qPCR vs. ELISpot: Which Assay is Better to Assess Antigen-Specific IFN-γ Secretion in PBMCs?

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INTRODUCTION

The cell-mediated immune response is an important immunogenicity endpoint in the development of biologics and vaccines. A frequently used read-out is the assessment of antigen-specific interferon-gamma (IFN-γ) secretion in peripheral blood mononuclear cells (PBMCs), which is used as a pharmacodynamic or immunogenicity endpoint in various immunomodulatory and novel therapies. In vaccine development and in cancer immunotherapy it is frequently used to assess the specific activation of T-cells targeted towards the respective viral or neoantigenic peptides. In gene therapy development, it is used to assess cellular immunogenicity against the viral gene vector or the transgene product, which could hamper therapy efficacy and safety. Protein-level immunoassays are routinely used to detect PBMCs response to stimulation and, among them, Enzyme-Linked Immunospot (ELISpot) assay has been extensively applied as the "gold standard". ELISpot is a functional cell-based assay capable of detecting activated IFN-γ-secreting cells at the single cell level. Using the extended Cytomegalovirus, Epstein Barr, and Influenza virus (CEF) peptide pool as a specific antigen stimulus and the mitogen Phytohemagglutinin-L (PHA-L), we demonstrate that IFN-γ secretion in PBMCs can also be efficiently assessed by standard reverse transcription quantitative PCR (RT-qPCR) analysis. We show a high degree of results correlation between independently optimized ELISpot and RT-qPCR assays, both between different PBMC donors and within individual donors.

ANALYTICAL METHODS

ELISpot setup

The ELISpot assay was independently optimized following current industry recommendations and harmonization guidelines (Janetzki et al. 2008). Healthy human PMBCs were isolated from whole blood by standard Ficoll Paque Plus centrifugation, PBMCs were rested overnight at high density, plated at optimal density (50 000 cells/well for PHA-L and 200 000 cells/well for CEF and untreated) and stimulated for approx. 22h with CEF (1 µg/mL in 0.2% DMSO medium) and PHA-L (10 µg/mL in 0.2% DMSO medium) concentrations in quadruplicate. Commercially optimized serum-free medium (by Cellular Technology Limited) was used during all steps. Plate development was performed using a commercial Human IFN-γ ELISpotPlus kit (supplied by Mabtech) following the kit instructions. Plates were analyzed with a Bioreader-4000-Pro-X instrument with EazyReader software (BIO-SYS). Camera and software spot analysis settings were optimized following published guidelines (Janetzki et al. 2015).

RT-qPCR setup

The RT-qPCR assay was independently optimized following current recommendations (Wissel et al.). PBMCs were thawed, rested and stimulated the same way as for the ELISpot assay. Of notice, PBMCs were plated at optimal density (200 000 cells/well for all experimental conditions) and stimulated for approx. 6h. Each condition was assessed in duplicate. The RT-qPCR specific experimental steps were optimized from a published method (Browne et al.). Briefly, RNA isolation was performed using a commercial MagMAX mirVana Total RNA Isolation kit (by Applied Biosystems), followed by RNA cleanup using RNeasy MiniElute Cleanup Kit (by QIAGEN). 20 ng of RNA were subsequently used for cDNA synthesis using SuperScript IV First-Strand Synthesis System (by ThermoFisher). qPCR was run on QuantStudio5 RealTime PCR System (by Applied Biosystems) with the SYBR Green master mix (by BioRad). The expression of the target gene IFN-γ and the reference genes ribosomal protein S18 (RPS18), ubiquitin Conjugating Enzyme E2 D2 (UBE2D2) and ribosomal protein L13a (RPL13a) were assessed in technical duplicates.

RESULTS

ELISpot setup

Four independent runs were performed under the same conditions, except for the plate development incubation, which was varied slightly to assess robustness. The software spot sensitivity was adapted accordingly and was optimized for each plate, while all other analysis parameters were kept constant. Inter- and intra-run precision for four different donors were <15% CV (<30% CV replicate precision for 30-100 spots/well) over four runs, demonstrating excellent precision and robustness. %CV was not assessed for results <30 spots/well. Results are presented in Figure 1.

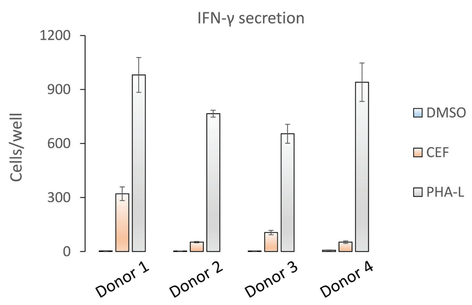

Figure 1. ELISpot assay. Results for each individual donor are expressed as IFN-γ secreting cells per well and are reported as mean ± SD.

RT-qPCR setup

Two independent runs were performed under the same conditions. A strong IFN-γ induction was detected as expected upon PHA-L treatment for all donors, and a milder effect was observed upon CEF stimulation. In order to assess the technical inter-run precision of the RT-qPCR assay, a complete analysis was performed on the same cDNA, and on newly synthetized cDNA starting from the same batch of isolated RNA. In each individual analysis, the samples were assessed in technical duplicates and the threshold cycle (CT) %CV <2% was used as acceptance criteria (Wissel et al.). Intra-run precision of biological duplicates was <30% CV for relative expression values, and the analysis performed on the same cDNA as well as on newly synthetized cDNA showed good inter-run precision with <20% CV for relative expression values. Precision analysis of the two completely independent runs showed a good degree of inter-run precision with <25% CV for relative expression values. Results are presented in Figure 2.

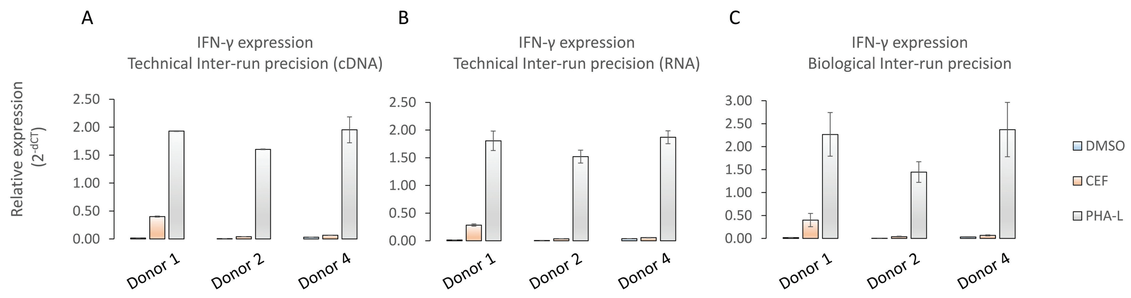

Figure 2. RT-qPCR assay. Results are expressed as relative expression and are reported as mean ± SD. A. Results of the analysis performed on the same cDNA samples. B. Results of the analysis performed on cDNA samples obtained by independent cDNA synthesis reactions, performed on the same RNA sample. C. Results of the analysis performed on two independent experiments. Due to technical reasons during cell stimulation, precision comparison data are not available for Donor 3.

RT-qPCR and ELISpot comparison

ELISpot has been extensively applied as the "gold standard" method for assessment of antigen-specific secretion in PBMCs, therefore we performed a comparison between the results obtained by the RT-qPCR assay and the ones obtained by the ELISpot assay. The experiments were conducted independently with the same PBMC batches isolated from the four donors.

An inter-donor comparison in response to the specific stimuli was conducted and the analysis of IFN-γ expression/secretion upon CEF or PHA-L stimulation showed a high degree of correlation as highlighted by the correlation coefficient R^2 (CEF R^2 = 0.9899 and PHA-L R^2 = 0.9653). The results of the inter-donor comparison are reported in Figure 3.

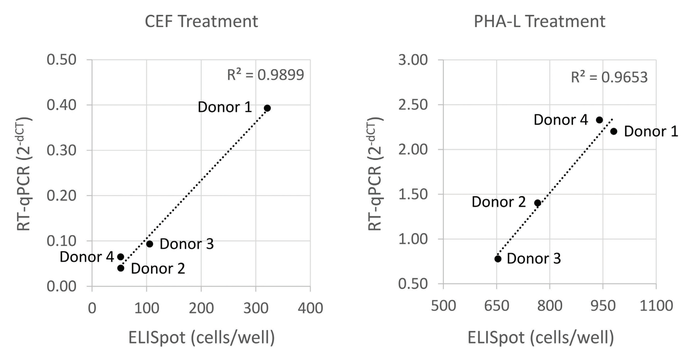

Figure 3. RT-qPCR and ELISpot results: inter-donor comparison. IFN-γ mRNA expression, assessed by RT-qPCR, correlates to measurement of protein secretion assessed by ELISpot. Results of the CEF and PHA-L stimulation are shown for all donors. For RT-qPCR, results are reported as relative expression; for ELISpot, results are reported as total number of cells per well.

In addition to the inter-donor comparison, an individual donor comparison between untreated, CEF- and PHA-L-treated PBMCs was performed and confirmed the high degree of correlation between the two methods for all four donors (R^2 >0.97), as reported in Figure 4.

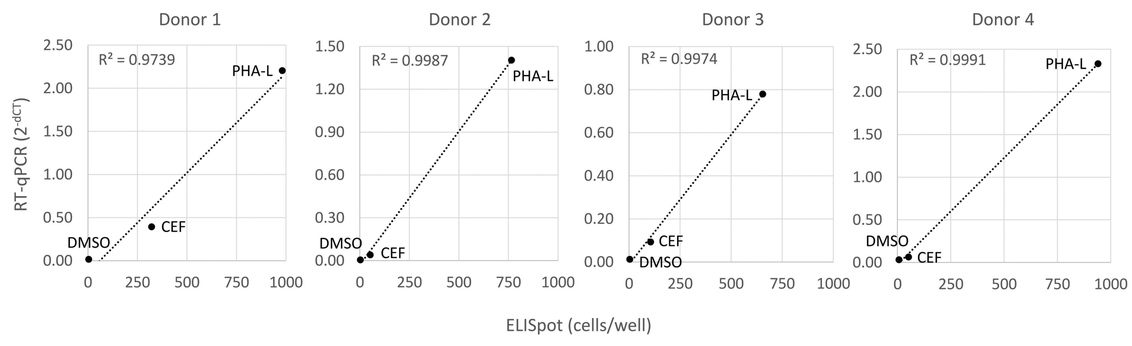

Figure 4. RT-qPCR and ELISpot results: individual donor comparison. IFN-γ mRNA expression, assessed by RT-qPCR, correlates to measurement of protein secretion by ELISpot. Results of the CEF and PHA-L stimulation are shown for each individual donor. For RT-qPCR, results are reported as relative expression; for ELISpot, results are reported as total number of cells per well.

DISCUSSION & CONCLUSIONS

Herein we show the optimization of a RT-qPCR method for the analysis of IFN-γ induction in PBMCs upon stimulation with the CEF peptide pool as well as with the mitogen PHA-L. We demonstrate a high degree of results correlation between independently optimized ELISpot an RT-qPCR assays, both between different PBMC donors and within individual donors. The two methods employ the same cell culture treatment protocol with different stimulation length, due to the different kinetics of cell responses at the RNA and protein levels.

Focusing on the advantages and disadvantages of the two assays (Table 1), both methods showed comparable precision between replicates and between runs. Both assays require roughly the same time to complete a 96-well plate. From a reagent point of view, ELISpot costs are considerably lower per run. However, the current RT-qPCR setup employed a relatively long cell stimulation protocol for gene expression analysis (6h) and it was completely manual, so it could still be further optimized and streamlined by testing shorter incubation times and by full sample extraction and pipetting automation. Most importantly, the RT-qPCR method allows reanalysis of the RNA and cDNA samples, which can be stored at -80°C and -20°C respectively, while reanalysis is not possible for ELISpot when a single PBMC sample aliquot is supplied.

For both methods the high variability of the cell-based stimulation and the need to evaluate untreated and positive controls wells (CEF, PHA-L, or both), in addition to the specific antigen-stimulated wells, limit the throughput of the assays. Of note, the ELISpot assay needs specific statistical considerations and response definition criteria that require all conditions to be evaluated in triplicates (Moodie et al.), leading to a maximum throughput of 8 samples per plate. For RT-qPCR, thanks to the extremely high precision of technical replicates, the analysis can also be done in duplicates. The need to evaluate at least one reference gene for all conditions limits the throughput when using singleplex SYBR Green detection technology to maximum 6 samples/plate. However, multiplexing the target and reference gene(s) using TaqMan-based probes would increase the throughput to 12 samples per plate, which is 1.5x higher throughput than ELISpot. Of note, both ELISpot and RT-qPCR allow multiplexing to detect multiple analytes of interest in a single run.

Parameter	ELISpot	RT-qPCR
Replicate precision	<30% CV (30-100 spots/well) <15% CV (>100 spots/well)	<2% CV (CT, technical replicates) <30% CV (relative expression, biological replicates / intra-assay precision)
Inter-run precision	<15% CV (>30 spots/well, 4 independent runs)	<20% CV (relative expression, 3 technical repeats) <25% CV (relative expression; 2 independent runs)
Reanalysis from a single PBMC aliquot	Not possible	Reanalysis of both RNA and cDNA
Assay length	3.5 days	3.5 days
Cost/plate	~210 Euro	~1500 Euro (SYBR Green) ~3100 Euro (TaqMan)
Maximum throughput with 4 treatment conditions	8 samples/plate in triplicates	6 samples/plate (SYBR Green) in duplicates 12 samples/plate (TaqMan) in duplicates
Multiplexing possibility	Fluorospot	TaqMan probes

Table 1. Assay comparison

Overall, we showed that IFN-γ expression in PBMCs can be efficiently assessed by standard RT-qPCR analysis leading to results comparable to the ELISpot assay. Besides excellent precision, RT-qPCR has the major advantage of storing RNA and cDNA for sample reanalysis. Throughput and assay length were comparable between the qPCR and ELISpot methods, with significant scope for improvement for the RT-qPCR assay in terms of assay optimization, duration, and automation. Therefore, we conclude that RT-qPCR is a viable alternative analytical platform to ELISpot for the detection of antigen-specific IFN-γ induction in PBMCs, as well as for any other analytes where gene expression analysis is expected to be faster, more sensitive, and more robust compared to protein secretion.

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