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Detection of immune cell response to *M. tuberculosis*–specific antigens by quantitative polymerase chain reaction☆

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Abstract

One third of the world's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*) and up to 10% of infected individuals develop active tuberculosis (TB) in their lifetime. Among the major challenges in the control of TB is the implementation of sensitive methods for detection of latent tuberculosis infection (LTBI). Currently, in vitro interferon gamma release assays, yielding single value readout, are used as an alternative to the traditional tuberculin skin test for the diagnosis of LTBI. More complex characterization of immune status of LTBI individuals, however, is desirable for indication of LTBI subjects for preventative chemotherapy. Here we describe a quantitative polymerase chain reaction (qPCR) for determination of expression levels of 14 genes, additional to interferon gamma, which was applied for comparison of the specific *Mtb*-antigen immune response of blood cells from healthy, latently infected, and TB individuals. With the use of principal component analysis and discriminant analysis, a pattern of mRNA levels of 6 genes was identified, allowing discrimination of healthy individuals from active TB and LTBI subjects. These results open the way to development of multimarker qPCR for the detection of LTBI.

Keywords: qPCR; Latent tuberculosis infection; Blood sample processing; Reference gene

1. Introduction

Tuberculosis (TB) is a major public health problem that contributes considerably to morbidity and mortality from infectious diseases around the world. It is estimated that approximately one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB in humans (World Health Organization, 2010) and some 10% of those develop active TB (Kaufmann and McMichael, 2005), while 90% of the *Mtb* carriers are asymptomatic individuals with latent tuberculosis infection (LTBI). Of LTBI donors, the recent contacts of TB patients and immunosuppressed individuals (e.g., HIV-infected individuals, organ transplant patients, patients undergoing anti–TNF-α therapy, etc.) are at particular risk of developing active TB (Krejsek and Kopecky, 2004; Kroesen et al., 2003). Treatment of LTBI with chemoprophylaxis substantially reduces the risk that TB infection progresses to disease (Menzies et al., 2011). Therefore, targeting and treating LTBI subjects with high risk of disease progression are a key strategy for effective control of the spread of TB. Such efforts are, however, complicated by the lack of a gold standard diagnostic test for the detection of LTBI. Most often, identification of individuals infected with *Mtb* relies on the tuberculin skin

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test (TST), which reflects a delayed type of hypersensitivity to *Mtb* antigens and exhibits rather high ratios of false-positive results (Pai et al., 2004). Recently, so-called interferon gamma release assays (IGRAs) were introduced into clinical practice. These 'recall response' assays measure the number of interferon gamma (IFN-γ)–secreting T cells, or the amount of IFN-γ released by sensitized blood cells, in response to restimulation by specific *Mtb* antigens (Mazurek et al., 2010). Among the limitations of IGRA use is the requirement for blood samples containing normal levels of viable leukocytes. This limits the usage of IGRAs, particularly in immunocompromised individuals (e.g., HIV-infected), who are a major risk group in TB. Therefore, there is a considerable demand for development of a more specific and sensitive method for detection of LTBI than TST or IGRA tests (Mazurek et al., 2010).

Specificity and sensitivity of tuberculosis diagnostic tests should be significantly enhanced by the detection of additional diagnostic markers that are involved in *Mtb* antigen–specific immune response (Ruhwald et al., 2007). Recently, quantitative polymerase chain reaction (qPCR) was used to monitor mRNA levels in *Mtb* antigen–activated peripheral blood mononuclear cells and was claimed to enable distinguishing among healthy, LTBI, and active TB diseased individuals, respectively (Wu et al., 2007). The use of qPCR for study of gene expression, however, requires statistical considerations of all involved factors, including the natural variation among individuals (intersubject biological variance) and the processing variation introduced during sample processing (Tichopad et al., 2009). Moreover, gene expression studies require appropriate normalization.

In this study, we focus on the optimization of critical steps of whole blood sample processing prior to qPCR, for the analysis of alterations of gene expression levels in antigen-stimulated T cells contained in whole blood samples. Contribution from the individual preanalytical steps to the total error of analysis was assessed, an optimal set of reference genes for normalization was defined, and the alteration of expression levels of marker genes involved in specific *Mtb*-antigen immune response was analyzed in blood samples from healthy, TB-diseased, and LTBI donors. Principal component analysis (PCA) and discrimination analysis of multimarker gene expression profiles allowed subject classification and discrimination of healthy and *Mtb*-infected individuals.

2. Materials and methods

2.1. Sample preparation

A total of 100 clinical samples were obtained. Blood was collected in intervals of 3 months from 19 healthy individuals (n = 64 samples) that scored negative in the commercial test QuantiFERON®-TB Gold In-Tube (QFT) (Cellestis, Victoria, Australia). A further 36 additional samples were repeatedly taken from 7 *Mtb*-infected individuals scoring positive for QFT. Active tuberculosis was diagnosed in 3 QFT-positive individuals by chest radiography and the other 4 QFT-positive individuals were latently infected. In order to reflect diversity among donors, we chose individuals of different age (from 24 to 86 years) and gender. Informed consent was obtained from the patients, and the study was approved by the relevant hospital ethical committee.

Whole-blood sample (3 mL) from each donor was aliquoted into 3 heparin-containing tubes (1 mL) of the QFT commercial test and contained *Mtb*-specific antigens; the positive control, mitogen; and the negative control, nil, respectively. Blood was incubated at 37 °C for 6, 9, 12, 15, or 18 h to establish the length of time for restimulation by *Mtb*-specific antigens. Standard incubation was 15 h. Aliquots of stimulated blood (0.5 mL) were transferred into microtubes and lysed by the addition of 1 mL of Tri-reagent solution (Molecular Research Center, USA). An additional 3-mL samples of whole blood were collected from each of 5 individuals (2 QFT-positive, 3 QFT-negative) into heparin-containing tubes for immediate lysis. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), including a treatment with DNase I (Promega, Wisconsin, USA). RNA integrity was checked by electrophoresis on formaldehyde agarose gels according to recommendations in the RNeasy Mini Kit protocol. Concentration and purity of RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). To study the effect on gene expression, duplicates of 200 ng of total isolated RNA were reverse transcribed into cDNA following the manufacturer's instructions in a 25-μL reaction using the Reverse Transcription System (Promega).

2.2. Quantitative PCR

Primers for the 17 selected genes (Table 1) were designed with Primer-BLAST (http://ncbi.nlm.nih.gov/tools/primer-blast), except for the genes encoding interleukin 18 (IL-18) and monocyte chemotactic protein-1 (MCP-1), whose sequences were already published (Mallat et al., 2001; Oberbach et al., 2010). The primers were analyzed for secondary structures including hairpins and self-dimers, and for cross-dimers in primer pairs with the Netprimer software (http://premierbiosoft.com/netprimer/index.html).

All primers were designed to anneal at 60 °C, and primer specificities and assay efficiencies were tested on control cDNA, prepared by pooling of cDNA from several individuals. Primer acceptance criteria were set as follows: i) quantification cycle (Cq) value in specific amplification of control cDNA shall be below 35 (Lefever et al., 2009); ii) single dominant peak in the derivative of the melting curve; iii) no amplification of nontemplate controls (Abramo et al., 2006); iv) reaction efficiency (E) between 90% and 100% (Rasmussen, 2001).

Quantitative PCR was performed using 200 nmol/L of each primer in the Bio-Rad CFX96 or CFX384 instruments

Table 1
Oligonucleotide primers used for quantification of gene expression

Gene		Oligonucleotide sequence (5′→3′)	Amplicon size (bp)	*E* (%) Bio-Rad CFX96	*E* (%) Bio-Rad CFX384	GenBank accession number
Interferon gamma	IFN-γ	F: GTTTTGGGTTCTCTTGGCTGTTA R: AAAAGAGTTCCATTATCCGCTACATC	112	95	97	NM_000619.2
Interleukin-12 subunit β	IL-12β	F: CGTTTGTCTGCCAGGATGTA R: TATCTCTTGCGTTCCCATCC	216	99	94	NM_002187.2
Interleukin-10	IL-10	F: GAGATGCCTTCAGCAGAGTG R: TGGGTCTTGGTTCTCAGCTT	172	99	99	NM_000572.2
Interleukin-2	IL-2	F: ACCTCAACTCCTGCCACAAT R: TGAGCATCCTGGTGAGTTTG	199	97	96	NM_000586.3
Interleukin-4	IL-4	F: TGCCTCCAAGAACACAACTG R: ACTCTGGTTGGCTTCCTTCA	242	95	99	NM_000589.2
Forkhead box P3	Foxp3	F: TGTCACAATCCTGTCCCTCA R: TGACGCTGCTTCTGTGTAGG	220	97	96	NM_014009.3
Interleukin-8	IL-8	F: TCTGGACCCCAAGGAAAACT R: TCCTGGGCAAACTATGTATGG	249	91	94	NM_000584.3
Interferon gamma–inducible protein 10	IP-10	F: CCACGTGTTGAGATCATTGCT R: ATTTTGCTCCCCTCTGGTTT	125	98	97	NM_001565.3
Tumor necrosis factor α	TNF-α	F: TCCTTCAGACACCCTCAACC R: AGGCCCCAGTTTGAATTCTT	173	94	95	NM_000594.2
β-2-Microglobulin	β2M	F: GCCTGCCGTGTGAACCAT R: CGGCATCTTCAAACCTCCAT	119	99	98	NM_004048.2
TATA Box–binding protein	TBP	F: ACTTCGCTTCCGCTGGC R: AGCATCTCCAGCACACTCTTCT	121	92	91	NM_001172085.1
Interleukin-18 (Mallat et al., 2001)	IL-18	F: CAAGGAATTGTCTCCCAGTGC R: CAGCCGCTTTAGCAGCCA	79	90	90	NM_001562.2
Interleukin-15	IL-15	F: CATGGAGCACAGAAATCAATG R: TGGCTATGGCAAGGGGT	117	99	99	NM_172174.2
Monocyte chemotactic protein-1 (Oberbach et al., 2010)	MCP-1	F: CCC CAG TCA CCT GCT GTT AT R: TGG AAT CCT GAA CCC ACT TC	171	95	98	NM_002982.3
Interferon-induced 35-kDa protein	IFI35	F: CAAACAGACCCGAGACCCAT R: TGGGACCTTGTCTTTGGGG	140	90	92	NM_005533.4
Transforming growth factor β 1	TGF-β1	F: CGACTACTACGCCAAGGAGGTC R: AGAGCAACACGGGTTCAGGT	148	90	91	NM_000660.4
Antigen peptide transporter 1	TAP1	F: CTCTGGAAACCCTGTGCGT R: CGGCCCGTAAAGAATGGAA	127	90	90	NM_000593.5

E = Reaction efficiency for each qPCR instrument; F= forward; R = reverse.

(Bio-Rad, Philadelphia, PA, USA) according to the standard protocol recommended by the manufacturer of SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, St. Louise, MO, USA). Briefly, 8 ng of reverse transcribed RNA was used as template in a 20-μL (Bio-Rad CFX96) or 10-μL (Bio-Rad CFX384) qPCR reaction volume, with an initial step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and recording melting curve.

2.3. Impact of sample processing on the accuracy of measuring gene expression levels

The impact of variance introduced by the individuals and by the processing steps on the final Cq value was analyzed with a hierarchical nested experiment as described by Tichopad et al. (2009). Briefly, 3 blood samples were collected from each of 3 QFT-negative donors and stimulated 15 h with *Mtb*-specific antigens at 37 °C. Total RNA was extracted and split into 3 aliquots for reverse transcription (RT) reaction. The cDNA was further split into 3 qPCR aliquots. This resulted in 3 subjects × 3 samples × 3 RTs × 3 qPCRs = 81 Cq values per gene. The analyzed genes were IFN-γ, IL-2, β-2-microglobulin (β2M), and TATAA-box binding protein (TBP). Nested ANOVA was used to model the experiment and estimate the variance contributions and standard deviations (SDs) of each sample processing level (SAS software, version 9.1 for Microsoft Windows, SAS Institute, Cary, NC, USA).

2.4. Impact of blood sample incubation at room temperature on mRNA levels

The impact on mRNA stability of the delayed initiation of incubation at 37 °C after blood sampling was analyzed as follows. Nine milliliters of blood was collected from each of 3 donors (2 LTBI, 1 healthy) and aliquoted into 1-mL QFT tubes containing *Mtb* antigens. Three aliquots from each donor were kept at room temperature for 0, 3, or 6 h, respectively, prior to incubation for 15 h at 37 °C. Next, Cq values were measured by RT-qPCR for all 17 genes. Mean

Table 2
Parameters resulting from processing of blood samples

		Procedure A[a]				Procedure B[b]			
		IFN-γ	IL-2	β2M	TBP	IFN-γ	IL-2	β2M	TBP
Mean Cq		25.43	27.12	18.52	29.40	25.76	27.65	18.44	29.16
SDs[c]	Intersubject variation	0.63	0.87	0.20	0.44	0.30	0.63	0.15	0.13
	Processing noise								
	Sampling	0.29	0.31	0.20	0.38	0.38	0.31	0.25	0.13
	RT	0.29	0.24	0.19	0.30	0.38	0.32	0.22	0.13
	qPCR	0.20	0.14	0.24	0.20	0.21	0.23	0.21	0.19
	Total processing noise[d]	0.46	0.42	0.37	0.53	0.58	0.50	0.39	0.26
Total SD[e]		0.78	0.97	0.42	0.69	0.65	0.80	0.42	0.29

[a] Procedure A employed the RNeasy Mini Kit.
[b] Procedure B employed the NucleoSpin® RNA II Kit.
[c] SDs were calculated by SAS software for Windows, version 9.1.
[d] Total processing noise = $\sqrt{SD^2_{sampling} + SD^2_{RT} + SD^2_{qPCR}}$.
[e] Total SD = $\sqrt{SD^2_{sampling} + SD^2_{RT} + SD^2_{qPCR} + SD^2_{subject}}$

Cq values were calculated for each gene at each of the 3 incubation times for each donor. ΔCq values were calculated and compared with paired *t* test using GenEx Software (MultiD, version 5.1, Göteborg, Sweden).

2.5. Selection of reference genes

The gene expression levels (Cq values) of 10 candidate reference genes were studied using the Human Reference Gene Panel (TATAA Biocenter, Goteborg, Sweden). This included the genes for GAPDH, cyclophilin A (PPIA), actin β (ACTβ), 18S rRNA (RRN18S), β2M, ubiquitin C (UBC), TBP, 60S acidic ribosomal protein P0 (RPLP), β-glucuronidase (GUSβ), and hypoxanthine–guanine phosphoribosyltransferase (HPRT1), respectively. The assay was performed on 32 samples in total, where 16 samples were from 16 QFT-negative individuals and 16 samples were from 7 QFT-positive individuals. Eight of the samples were stimulated with *Mtb* antigens, 14 samples with mitogen, and 10 were unstimulated (negative control). The NormFinder and geNorm algorithms (GenEx Software) were used to identify the optimum reference gene(s).

2.6. Relative quantification of gene expression

Specific amplification of each targeted cDNA was confirmed by melt curve analysis and gel electrophoresis. The gene expression ratio (*R*) of a target gene to a reference in a test sample (stimulated by *Mtb* antigens) relative to control sample (negative control sample) was calculated

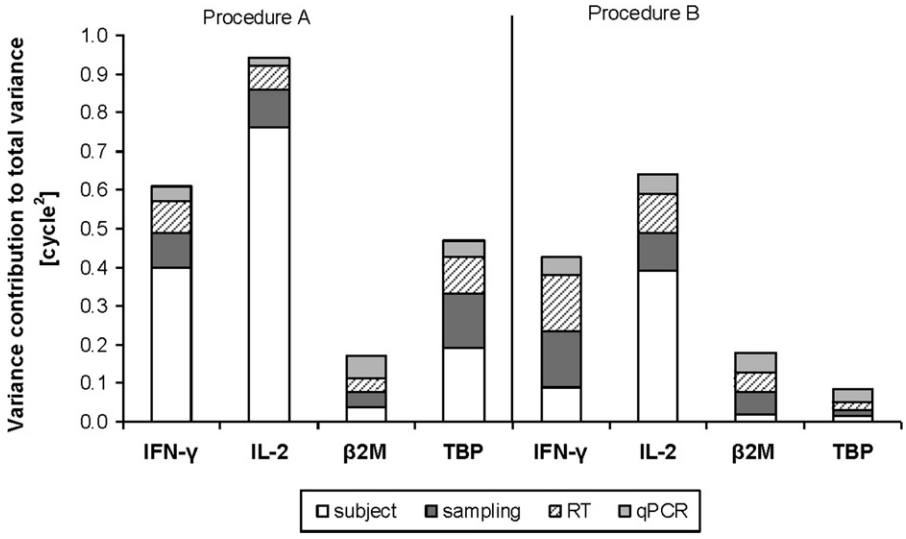

Fig. 1. Variance contributions of intersubject variation and sample processing steps to total variance of Cq. Variance contributions to total variance for 2 procedures, A and B (A: RNeasy Mini Kit, B: NucleoSpin® RNA II), are plotted in this figure. The total variance was lower in reference genes compared to marker genes in both procedures. In contrast, the inherent intersubject variation contributed more importantly to the total variance in procedure A, while this procedure exhibited a lower variance in processing noise steps (sampling, RT, qPCR) compared to procedure B, except for TBP. Total variance was calculated as follows: total variance = $SD^2_{subject} + SD^2_{sampling} + SD^2_{RT} + SD^2_{qPCR}$ (Tichopad et al., 2009).

based on estimated PCR efficiencies, E, and measured ΔCq between the test and control samples (Kubista et al., 2006).

$$R = \frac{\left(1 + E_{\text{target}}\right)^{\Delta \text{Cq}_{\text{target}}(\text{control-sample})}}{\left(1 + E_{\text{ref}}\right)^{\Delta \text{Cq}_{\text{ref}}(\text{control-sample})}}$$

Median gene expression of QFT-positive and QFT-negative donors was calculated for each target gene and compared using the Wilcoxon–Mann–Whitney U test. $P \leq 0.05$ was considered significant. All analyses were conducted with SigmaPlot 11 software (Systat Software Inc., California, USA).

3. Results

3.1. Impact of sample processing on the accuracy of measuring gene expression levels

Biological variation and technical noise introduced at various sample processing steps confound the Cq determination in gene expression measurements (Tichopad et al., 2009). We determined standard deviations (SDs) and the variance contributions of blood sampling, RT, and qPCR steps to the measured Cq values in 2 tested procedures (A and B). A fully nested design experiment was performed with samples from 3 donors, split into sets of triplicates at every processing step (3 subjects × 3 samples × 3 RTs × 3 qPCRs = 81 Cq values) to assess SDs and the variance contributions of gene expression measurements of IFN-γ, IL-2, and the reference genes β2M and TBP. Moreover, 2 commercial kits for total RNA extraction were compared: RNeasy Mini Kit (Qiagen) in procedure A and NucleoSpin® RNA II (Macherey Nagel, Düren, Germany) in procedure B.

The estimated SDs for the various sample processing steps, intersubject variations, total processing noise, total SD of the Cq determination, and mean Cq of tested genes are respectively summarized in Table 2. SD values are labeled with a respective subscript. For all genes, mean Cq values in the range of 18.4–29.4 cycles were obtained, which reflects expression levels sufficiently high for reliable determinations (Table 2). As expected, similar SD_{qPCR} values (0.14–0.24 cycles) were obtained for all genes and were lower than the SD values of other steps, revealing that qPCR is the most reproducible step of the test.

The SDs of inherent intersubject variation were lower in the reference genes β2M and TBP compared to the marker genes IFN-γ and IL-2 (Table 2), as expected, and the total variance was lower in reference genes compared to marker genes in each procedure (Fig. 1).

In general, for an optimized procedure, the total processing noise should be lower than $SD_{subject}$. As shown in Table 2, the $SD_{subject}$ of marker genes was higher compared to total processing noise, except for IFN-γ in procedure B, for which total processing noise was higher

Table 3
Impact of blood sample incubation at room temperature on mRNA levels

Donor	ΔCq of Cq mean values in time points	IFN-γ	TNF-α	IL-2	IL-4	IL-8	IL-10	IL-12β	FOXP3	IP-10	ß2M	TBP	IL-15	MCP-1	TAP1	IFI-35	IL-18	TGF-β1
A	Δ[Cq (0) − Cq (3)]	0.40	0.50	1.02	0.13	0.22	0.22	0.97	0.03	0.52	0.48	0.40	0.33	0.43	0.95	0.92	0.40	0.33
	Δ[Cq (0) − Cq (6)]	0.16	0.63	0.83	0.15	0.53	0.48	0.47	0.58	0.33	0.35	0.62	0.52	0.52	0.67	0.03	0.20	0.43
B	Δ[Cq (0) − Cq (3)]	0.08	0.68	0.33	0.18	0.50	0.73	0.58	0.65	0.32	0.13	0.75	0.45	0.65	0.22	0.17	0.03	0.35
	Δ[Cq (0) − Cq (6)]	0.50	0.45	0.30	0.28	0.63	0.57	0.35	0.33	0.13	0.25	0.53	0.70	1.15	0.98	0.75	1.00	0.84
C	Δ[Cq (0) − Cq (3)]	0.23	0.43	0.05	1.15	1.00	0.70	1.02	0.10	0.52	0.02	0.42	0.52	0.82	0.47	0.27	0.18	0.15
	Δ[Cq (0) − Cq (6)]	0.30	0.57	0.63	0.97	0.15	0.57	0.05	0.43	0.60	0.62	0.30	0.28	0.28	0.67	0.15	0.12	0.20
Threshold value of $P = 0.00285$	P value	0.72	0.55	0.34	0.34	0.43	0.25	0.70	0.02	0.43	0.68	0.40	0.01	0.94	0.11	0.15	0.38	0.54

Mean values of Cq from triplicates in time 0, 3, and 6 h of delay at room temperature were calculated for all genes and each donor (A, B, C). Differences between ΔCq of Cq mean values were determined using multiple testing by paired t test with threshold value of $P \leq 0.00285$, in order keep the overall risk of type I error at a $P \leq 0.05$ level.

compared to $SD_{subject}$, probably because of the differing purity of samples.

On the basis of the lower total processing noise values of each individual gene in procedure A compared to B (except for TBP), procedure A was used for further assessment of gene expression in the samples from the test group of donors (Table 2).

3.2. Impact of blood sample incubation at room temperature on mRNA levels

We assessed the effect of room temperature incubation of the blood samples before the 15-h stimulation at 37 °C. Cq values were measured for all 17 genes in triplicate blood samples from 3 donors that were stimulated with antigen after room temperature incubation of 0, 3, or 6 h, respectively. Multiple testing was performed using paired *t* test and a threshold value of $P = 0.0028$, to keep the overall risk of type I error at a $P \leq 0.05$ level, according to the Bonferroni correction (Bland and Altman, 1995). No statistically significant difference was observed between individual ΔCq values with samples stimulated immediately and after a delay of 3 or 6 h before initiation of the antigenic stimulation at 37 °C (Table 3). Hence, the 6-h room temperature incubation of the blood sample before stimulation at 37 °C had no significant influence on the measured Cq values for the 17 genes.

3.3. Blood restimulation time

The length of time for antigenic restimulation of blood cells was optimized by repeatedly collected blood samples from 4 QFT-positive individuals ($n = 18$ samples) and from 6 QFT-negative individuals ($n = 21$). The blood samples were stimulated for 6, 9, 12, 15, or 18 h at 37 °C and processed for determination of Cq values for the 15 marker genes. The time course of relative gene expression of the 15 genes in blood samples stimulated with *Mtb* antigen is shown in Fig. 2. Stable expression of IFN-γ and IL-2 indicating activation of T cells was observed between 6 and 18 h (Fig. 2A and B). This agrees with instructions for the QuantiFERON®-TB Gold In-Tube kit that recommend a minimal stimulation of 16 h prior to determination of IFN-γ secreted from activated T cells. When this recommendation was corroborated using flow cytometry analysis of *Mtb* antigen–stimulated blood cells, the levels of IFN-γ secretion by specifically activated $CD3^+CD4^+$ T cells from QFT-positive donors were found to be maximal between 15 and 18 h. At this time point, the contribution to unspecific IFN-γ production by $CD3^-$ blood cells (NK cells) was already minimal (data not shown). Furthermore, when expression of the 15 marker genes was analyzed within 6 to 18 h of stimulation, the expression levels of most of genes were found to reach saturation already after 6 h of stimulation (Fig. 2C). Therefore, the time of 15 h, at which levels of expression of most marker genes appeared to reach a maximum, was arbitrarily taken as a standard blood cell stimulation time for further experiments.

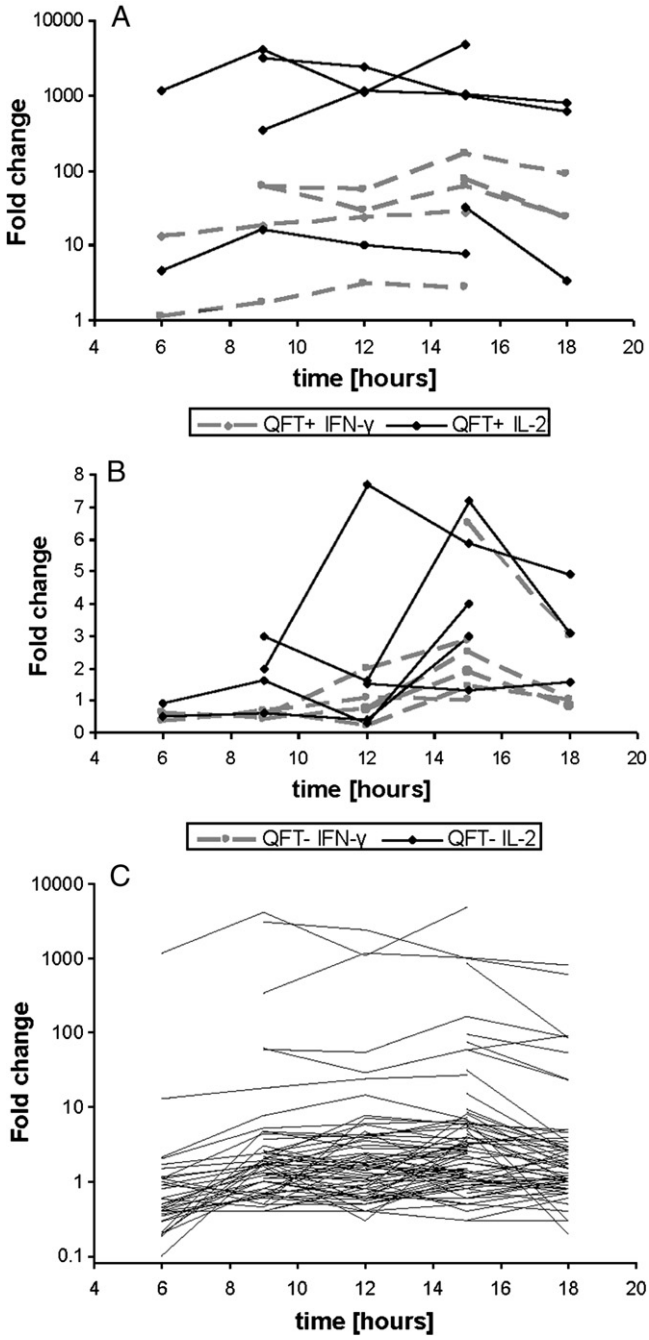

Fig. 2. Suitable time of restimulation. Whole blood samples were incubated for indicated times with *Mtb* antigens, mitogen, and negative control, and relative gene expression levels were determined as described in Materials and Methods. (A) Fold change of antigen-specific expression of IFN-γ and IL-2 genes in blood samples from QFT-positive (QFT+) donors (B) or QFT-negative (QFT−) donors. (C) Fold changes of expression of 15 marker genes in time.

3.4. Selection of reference genes

For gene expression studies using qPCR, it is necessary to compensate for variance in the amount of material used, yield variations, and inhibition. Data can be normalized to total amount of RNA used or to reference gene (Karge et al.,

1998). There is no universal reference gene that is expressed at a constant level under all conditions, in all tissues, and at all phases of cell cycle. Therefore the choice of reference gene(s) needs to be validated for each specific purpose and study (Dheda et al., 2004).

Towards this aim, we first analyzed expression levels of the frequently used reference genes GAPDH, PPIA, ACTβ, RRN18S, β2M, UBC, TBP, RPLP, GUSβ, and HPRT1 to identify the most suitable ones for our study.

The expression of the genes in representative samples, as described under Materials and Methods, was analyzed using the NormFinder algorithm (Andersen et al., 2004) as implemented in GenEx. First, the data were analyzed considering the QFT status of the donors or stimulation agents (*Mtb* antigens, mitogen, or no stimulation) to identify differentially expressed genes that would introduce a bias. ACTB, RRN18S, UBC, and HPRT1 were excluded from the panel because of too high a bias (intergroup variance ≥0.3 cycle). Analysis was then repeated without considering groups (Fig. 3A), since the remaining genes did show negligible bias. β2M and TBP were now found to be the most stable genes. This result was confirmed using the geNorm algorithm (Vandesompele et al., 2002) (Fig. 3B). Total RNA amount was included for analysis to compare differences between total RNA and reference gene to normalization. As shown in Fig. 3A, total RNA stability value was higher than those of β2M and TBP, confirming the differences between samples due to various factors and the need for using reference genes for normalization.

3.5. Differences in gene expression levels in response to Mtb-specific antigen restimulation of blood cells from QFT-positive and QFT-negative individuals

Gene expression after specific *Mtb*-antigen restimulation in blood cells was compared between 11 whole blood samples from QFT-positive and 27 samples from QFT-negative individuals. The blood samples were stimulated for 15 h at 37 °C before expression profiles for 15 immune-related genes were measured, as described under Materials and Methods.

Statistically significant ($P < 0.001$) differences were observed for IFN-γ, IL-2, IP-10, TNF-α, Foxp3, IFI-35, TAP-1, MCP-1, IL-15. IFN-γ, IL-2, and IP-10 genes, which exhibited a median expression increase of between 58- and 153-fold more in QFT-positive compared to QFT-negative samples (Fig. 4A). A 2- to 6-fold difference was then observed for TNF-α, Foxp3, IFI-35, TAP-1, MCP-1, and IL-15 genes (Fig. 4B). For IL-10, a significantly lower expression ($P < 0.015$) was observed in QFT-positive individuals. No significant difference in expression was observed for IL-12β, IL-18, IL-8, and TGF-β1, respectively.

3.6. Classification of individual responses by PCA and discriminant analysis

To find a gene expression signature characterizing the response to restimulation with *Mtb* antigens for samples

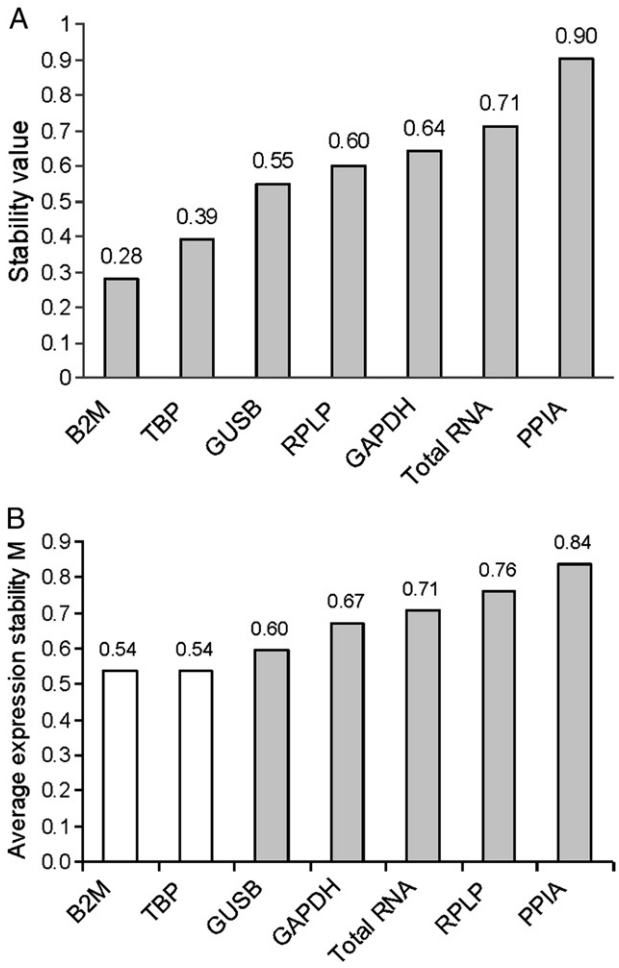

Fig. 3. Selection of optimal reference gene(s) for normalization of gene expression in blood. (A) The stability value of reference genes was determined using the NormFinder algorithm. β2M and TBP were the most stable reference genes in whole blood, with stability values of 0.28 and 0.39, respectively. (B) The last pair of candidates, β2M and TBP, remaining from 10 reference genes calculated by the GeNorm algorithm was recommended as the optimal pair of reference genes (M value indicating the average expression stability of the gene). This result is basically consistent with the NormFinder calculation.

from a specific group of donors, the data were analyzed by PCA and discriminant analysis using the XLSTAT software (http://xlstat.com). The multidimensional information on gene expression profiles was transformed into a 2-dimensional plot by the use of PCA (Jolliffe, 2002). This yielded an orthogonal vector defining a space of lower dimension, while accounting for the maximum variation in the original space. Discriminant analysis (Kender et al., 2003) was used to classify individuals based on the PCA.

Six classification genes (IL-10, IL-2, TNF-α, IFI-35, Foxp3, and IFN-γ) were identified on the basis of the correlation biplot, which allowed differentiation of the 26 tested individuals from which blood samples were repeatedly collected. First, 90% of randomly chosen samples were taken and analyzed by PCA, yielding classification into 3 groups. Next, the rest (10%) of the samples were used to test the

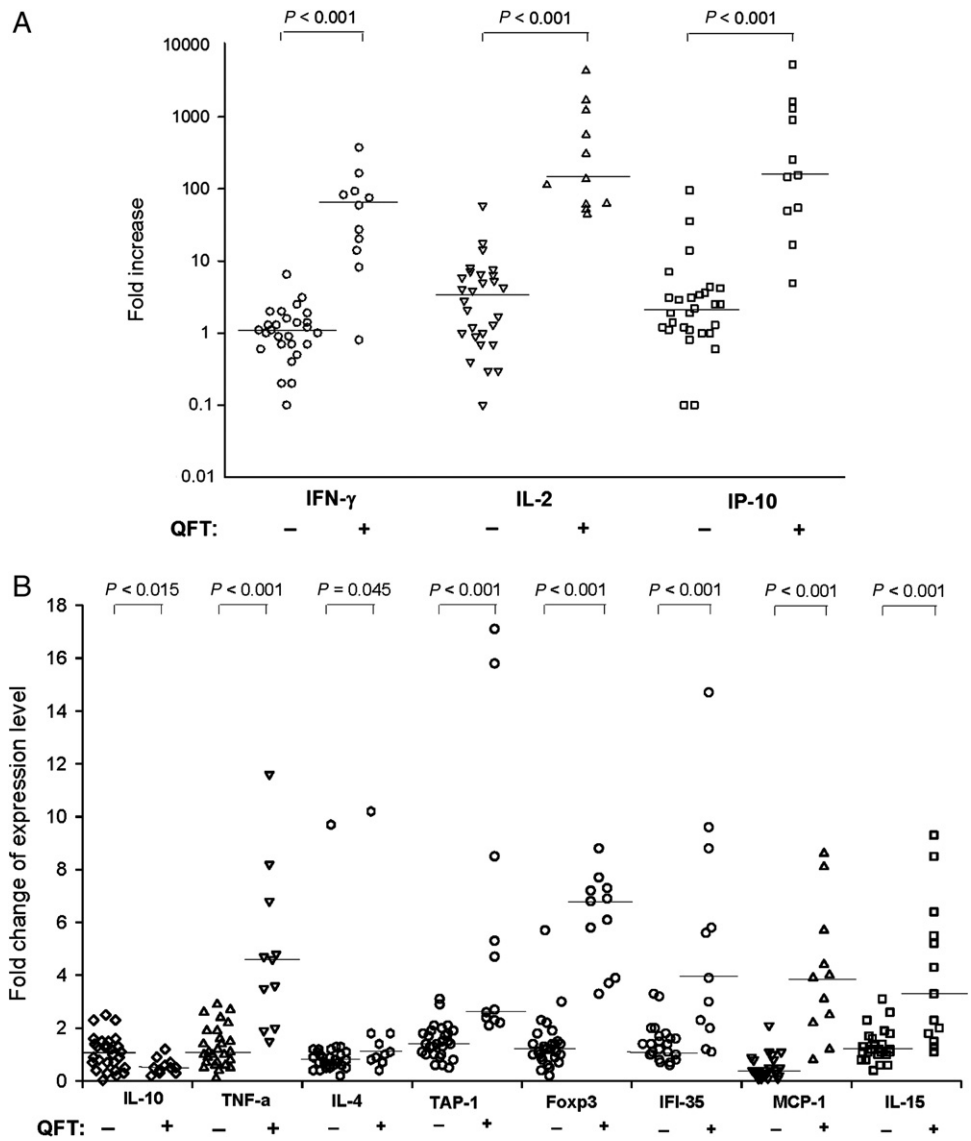

Fig. 4. Differences in gene expression levels in response to specific *Mtb* antigenic restimulation of blood cells from QFT-positive and QFT-negative individuals. Gene expression levels of TB immune–related genes were assayed in *Mtb* antigen–activated whole blood cells from QFT positive/negative groups of individuals by qPCR. The horizontal line represents the median for each group and differences between groups were determined using the Wilcoxon–Mann–Whitney *U* test (SigmaPlot 11 software). In all instances, a *P* value of <0.05 was considered significant. (A) The fold increase of the expression levels of IFN-γ, IL-2, and IP-10 was significantly higher ($P < 0.001$) in whole blood from QFT-positive (QFT+) subjects as compared to QFT-negative controls (QFT−). Genes exhibited an increase in median gene expression level between 58- and 153-fold. (B) The fold change of the expression levels of TNF-α, TAP-1, Foxp3, IFI-35, MCP-1, and IL-15 was significantly higher ($P < 0.001$) in whole blood from QFT-positive subjects as compared to QFT-negative controls, ranging between 2- and 6-fold enhancement. Gene expression of IL-10 was significantly decreased ($P < 0.015$) in whole blood from QFT-positive as compared to QFT-negative controls. Please note that Panel A has a logarithmic scale, while panel B does not.

model and assess whether the classification was nonrandom. This testing was repeated 5 times and, always, the samples were found to classify into 3 groups. Furthermore, the classification of individuals based on PCA results was confirmed by discriminant analysis, in which a score of 94.7% was reached for the confusion matrix. As a result, the samples ($n = 38$) could be classified into 3 groups (Fig. 5), where the first group included 25 samples from QFT-negative individuals, the second comprised most of the samples ($n = 10$) from QFT-positive individuals, and the third group comprised a heterogenous group of samples ($n = 3$) exhibiting borderline response. This shows that the developed qPCR assay panel differentiated between samples from QFT-positive and QFT-negative donors.

4. Discussion

In this report, we describe an expression panel of 6 genes that sensitively reflects the antigen-specific response of human immune cells restimulated ex vivo in whole blood by the specific *Mtb* T-cell antigens. Moreover, an indication of

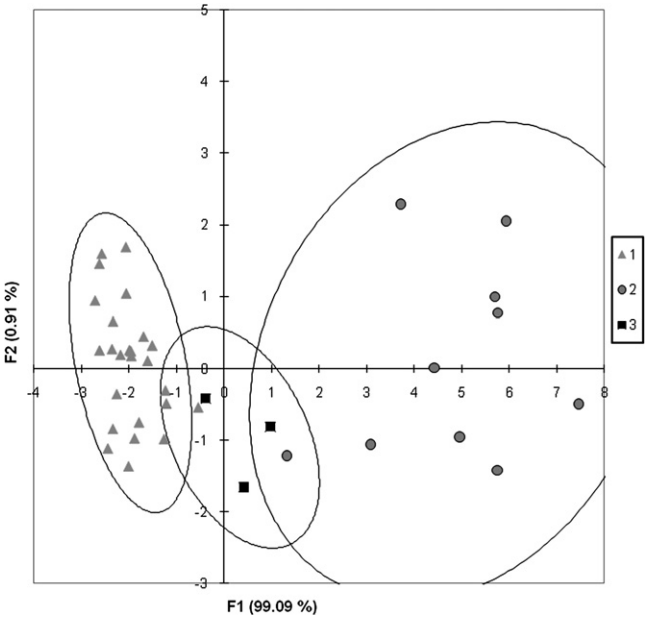

Fig. 5. Classification of individual responses by PCA and discriminant analysis. Tested samples ($n = 38$) were classified into 3 groups by using PCA (XLSTAT software), by which 6 classification genes (IL-10, IL-2, TNF-α, IFI-35, Foxp3, and IFN-γ) allowing differentiation of samples were identified. The group on the left side of the figure includes a compact group of QFT-negative samples ($n = 25$), despite individual differences. The second group (on the right side of the figure) comprised most of the QFT-positive samples ($n = 10$) with more value dispersion due to individual and TB infection variation in QFT-positive donors. The third group (in the middle of the figure) comprised the heterogenous group of samples ($n = 3$), from which 1 sample belonged to a LTBI donor, another belonged to a blood donor cured of TB, and the last one to a patient treated for active TB with lower response in the QFT test. F1 and F2 represent the 2 main components.

an intermediate response, opening for potential differentiation of latently infected (LTBI) from active TB patients, was obtained. The assay was further analyzed for contribution of individual processing steps to the total variance of Cq determination, which was found to be below 1 cycle^2. This shows that, upon validation on sufficiently large sets of samples, the assay might be clinically applicable. Continued study in this direction is underway.

Despite intensive attempts to define new biomarkers for the diagnosis of LTBI that would also be prognostic of progression towards active TB (Abramo et al., 2006; Agranoff et al., 2006), *Mtb*-specific IGRAs still remain the only choice despite complications in immunocompromised subjects and high indeterminate rate. Current clinical use of the IGRAs, however, clearly shows that more biomarkers than IFN-γ expression and/or release need to be assessed in LTBI diagnostics, in order to obtain a more complex and accurate predictive readout (Ruhwald et al., 2007). Recently, in a large study by Berry et al. (2010), whole genome expression profiling using microarrays was performed on whole blood samples from active TB patients and subjects living in high TB-burden setting. An expression profile signature of 393 genes was defined that differed between diseased and healthy subjects, and an 86-gene expression signature specific for TB was derived. This discriminated active TB from other inflammatory and infectious diseases (Berry et al., 2010). Moreover, a subsignature potentially predictive of an increased risk of progression from LTBI towards active TB was reported. Whole genome expression profiling is, however, far from clinical applicability and a much simpler multiparametric but cost-effective assay is needed for improvement of LTBI diagnostic and prediction of progression towards active TB, in order to enable early chemoprophylactic treatment of subjects at risk.

We aimed here to develop a multimarker qPCR test discriminating *Mtb* infected and healthy donors. Some of the genes in the 86 gene TB signature of Berry et al. (2010) were used also in our panel (TAP-1, IFI-35, IP-10). The expression of IFN-γ–inducible TAP-1, IFI-35, and IP-10 genes was, indeed, found to be specifically enhanced in response to *Mtb*-specific antigenic stimulation of blood cells from TB-diseased or LTBI subjects (QFT-positive). Previously, the expression of IP-10 was monitored and elevated levels were found in both adult TB patients and LTBI household contacts of TB patients (Azzurri et al., 2005; Ruhwald et al., 2007). Moreover, IP-10 was found to be produced in significantly higher levels than IFN-γ in response to *Mtb* antigens also in children with both active TB and LTBI, demonstrating its potential as a TB biomarker in children, while failing to discriminate active TB and LTBI (Whittaker et al., 2008). Further genes involved in our profiling analyses were the cytokines IFN-γ and IL-2 known to be upregulated in blood cells from patients with active TB and LTBI compared to uninfected controls (Biselli et al., 2010). Expression of IL-12 was analyzed by qPCR here, as its expression is critical for the induction of IFN-γ, the master cytokine involved in the control of TB infection. MCP-1 and IL-15 expression was tested by qPCR and found to be significantly enhanced in response to *Mtb*-specific antigenic stimulation in samples from *Mtb*-infected (active or latent) compared to uninfected subjects (Frahm et al., 2011). Similarly, elevated serum TNF-α levels have been reported by others in advanced TB patients when compared to those with mild TB and healthy individuals (Fiorenza et al., 2005). Therefore, expression of TNF-α was analyzed as well, taking into account that TNF-α levels are important in immune response to TB and affect the outcome of the disease (Marino et al., 2007; Ray et al., 2009).

Additional genes analyzed as markers of regulation in TB and LTBI were Foxp3 and IL-10 genes, known to reflect expansion of T_{reg} cells and serve as an important clinical biomarker of disease progression (Jamil et al., 2007). Indeed, it is becoming increasingly clear that the transition from latent infection to active TB disease is not merely reflecting a "switch" from a Th1 to a Th2 response and involves also enhanced levels of specific T_{reg} cells (Fontenot et al., 2005; Walker et al., 2003). Finally, the set of marker genes analyzed was complemented with IL-4, IL-8, IL-18, and TGF-β1 that play important role in immune response to

infection in general and in TB infections in particular (Ameixa and Friedland, 2001; Hernandez-Pando et al., 2009; Schluger and Rom, 1998; Yamada et al., 2000).

Out of the set of the 15 tested markers, for which expression levels were assessed following antigenic stimulation of blood cells with *Mtb* antigens, 11 genes exhibited altered expression. Six of those (IL-10, IL-2, TNF-α, IFI-35, Foxp3, and IFN-γ) formed an expression signature that allowed classification of the tested samples by PCA and discriminant analysis into 3 distinct groups, depending on the QFT status of the blood donor (QFT-positive versus QFT-negative). The remaining marker genes could be excluded from the panel because their expression did not change in response to *Mtb* antigens (IL-12β, IL-18, IL-8, and TGF-β1), or there was no influence on discrimination between QFT-positive/negative individuals (MCP-1 and TAP-1), or because the expression level correlated with that of markers already included in the transcriptional signature and did not improve the discriminating power of the assay (IL-15, IL-4, and IP-10), respectively.

The assays were also assessed for the impact of intersubject variation and processing steps on the total SD of the Cq determination (Tichopad et al., 2009). As expected, the noise from the RT and sampling steps contributed more importantly to the total processing noise than that from the qPCR step. We opted for the use of sample and RT replicates preferentially to any other replicates when working with blood. The impact of intersubject variation, reflecting the biological variation among donors, was difficult to estimate in this study. It was the top level of the nested design, and its contribution was confounded by the contributions of downstream processing steps. Therefore, this source of the variance cannot be estimated with reasonable precision (Tichopad et al., 2009). This could be the reason for higher values of intersubject variations observed in procedure A, as compared to procedure B, for all genes. Moreover, the intersubject variation was lower for the reference genes β2M and TBP as compared to IFN-γ and IL-2, suggesting that intersubject noise was gene dependent, as expected.

An important aspect of the here-described assay was the careful selection of reference genes used for normalization of expression levels of marker genes. As numerous reports indicating differences in the expression levels of commonly used endogenous reference genes can vary considerably between different tissues and different experimental conditions, we performed qPCR validation for 10 candidate reference genes previously found to be stably expressed in different tissues (Li et al., 2009; Vandesompele et al., 2002).

β2M and TBP were chosen for normalization because they were found to be the most stable by geNorm and NormFinder. This goes well with a previous observation of Stamova et al. (2009) who identified the β2M as a stable reference gene in human peripheral blood without specific antigenic stimulation.

In summary, we used the QFT blood sampling and antigenic stimulation format, where ELISA detection of secreted IFN-γ was replaced by multimarker qPCR. This allows detecting several TB immune-related markers based on which TB-infected individuals can be distinguished from healthy uninfected donors. Tested samples could be classified into 3 groups. QFT-negative samples created 1 compact group, regardless of the individual differences. The second group comprised most of the QFT-positive samples with more scatter due to individual and TB infection variation. The third group comprised heterogenous samples with intermediate response.

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