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Interferon gamma transcript detection on T cells based on magnetic actuation and multiplex double-tagging electrochemical genosensing

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ABSTRACT

Interferon-γ is a proinflammatory cytokine, and its production is related with effective host defense against intracellular pathogens. Therefore, the level of interferon-γ is considered a good biomarker for intracellular infections. It is also useful for the assessment, treatment progression and follow-up of non-communicable diseases, including cancer and autoimmune disorders, among others. This work addresses the development of a novel interferon-γ release assay (IGRA) to evaluate the expression of interferon-γ transcripts as biomarker produced by isolated T cells, as a main advantage. The method sequentially combined three different types of magnetic separation, including the immunomagnetic separation of the T cells performed on antiCD3 modified magnetic particles, the retrotranscription and multiplex double-tagging PCR on polydT-modified magnetic particles and, finally, the electrochemical genosensing on streptavidin magnetic particles as a support. This approach is able to quantify the levels of cellular interferon-γ produced by as low as 150 T cells with outstanding analytical features. The detection of interferon-γ transcripts is performed from only 100 μL of whole blood which can be potentially obtained by fingerprick, demonstrating a further clear advantage to be considered as a promising strategy for the quantification of this important biomarker in several clinical applications.

1. Introduction

Interferon gamma (IFN-γ) is a proinflammatory cytokine produced by activated NK cells, NKT cells, effector T $CD4^+$ cells (T helper 1 cells, Th1) and $CD8^+$ T cells (cytotoxic T cells) during the immune response against intracellular pathogens, such as virus and bacteria (Van de Vosse et al., 2004). Individuals with deficiency in either IFN-γ production, receptor expression or the signaling pathway (IL-2/IL-23/IFN-γ) show high incidence of infections due to *Salmonella* or intracellular bacteria as *Mycobacterium* (Lamhamedi et al., 1998; Noordzij et al., 2007) and viruses (Dammermann et al., 2015). On the contrary, the increased production of IFN-γ is associated to an improved anti-tumor response (Dunn et al., 2006), and plays also an important role in autoimmune disorders (Baccala et al., 2005; Pollard et al., 2013), for instance in Systemic Lupus Erythematosus (Welcher et al., 2015) and psoriasis (Latorre et al., 2014), among others. Interferon-γ release assay (IGRA) is based on the production and quantification of IFN-γ upon *in vitro* stimulation of the T lymphocytes with specific antigens. In detail, the assay is performed by 24 h incubation of 1 mL whole blood from the patients, containing the T lymphocytes, with the antigen, followed by centrifugation and the quantification on the supernatant of the released IFN-γ by ELISA. This test can be potentially used in the diagnosis of all the diseases based on cell-mediated immunity, and the specificity is provided by the stimulation of the T lymphocytes with the antigens driving the immune response. However, it is especially useful in routine clinical practice for the diagnosis of latent tuberculosis (TB) (Horsburgh and Rubin, 2011). Latent TB infection is a non-communicable asymptomatic condition which can turn into active TB in people who have a weak immune system. Lately, TB incidence is increased due to HIV coinfection (Chiacchio et al., 2017), and anti-TNF alpha immunotherapy in rheumatic diseases (Vassilopoulos et al., 2011). The diagnosis of latent TB is usually performed by the Tuberculin skin test based on the intradermic injection of a purified protein from *Mycobacterium*. However, this test can provide false positives due to exposure to non-tuberculous *Mycobacteria* as well as the previous vaccination with BCG (Bacillus Calmett-Guérin). Besides, this test is time consuming (up to 48–72 h). Identification and treatment of latent TB can substantially reduce the risk of developing active TB and is a worldwide major focus of TB control. However, identification of all persons with latent TB would require screening large numbers of low-risk individuals that would not be cost-effective with the current IGRA test (Horsburgh and Rubin, 2011). There are two IGRA kits currently in the market for

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the diagnosis of Latent TB: QuantiFERON-TB Gold (Qiagen, The Netherlands) and T-SPOT.TB (Oxford Immunotec, UK). Several studies compare the efficiency of both methods (Domínguez et al., 2008; Ayubi et al., 2017; Sharma et al., 2017) and their principles are detailed discussed in Supp. data and Table S1. Individuals infected with *Mycobacterium tuberculosis* usually have lymphocytes in their blood that recognize the mycobacterial antigens, generating and secreting IFN-γ. Another commercial application of IGRA test is the QuantiFERON-CMV (Qiagen), which was developed for the detection of human cytomegalovirus (CMV) reactivation after transplantation (Nesher et al., 2016). The transplanted patients at risk to developed CMV disease produced a minimal IFN-γ response.

In this paper, a novel IGRA test based on electrochemical for the detection of IFN-γ transcripts produced by isolated T lymphocytes is described. To the extent of our knowledge, this is the first time that a biosensor for the detection of IFN-γ transcripts produced by isolated T cells is reported. This approach combines the advantages of the integration of magnetic particles (MPs) for the preconcentration and isolation of different targets (including whole T cells, mRNA transcripts and double-tagged DNA) for the assessment of IFN-γ expression by T cells. Firstly, the method involves the preconcentration of T lymphocytes from whole blood by immunomagnetic separation on magnetic particles modified with the antiCD3 antibody (Carinelli et al., 2015). The messenger RNA is then released and isolated on polydT-MPs, followed by retrotranscription to obtain cDNA. The cDNA is amplified on beads by a multiplex double-tagging PCR using a double-tagging set of primers specific for IFN-γ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene control. Finally, the simultaneous electrochemical magneto-genosensing of the two transcripts is performed on streptavidin-magnetic particles as a support (Brandão et al., 2015; Ben Aissa et al., 2017). As summarized in Table S1 (Supp. data) the main advantages of this method compared with the commercial available IGRA test is that the T cells producing IFN-γ are specifically separated from whole blood by immunomagnetic separation, thus increasing the sensitivity of the assay and removing potentially interfering substances that can affect test results. Moreover, the electrochemical genosensor detects the IFN-γ mRNA while other methods are based on the determination of IFN-γ (protein). Furthermore, the electrochemical genosensor has the advantage of combining high sensitivity/specificity as well as simplicity of instrumentation, and can be easily expanded into multiplex detection platform. Finally, the detection of IFN-γ transcripts is performed from only 100 µL of blood which can be potentially obtained by fingerprick, demonstrating a further clear advantage over commercial IGRA test requiring up to 4–5 mL of blood obtained by extraction (Table S1, Supp. data). Therefore, the development of the electrochemical sensing platform is a significant step towards a low cost, sensitive, and specific transcript detection of IFN-γ.

2. Materials and methods

2.1. Chemicals and Biochemicals

Magnetic particles (MPs) modified with antiCD3 antibody (antiCD3-MPs) (Dynabeads™ CD3 Prod. No. 111-51D, 4.5 µm diameter), MPs modified with polydT (polydT-MPs) (Dynabeads® Oligo $(dT)_{25}$ Prod. No. 610.02, 2.8 µm diameter) and MPs modified with streptavidin, (streptAv-MPs) (Dynabeads™ M-280 streptavidin Prod. No. 112.05, 2.8 µm diameter), SuperScript™ III Reverse Transcriptase (Prod. No. 18080-044) and RNaseOUT recombinant Ribonuclease Inhibitor (Prod No. 10777-019) were purchased from Thermo Fisher. *Taq* polymerase 1U μL^{-1} (Prod. No. 10002-4100) was purchased from Biotools. Anti-digoxigenin-peroxidase Fab fragments (antiDIG-HRP) (Prod. No. 1214667) and anti-fluorescein-peroxidase Fab fragments (antiFLU-HRP) (Prod. No. 11426346910) were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

The primers for the multiplex double-tagging RT-PCR amplification were obtained from Sigma-Aldrich, and are described in Table S2, Supp. data. These primers were selected for the specific amplification of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and IFN-γ genes (Genbank reference NM_002046.4 and NM_000619.2, respectively, described in Supp. data).

The T cell clone PB100.2i was obtained by cloning the infiltrating T lymphocytes from a pancreatic donor organ. The expansion method is detailed described in Supp. data (Xufré et al., 2013).

All buffer solutions were prepared with milliQ water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). Any solution used in RNA preparation was RNase-free by treatment with diethylpyrocarbonate (DEPC). The composition of these solutions is described in Supp. data.

2.2. Instrumentation

Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems Inc, USA). A three-electrode setup was used comprising a platinum auxiliary electrode (Crison Instruments 52-67), a double junction Ag/AgCl reference electrode (Orion 900200) with 0.1 mol L^{-1} KCl as external reference solution and a magneto electrode (m-GEC) as working electrode (Pividori and Alegret, 2005). Cell concentration was determined by Neubauer chamber using Nikon Eclipse TS100 microscopy (Nikon Instrument, USA). Retrotranscription and polymerase chain reaction were carried out in My-Cycler Thermal Cycler (Bio-Rad). After extraction, RNA was quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA).

2.3. Immunomagnetic separation, multiplex double-tagging reverse transcription PCR of IFN-γ transcripts on magnetic beads and electrochemical genosensing

This approach sequentially combines three different types of magnetic separations, as depicted in Fig. 1. Firstly, the method involves the preconcentration of T lymphocytes from whole blood based on the CD3 receptor by immunomagnetic separation on magnetic particles modified with the antiCD3 antibody (Carinelli et al., 2015) (Fig. 1, panel a1). The messenger RNA was released (Fig. 1, panel a2) and isolated on polydT-MPs based on the poly(A) tail present in the transcript, followed by retrotranscription to obtain cDNA (Fig. 1, panel b1). After that, the cDNA was amplified by multiplex double-tagging PCR on beads (Fig. 1, panel b2), using a double-tagging set of primers specific for IFN-γ and GAPDH as housekeeping gene control. During PCR, the cDNA is not only amplified but also labeled at the same time with biotin/fluorescein (BIO/FLU) or biotin/digoxigenin (BIO/DIG) tags for IFN-γ and GAPDH transcript, respectively. Finally, the electrochemical magneto-genosensing was performed on streptavidin-magnetic particles as a support, based on the BIO tags on both amplicon through biotin-streptavidin interaction. The FLU and DIG-tags, carried by the other primer, were used for the labeling with the specific antibodies, antiFLU-HRP and antiDIG-HRP, coding for interferon-γ and GAPDH, respectively, and performed in two separated reaction chambers (Fig. 1, panel c1). The simultaneous electrochemical readout of the two transcripts was based on peroxidase (HRP) enzyme as electrochemical reporter and performed in the same electrochemical cell on m-GEC electrodes, as previously reported (Brandão et al., 2015; Ben Aissa et al., 2017) (Fig. 1, panel c2). The experimental details are described in the next sections.

2.3.1. Immunomagnetic separation of T lymphocytes

100 µL of T cells (at different concentration ranging from 0.1 to 1000 cells μL^{-1}) were incubated with 8×10^5 antiCD3-MPs for 30 min at 4 °C while shaking, followed by a washing steps with PBS 0.1% BSA. The content of the preconcentrated cells on antiCD3-MPs was released by resuspending them on 1 mL of Lysis/Binding buffer, and disrupted using a syringe.