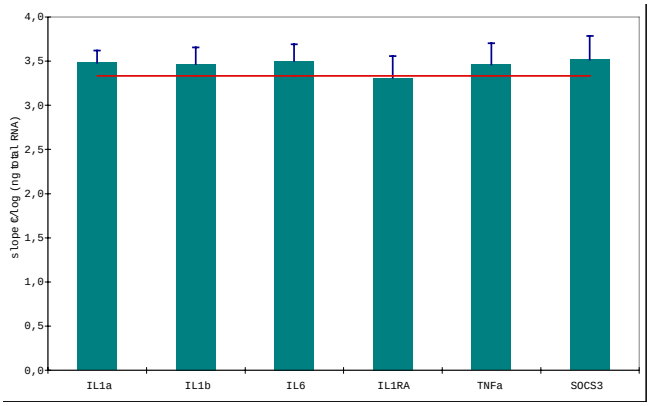

Figure 2
PCR efficiency of cytokines and related molecules among rat organs and structures. Slope values are related to PCR efficiencies (*Ex*) using the equation $Ex = (10^{-1/slope}) - 1$. The mean efficiency is 0.965 ± 0.085. The red line matches PCR efficiency of 1 (slope = -3.32). Slope values are assessed using LightCycler 3 software (ROCHE), from templates coming from two brain structures (hypothalamus, hippocampus) and five organs (ileum, liver, lung, spleen, skin). RNA was isolated using phenol RNA extraction. The primer sets are listed in Tables 1, 2 and amplification conditions are described in "methods". Each value is the mean of fourteen independent experiments. Data are means ± SEM.

transferase (HPRT)) with the same amplification efficiencies as target genes described above, are shown in Table 2.

Quantification

As previously shown (Figures 1 and 3), amplification efficiency and linearity of PCR assays allow the quantification in a useful range in many tissues, i.e. with a higher C_T than that of cytokine mRNA in control rats. Linearity and efficiency of PCR allow the quantification of mRNA with both methods used, i. e. the standard curve method and the comparative threshold cycle (ΔC_T) method.

The internal standard curve quantification method is the most accurate of quantifying mRNA. Indeed, the precise efficiency of each individual amplification is taken into account for each mRNA measurement. Recombinant DNA, i.e. plasmid or overlapping PCR products can be used to generate standards although the PCR efficiency slightly changes with the chemical properties of the template. The assay's accuracy is improved by using cDNA standards handled (RNA extraction and reverse transcription) in the same way as the samples. In our hands, the best results were obtained working with a pool of all tested samples. The standard pool was diluted over a 100-fold range, in a five point standard curve, centred on the concentration of the tested samples. Similar results were obtained with LPS-stimulated spleen cells prepared as described in materials and methods. This method is still time-consuming and, depending on the PCR efficiency, can be replaced with the easier ΔC_T method. Briefly, the C_T indicates the fractional cycle number for which the amount of amplified target reaches a fixed threshold. This amount is a constant depending on the primer set. The

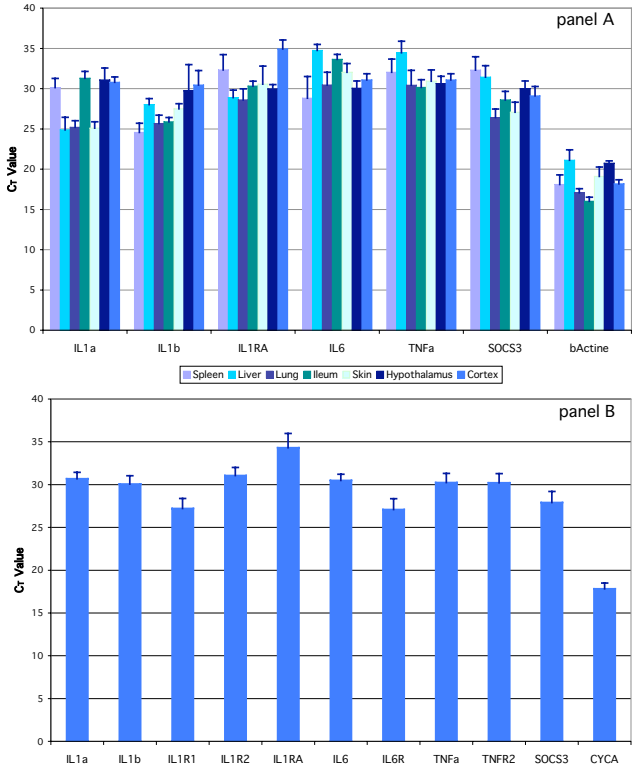

Figure 3
Expression levels of cytokines and related molecules in control rats. A) Cycle threshold (C_T) values of pro-inflammatory cytokines and SOCS3 mRNA in various structures and organs in Lewis rats. Quantifications were carried out from 30 ng of RNA (phenol extraction). Each value is the mean of seven values. Data are means ± SEM. B) C_T values of pro-inflammatory cytokine and receptor mRNAs in hypothalamus from Sprague-Dawley rats. Quantification was carried out from 30 ng of RNA (RNeasy extraction; QIAGEN). Each value is the mean of seven values. Data are means ± SEM.

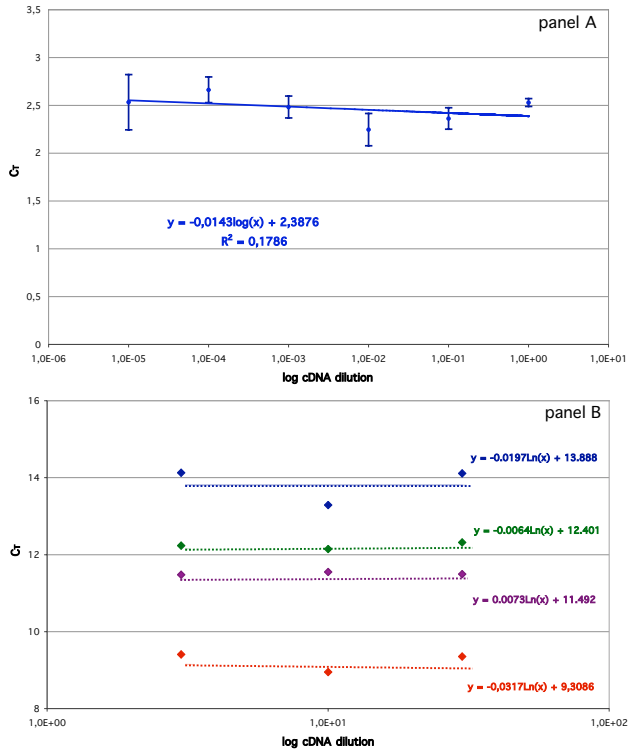

Figure 4
Validation of the 2-ΔCT method for cytokine mRNAs quantification. The ΔC_T method for relative quantification requires that the efficiency of targets (cytokines and related molecules) and reference (CypA) amplified in different tubes is approximately the same. Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers sets. The difference (ΔC_T) between cycle threshold (C_T) from target and reference was calculated for each dilution. The absolute value of the slope of ΔC_T in relation to the logarithm of cDNA concentration should be less than 0.1 [14]. Panel A shows the results of an IL1b/CypA assay where a cDNA preparation was diluted over a 10.000-fold range in three independent experiments. The data were fit using least-squares linear regression analysis (n = 3). Data are means ± SEM. Panel B: The validity of the ΔC_T method was controlled for each experiment: using a 10-fold range standard curve using sample pools as calibrators (green diamond) IL1a/CypA, (blue diamond) IL6/CypA, (red diamond) SOCS3/CypA, (purple diamond) TNFa/CypA.

difference (ΔC_T) between the C_T of the target gene (C_{Tt}) and the reference gene (C_{Tr}) depends on the RNA relative copy number between the target and the reference gene. When the PCR have been properly optimized, the PCR efficiencies are close to one and the amount of target (X_N), normalized to an endogenous reference is given by the equation: $X_N = K \times 2^{-\Delta CT}$ were K is a constant according to the target and reference primer sets [12]. The normalization to a calibrator allows to reduce the previous equation in removing the constant K. In these conditions, the amount of target normalized to an endogenous reference and relative to a calibrator ($X_{N,C}$) is given by the equation: $X_{N,C} = 2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T$ is the difference between the ΔC_T of the sample and the ΔC_T of the calibrator. So the ΔC_T method is based on i) similar amplification efficiency rates between target and reference genes and ii) PCR efficiency close to 1 [13]. This hypothesis should be taken into account by checking the influence of template dilution on the ΔC_T variation. Figure 4 (panel A) shows the results of an IL1b/CypA assay where a cDNA preparation was diluted over a 10.000-fold range in three independent experiments. ΔC_T calculation can be used when the slope

obtained is close to zero. An absolute value of the slope less than 0.1 is adequate [14] although higher values are acceptable [15]. The validity of the ΔC_T method was controlled for each experiment: the PCR efficiency was controlled with a 10-fold range standard curve (two or three points) using sample pools as calibrators (Figure 4, panel B).

In the vast majority of the experiments, we chose the ΔC_T method due to the facility and speed for set up and analysis. The standard curve method was favoured with low-level mRNA samples.

Low-level mRNA samples

Although RT-PCR assays described above are able to assess basal mRNA levels in most rat tissues, quantification problems could appear in small samples such as isolated skeletal myofibres or small numbers of cells. At low copy number of target DNA the well-known problem of increased variability in target sampling is associated with other drawbacks: i) primer dimers and non-specific products are more readily generated [16], ii) slight decreases in PCR efficiencies are observed in standard curve for the lowest concentrations.

In order to avoid these difficulties, low mRNA expression can be detected using cycle-limited nested RT-PCR [16]. Another way is to allow the denaturation of unspecific products before reading the fluorescent signal. For this purpose, the fluorescence is measured at the end of a 2–3 seconds incubation at 5°C below the product melting temperature. This step is added at the end of elongation step at 72°C. However, if problems occur in proportion to low levels of total cDNA in templates (i.e. for small samples), quantification must be assessed using the standard curve method instead of the ΔCT method. We either used an optimised curve-fit through the data of the standard, or we optimized the amplification conditions according to the specific template [11].

Real Quant software (Roche Applied Science, Mannheim, Germany) allows the quantification using a two sections curve-fit, i) a linear section describing the upper concentration range and, ii) a curved section for the non-linear part, on the lower concentration range of the standard curve. As we previously described in isolated skeletal myofibres [17], use of a sample pool slightly enriched in recombinant DNA as a standard, strongly increases the accuracy of the PCR assay. A complementary way is to optimise the amplification conditions directly on a sample-pool i) by decreasing the primer annealing temperature in a 1–5°C range, ii) on a LightCycler (Roche Applied Science, Mannheim, Germany) by decreasing the temperature ramping between annealing and elongation steps (from 20°C/ to 1–3°C/s) to reduce non specific hybridisation. The specificity of each PCR product is controlled using melting curves. This method which lessens C_T in a 1–3 cycle range, allowed us to quantify weakly expressed transcription factors in small amounts of CD34+ cells [18].

Cytokine mRNA quantification in various physiopathological states

We improved our PCR assay in numerous tissues in a broad range of physiopathological states such as γ-irradiation, thermal injury, contention stress, heat stroke and in *ex vivo* LPS or lectin total-blood stimulation (data not shown). This method enables numerous fold increases in cytokine and related molecule mRNAs (Figure 5.A) to be displayed as well as slight increases (Figure 5.B1,5.B3). Moreover, the sensitivity of the method ensures the detection of rare cases of decrease of basal levels of cytokine mRNA as shown in Figure 5.B.2.

Discussion

RT-PCR has proved to be a powerful method of studying gene expression in mammalian tissues. Cytokine mRNA quantification was one of the earliest examples using this method [19]. Indeed, RT-PCR is advantageous for cytokine transcript analysis because it can be used to quickly monitor the simultaneous expression of an array of cytokines from a single sample and requires only small quantities of template material. Up to now, such studies by techniques such as ELISA are not possible at the protein level, due to the lack of sensitivity of ELISA kits and the availability of high sensitivity ELISA kits for rats.

In this report we describe the application of a novel SYBR Green real-time RT-PCR assay for the quantification of a large panel of rat cytokines and related molecules. This method allows an accurate determination of basal cytokine and a related molecule pattern in most of the control rat organs and therefore, easily detects increased mRNA levels. Moreover, the assay sensitivity is adequate to analyse i) mRNA levels in small samples and ii) rare cases of decrease of basal cytokine levels in pathophysiological conditions. Experimental variations in different samples are corrected through normalization to a housekeeping gene. As the reliability of the results depends on the choice of the most relevant housekeeping gene according to the specific experimental conditions, we propose primer sets, able to quantify the most common housekeeping genes, Furthermore, the low differences in amplification efficiencies of PCR assays, allow the use of the fast ΔC_T quantification method.

Although mRNA quantification of cytokines and cytokine receptors is an essential tool, it is only a rough estimate of cytokine profiles at the protein level. Particularly post-transcriptional control is an important feature of cytokine

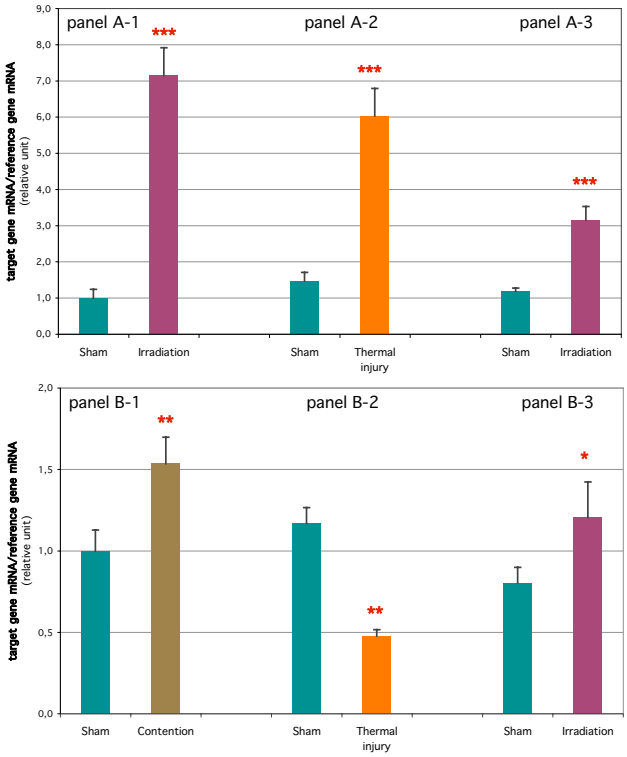

Figure 5

Quantification of cytokine-related molecule mRNAs in various pathophysiological conditions. Studies were in accordance with French ethical guidelines and approved by a Medical Committee of the French Army Medical Research Centre (CRSSA). Sham-animals were handled in the same conditions as injured animals without injury exposure. Conditions for 40% total body surface thermal injury (TI) and 8 Gy whole-body irradiation (WBI) were described previously [12,22]. A 30 minutes contention was achieved in the irradiation device. Data are means ± SEM, n = 6. Statistical significance was assessed using ANOVA or Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). A.) 1; increase in spleen TNFa mRNA 3 h after 8 Gy WBI, 2; increase in liver SOCS3 mRNA 24 h after 40% total body surface TI, 3; ileum increase in IL1RA mRNA 23 h after 8 Gy WBI. B.) 1; increase in hypothalamus IL1a mRNA 3 h after a 30 minutes contention, 2; decrease in liver IL6 mRNA 6 h after 40% total body surface TI, 3; increase in hypothalamus IL6-receptor mRNA 2 h after 8 Gy WBI.

receptor regulation. It may be useful to investigate the functionality of the cytokine network to overcome the impossibility of studying many cytokines at the protein level, by measuring mRNA encoding inducible proteins related to cytokine pathways, such as i) SOCS proteins, ii) cytokine-induced genes and iii) inducible transcription factors. SOCS proteins, in particular, which are cytokine-inducible regulators of signalling are of great interest [20]. Specifically, quantification of SOCS 3 mRNA allows the investigation of IL-6 signalling [21]. IL1-b, in the same way, induces both nitric oxide synthase 2 (NOS-2) and cyclooxygenase 2 (COX-2) gene expression [3,22,23]. Activation of transcription factors, such as AP1 and C/EBPβ, the rodent homologue of human NF-IL6, which play a critical role in gene regulation in response to inflammatory cytokines can be studied at the transcriptional level [24,25]. Primer sets, homogeneous with respect to PCR efficiency, designed for quantification of these cytokine-related mRNAs with real-time PCR assays are given in Tables 2, 3.

Conclusions

Herein we report an innovative SYBR Green real-time RT-PCR assay developed to detect rat pro-inflammatory cytokines and related molecules in a homogenous and reproducible manner. Due to its high sensitivity, this assay is suitable for, i) analyzing basal levels, and even physiopathological decreases below the basal level of target mRNA in most of rat organs, ii) quantifying mRNA from very small samples. Real-time RT-PCR is currently the best way to investigate the cytokine network, while waiting for the development of more sensitive assays for the detection of secreted cytokine and cytokine receptors in rats. In particular, real-time RT-PCR assays should be carried out to analyse i) genes involved in cytokine signalling as transcription factors or suppressors of cytokine signalling and ii) cytokine-induced genes.

Methods

Oligonucleotide primers

Oligonucleotide primers were synthesised at Eurogentec (Saraing, Belgium). Primer design and optimization concerning primer dimer, self-priming formation and primer melting temperature was done with MacVector software (Accelrys, San Diego, USA).

Total RNA isolation

Total RNA was isolated using either phenol or anion exchange resin. Phenol extraction was carried out using a protocol adapted from Chomczynski and Sacchi [26]. In short, samples were disrupted (5% weight/volume) in lysis buffer (4 M guanidine thiocyanate, 25 mM pH7.0 sodium citrate, 0.5% N-laurylsarcosine and 0.1 M β-mercaptoethanol) with a blender (Waring Blender, New Hartford, USA). RNA isolation was carried out from 900 μL of lysate with 90 μL of 2 M pH4.0 sodium acetate, 810 μL of phenol and 180 μL of 24/1 chloroform / isoamylic alcohol. Conversely, total RNA was isolated from 30 mg samples, using RNeasy mini kit (QIAGEN S.A., Courtabeuf, France) following the manufacturer's instructions with the optional RNase-free DNase step to avoid contamination with genomic DNA.

Table 4: Primer sequences and detailed PCR conditions used to generate standard recombinant DNA.

Gene name		5'-3' primer sequence	Position[a] (cDNA)	Annealing temperature[b]
IL1a	FW	GTGGTGGTGTCAGCAACATCAAAC	275–862	56°C
	RW	GAAATCTATCATGGAGGGCAGTCC		
IL1b	FW	TGAAAGCTCTCCACCTCAATGGAC	501–894	57°C
	RW	TGCAGCCATCTTTAGGAAGACACG		
IL1RA	FW	AAGACCTTCTACCTGAGGAACAACC	139–310	55°C
	RW	GCTTGGTGTCATCTCCAGACTTG		
IL1R1	FW	TGTCTACTGGAAGTGGAATGGGTC	1143–1500	56°C
	RW	GGGAAGAAAATCAGAGCAGGAGTC		
IL1R2	FW	CACCCAGTTCTTGGAGACGATTG	226–598	57°C
	RW	TGGAGGAGAGAGCTGAGATTTGC		
IL6	FW	TCTGGAGTTCCGTTTCTACCTGG	388–682	55°C
	RW	CATAGCACACTAGGTTTGCCGAG		
IL6R	FW	AGCAGGCAATGCTACCATTCAC	264–873	57°C
	RW	GTCGGTATCGAAGCTCGAATTG		
TNFa	FW	AGCACAGAAAGCATGATCCGAG	4–499	58°C
	RW	CCTGGTATGAAGTGGCAAATCG		
TNFR2	FW	TCAGATGTGCTGTGCTAAGTGTCC	93–512	58°C
	RW	GCCAGGATGCTACAAATGCG		
SOCS3	FW	ATGGTCACCCACAGCAAGTTTC	18–679	56°C
	RW	TACTGGTCCAGGAACTCCCGAATG		

Abbreviations: see Tables 1, 2. These primer sets allow generating recombinant DNA to ensure the specificity of the PCR amplification or to generate standard curves. [a] Position of amplification product within cDNA sequence. Genbank accession number are given in Tables 1, 2, [b] melting temperature of specific PCR product.

Reverse transcription

Reverse transcription of mRNA was carried out in a 60 μL final volume from 4 μg total RNA using 300 U M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instructions with 500 ng oligo $(dT)_{12-18}$ and 50 U ribonuclease inhibitor (RNase-Out, Promega).

Real-time PCR

PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 0.5 μL of cDNA, corresponding to 30 ng of total RNA in a 20 μL final volume, 3–4 mM $MgCl_2$ and 0,4 μM each primer (final concentration). Detailed PCR conditions are displayed in Table 3. Briefly, quantitative PCR was performed using LightCycler (Roche Applied Science, Mannheim, Germany) for 45 cycles at 95°C for 20 s, specific annealing temperature for 4–5 s and 72°C for 8 s. Amplification specificity was checked using melting curve following the manufacturer's instructions.

Result analysis and quantification

Results were analysed with LightCycler Software v.3.5 (Roche Applied Science, Mannheim, Germany) using the second derivative maximum method to set C_T. Quantification using either standard curves or the ΔC_T method was carried out with Real Quant Software (Roche Applied Science, Mannheim, Germany).

Standard preparation: LPS-stimulated spleen cells

Eight-week-old Wistar male rats were anesthetized under halothane. Spleens were aseptically removed and perfused with 5 mL of Hank's buffered saline (Sigma-Aldrich). Spleens were then prepared as single-cell suspensions in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum and antibiotics, all purchased from Sigma. Suspensions were adjusted to 5×10^6 viable cells/mL. Spleen cell cultures were carried out in 25 cm^2 tissue-culture flasks (Falcon, BD Biosciences) at 50×10^6 cells in 10 mL of RPMI-FCS complete medium. They were stimulated with 1 μg/mL of lipopolysaccharide (LPS, *Salmonella typhimurium*, Sigma-Aldrich) for 2 and 10 h, at 37°C, in a 5% CO2-95% air atmosphere. At the end of the incubation time, cells were harvested and stored in 500 μL of RNA Later (Ambion, Austin, USA) before RNA extraction.

Standard preparation: specific recombinant DNA

Specific recombinant DNA standards were synthesized using PCR. Reactions were performed using LightCycler (Roche Applied Science, Mannheim, Germany) for 45 cycles at 95°C for 20 s, specific annealing temperature for 5 s and 72°C for 10 s. PCR were carried out with the LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 0.5 μL of cDNA, corresponding to 30 ng of total RNA in a 20 μL final volume, 4 mM $MgCl_2$ and 0,4 μM each primer (final concentration).

Primer sets and annealing temperature are described in Table 3. Standard curves were achieved from ten-fold dilutions of PCR product in a 10^{-7}-10^{-12} range. Moreover, comparison between PCR products melting peaks obtained either with cDNA sample or recombinant DNAs, ensured the specificity of the PCR amplification.

DNA contamination

Genomic DNA contamination of total RNA was controlled either using RT-PCR specific to the first intron of vasopressin gene (Genbank X59496) or RT minus control. Vasopressin gene amplification is carried out in the same reaction conditions as IL1a mRNA. 5'to 3' forward and reverse primers are as follows, AGCATGTGGTCTTTGGGAAGGTG and TAGGCTCAAATCTGGTCAGGTCAC generating a 107 bp DNA fragment.

Authors' contributions

AP conceived the study and designed the primer sets. CM and OB carried out the experiments and designed the experimental procedures. AA helped design the experimental procedures and co-write the paper with AP. DA and YC carried out the thermal injury study. JM, DC carried out the irradiation studies and took part in work conception with EM. All authors read and approved the final manuscript.

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