RESEARCH ARTICLE

Development of a cytokine-secreting-based assay for the identification, sorting and transcriptomic analysis of polyfunctional human T cells

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ABSTRACT. Polyfunctional T cells that simultaneously produce the cytokines IFN-γ, IL-2 and TNF have been correlated with better clinical outcomes in various diseases. To date, cytokine polyfunctionality within T cells has been exclusively studied by intracellular cytokine staining coupled with flow cytometric analysis. Thus, further downstream interrogation of polyfunctional T cell characteristics such as transcriptomic analysis has not been possible. Here, we report the use of a flow cytometric method based on cytokine secretion assay technology to detect and isolate, for the first time, viable human polyfunctional T cells directly from *in vitro* stimulated whole blood samples. We demonstrate the successful application of this method to sort polyfunctional T cells obtained from human volunteers, which can be then used for downstream applications such as transcriptomic analysis using RT-qPCR. This assay will facilitate in-depth investigations of T cells with distinct cytokine polyfunctionality, including defining their molecular profile and understanding the mechanisms regulating their generation and function.

Key words: polyfunctional T cells, cytokine secretion, novel assay, cell sorting, transcriptomic analysis

T cell responses have been traditionally monitored using immunogenicity assays that measure cytotoxic and proliferative functions or cytokine production [1]. More recently, high-dimensional flow-cytometric studies have associated cumulative T cell effector functions with protective immunity in a number of diseases [2, 3]. In particular, polyfunctional T cells that simultaneously produce multiple cytokines following antigen-specific stimulation have been widely reported in the literature, and correlated with better clinical outcomes in various diseases, including Leishmania major [4], *Mycobacterium tuberculosis* [5] and HIV [6], as compared to monofunctional T cells secreting only one cytokine. However, very little is known about the basic characteristics of polyfunctional T cells. It has been proposed that cytokine polyfunctionality in T cells is dynamically regulated over time with epigenetic modifications occurring on the promoters controlling cytokine gene expression [7]. However, the molecular and transcriptomic profiles of polyfunctional T cells remain unexplored, and it is unclear whether polyfunctional cells are actually different from monofunctional cells, apart from their ability to secrete additional cytokines.

In all published reports, T cell cytokine polyfunctionality has been determined by intracellular cytokine staining followed by flow-cytometric analysis [8]. This process requires cell fixation that generally precludes the recovery of high quality RNA and downstream analysis, and thereby hinders the transcriptomic assessment of these polyfunctional T cells. Most recently, new technologies, such as the PrimeFlow^{TM} RNA Assay (eBioscience), which analyzes RNA transcripts by flow cytometry, and SmartFlare RNA Detection Probes (EMD Millipore), which uses nanoparticle technology to label RNA within viable cells, have been developed. However neither of these technologies allow for the detection and subsequent downstream analysis of polyfunctional cells since they are based on RNA expression, and there is no evidence that the cytokine protein is expressed.

Viable lymphocytes secreting a specific cytokine protein of interest can be detected and isolated using the cytokine secretion assay (CSA, Miltenyi Biotech), also called cell-surface affinity matrix technology [9]. This technology combines a cytokine-catch antibody and a cytokine detection antibody coupled to a fluorochrome to build an affinity matrix on the lymphocyte surface for capture of the secreted cytokine. After a defined time period of stimulation and secretion, cells producing the target cytokine can be analysed and sorted by flow cytometry [9-11]. This method has been successfully used to isolate antigen-specific $CD4^+$ T cells and $CD8^+$ T cells based on the secretion of IFN-γ [12, 13], or the combination of IFN-γ and TNF [14]. However, thus far the technology has

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been limited to the simultaneous detection of no more than two cytokines and therefore has not been used to detect polyfunctional T cell populations.

Herein, we report an *in vitro* assay based on cytokine secretion assay technology that allows for the successful identification and isolation of viable polyfunctional T cells directly from *in vitro*-stimulated human whole blood samples. We demonstrate that the sorted polyfunctional T cells can be used for downstream molecular analysis such as RT-qPCR and transcriptomics, allowing the first such analysis of T cell populations with distinct cytokine polyfunctionality.

METHODS

Sample collection

Peripheral blood was collected in lithium-heparin vacutainers (Becton Dickinson) from healthy, adult human volunteers, aged between 25 and 52 years. For the detection of influenza-reactive, cytokine-producing T cells, blood samples from five healthy volunteers were taken five weeks post-immunisation with the seasonal influenza trivalent vaccine (Fluvax 2015, CSL therapies). All samples were collected with informed consent with the approval of the QIMR Berghofer Medical Research Institute Human Research Ethics Committee. All volunteers were healthy and asymptomatic at the time of blood sampling.

Whole blood in vitro *stimulation*

Three millilitres of whole blood were diluted 2:1 in RPMI 1640 medium (Invitrogen) supplemented with 25 mM Hepes, 2 mL L-glutamine, 10 units/mL of penicillin (Life Technologies) and 10 μg/mL of streptomycin (Life Technologies), dispensed into tissue culture-treated, 6-well, flat-bottomed plates (Becton Dickinson) in the presence of 1 μg/mL of co-stimulatory antibodies anti-CD28 and anti-CD49d (Becton Dickinson) and 10 μg/mL mitogen PHA (Sigma Aldrich), or a pool of synthetic influenza peptides (5 μg/mL each of 9-mer peptides derived from influenza matrix protein epitope (residues 58-66) and influenza nucleoprotein epitope (residues 265-274) (synthesized at 95% purity by Mimotopes Pty Ltd.); both peptides can induce $CD4^+$ and $CD8^+$ T cell responses in healthy individuals [15]. Cultures were incubated for 16 h (PHA stimulation) or 6 h (peptide stimulation) at 37°C, in an atmosphere of 5% CO_2.

Three-colour, polyfunctional cytokine secretion assay

IFN-γ-FITC, IL-2-PE and TNF-APC cytokine secretion assay detection kits were purchased from Miltenyi Biotech. Following *in vitro* stimulation, red blood cells were lysed with an in-house hypotonic solution (1.55 M NH_4Cl, 0.1M $NaHCO_3$ and 0.5M EDTA adjusted to pH 7.3) for 10 min at room temperature, under continuous rotation (10-15 rpm), using a rotary tube mixer. Cells were washed twice in cold wash buffer (PBS with 0.5% BSA and 1 mM EDTA), resuspended in 100 μL of cocktail containing 20 μL of each catch antibody reagent and incubated for five mins on ice. Cells were resuspended in 10 mL tubes (Sarstedt) in 5 mL of pre-warmed medium (medium used for whole blood antigen stimulation further supplemented with 5% AB human serum (Sigma-Aldrich)) and incubated for one h at 37°C, under continuous rotation (10-15 rpm), using a rotary tube mixer. Cells were placed on ice for one min, and washed twice with five volumes of cold wash buffer followed by centrifugation at 300g and at 4°C for 10 mins. Cells were then resuspended in 100 μL of cocktail containing 20 μL of each cytokine detection antibody reagent, together with 20 μL of anti-human CD4-V500 and 2 μL of anti-human CD8-APC-H7 antibodies (Becton Dickinson) and 2 μL of anti-human CD14-PE Texas Red antibody (Beckman Coulter). Cells were incubated for 10 mins on ice in the dark, rinsed with ten volumes of cold wash buffer followed by centrifugation at 300 g and at 4°C for 10 min, and then resuspended in 500 μl of cold wash buffer containing 1 μg/mL of propidium iodide (Sigma-Aldrich) to allow for dead cell exclusion. Samples were run on a BD LSR Fortessa 4 (Becton Dickinson) and data acquired with DIVA software. Gating and frequencies were defined using FlowJo (version 9.4.4, FlowJo, LLC). Alternatively, cells were sorted using a BD Aria III cell sorter (Becton Dickinson) directly into 75 μL of RLT lysis buffer (Qiagen) supplemented with 1% of 2-mercaptoethanol (Sigma-Aldrich) and cell lysates were stored at -70°C for up to one month. Cytokine-secreting $CD4^+$ T cells were gated as $PI^-/CD14^-/CD8^-/CD4^+$ cells.

mRNA extraction, cDNA synthesis and specific target pre-amplification

Frozen cell lysates were thawed quickly on ice and mRNA was extracted using the RNEasy Micro kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesised using oligo-dT and Superscript III RT (Invitrogen) according to the manufacturer's instructions. Specific target amplification was performed with the Taqman PreAmp master mix (Life Technologies), and a pool of the primers to be used in qPCR according to the manufacturer's instructions. The cycling conditions used were: hot start for 10 min at 95°C, followed by 14 amplification cycles at 95°C for 15 s and 60°C for 4 min. Amplification was directly followed by a treatment with 24 Units of Exonuclease I (Thermo Fisher Scientific) according to the manufacturer's instructions. Amplified products were then diluted 1:5 in TE Buffer (Integrated DNA technologies) and stored at -20°C for up to seven days.

Cytokine gene expression by RT-qPCR

IFN-γ, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12B, IL-13, IL-17A, IL-23A, IL-24, TNF, TGFB and RPL13A gene expressions were measured from 1 μL of the pre-amplified cDNA samples using Taqman gene expression assays (Life Technologies) and Platinum Taq polymerase (Life Technologies) in a 10 μL volume reaction on a 384-well plate using the Light Cycler 480 Real Time PCR cycler (Roche), according to the manufacturer's instructions. Each gene expression was assayed in duplicate. Cycling conditions used were: 50°C for 2 min followed by 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Fold-changes were calculated with the ddCt value method [16] using RPL13A expression as the reference gene, and the expression values from the cytokine triple-negative $CD4^+$ T cells as baseline.

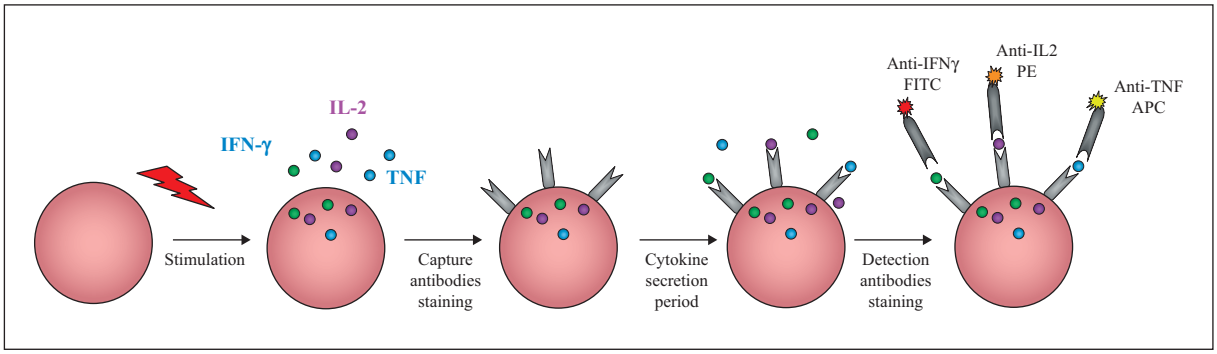

Figure 1
Principle of the three-colour, polyfunctional cytokine secretion assay. Following whole blood *in vitro* stimulation, cells are stained with a combination of IFN-γ, IL-2 and TNF capture antibodies (Miltenyi Biotech cytokine detection kits) and then incubated for one hour, during which time the cytokine is captured on the cell surface. The cells are then stained with a combination of IFN-γ, IL-2 and TNF detection antibodies (Miltenyi Biotech cytokine detection kits) and assessed by flow cytometry.

RESULTS

We adapted the commercially available cytokine secreting assay to increase the breadth of cytokine recognition in order to detect triple-cytokine-positive polyfunctional T cells (*figure 1*). Briefly, after *in vitro* stimulation to induce cytokine production, cells were stained with equal quantities of bispecific capture antibodies that simultaneously bind a ubiquitous cell-surface molecule and one of the three cytokines of interest (e.g., IFN-γ, IL-2 and TNF). The cells were then cultured for one hour to allow cytokine secretion and capture on the cell surface after which the surface-retained cytokines were detected with a mixture of antibodies specific for each cytokine coupled to different fluorochromes. Cytokine secreting cells could then be assessed or sorted by flow cytometer.

We assessed the capacity of this polyfunctional cytokine secretion assay to detect single-, double- and triple-cytokine producing cells using whole blood from five healthy volunteers (*figures 2A, B*). IFN-γ-, IL-2- or TNF-producing T cells were successfully detected after *in vitro* stimulation with either the mitogen PHA or influenza peptides (*figure 2C*). Triple-positive, double-positive and single-positive cytokine-producing T cell populations could be clearly distinguished (*figure 2D*).

We next determined whether this assay could be used for FACS sorting and transcriptomic analysis of polyfunctional T cells. $CD4^+$ T cells secreting IFN-γ, IL-2 and TNF (IFN-γ triple-positive) or $CD4^+$ T cells not secreting any cytokines (triple-negative), were isolated from PHA-stimulated whole blood (from five healthy volunteers) using the three-colour, polyfunctional cytokine secretion assay and sorted by FACS (*figure 3A*). Cell populations were sorted directly into lysis buffer in order to minimise sample manipulation and associated RNA degradation. The level of mRNA expression of the triple-positive cytokines was measured by RT-qPCR following a specific gene target pre-amplification, which was included as the number of sorted cells from each individual was small (ranging from 300 to 10,000 cells). The relative mRNA expression of each cytokine within the IFN-γ triple-positive population was significantly increased relative to the triple-negative population (*figure 3B*) confirming the triple-positive status of the sorted cells. We tested the mRNA expression of a further thirteen cytokine genes (*figure 3C*) and identified significant increases in IL-5, IL-10 and IL-12β; thereby providing the first glimpse of the transcriptomic profile of human polyfunctional $CD4^+$ T cells. These data establish that gene expression analysis can be performed on T cell populations sorted using our three-colour, polyfunctional cytokine secretion assay.

DISCUSSION

Numerous studies have reported the induction of polyfunctional T cells in various infection or immunisation models, and their clinical importance in some models; however, there is very limited information on the basic characteristics of these cells and how they differ from monofunctional T cells. All prior studies have used cell fixation and intracellular staining, which precludes further transcriptomic analysis. Indeed, our own desire to assess polyfunctional T cell development during studies of controlled human malaria encouraged us to develop a method to recover high quality RNA from human polyfunctional T cells. We therefore developed a flow cytometric method to isolate viable polyfunctional T cells directly from *in vitro*-stimulated human whole blood samples. We have validated the assay at the molecular level, identifying distinct transcriptomic profiles of isolated cell populations and providing the first insight into the molecular profile of T cells with distinct cytokine polyfunctionality.

Since this assay can be used directly on whole blood, without any introduced variation associated with sample manipulation, it provides the opportunity to analyse the gene expression profile of polyfunctional T cells from whole blood samples in prophylactic or therapeutic clinical trials. Such studies could directly address the current controversy over the importance of polyfunctional T cells in the clinical setting, as well as providing a potential immune surrogate of infection, vaccination or protective efficacy.

Finally, by taking advantage of the wide range of cell surface-staining antibodies and cytokine detection reagents now available, this assay could be applied to the study of specific lymphocyte populations. For instance, the addition of various surface activation markers such as CD137, CD38 or CD69, would allow for dissection of the molecular profile of recently activated, antigen-specific T cells present in the peripheral blood.

In summary, we report here the development and application of a three-colour cytokine secreting assay for the

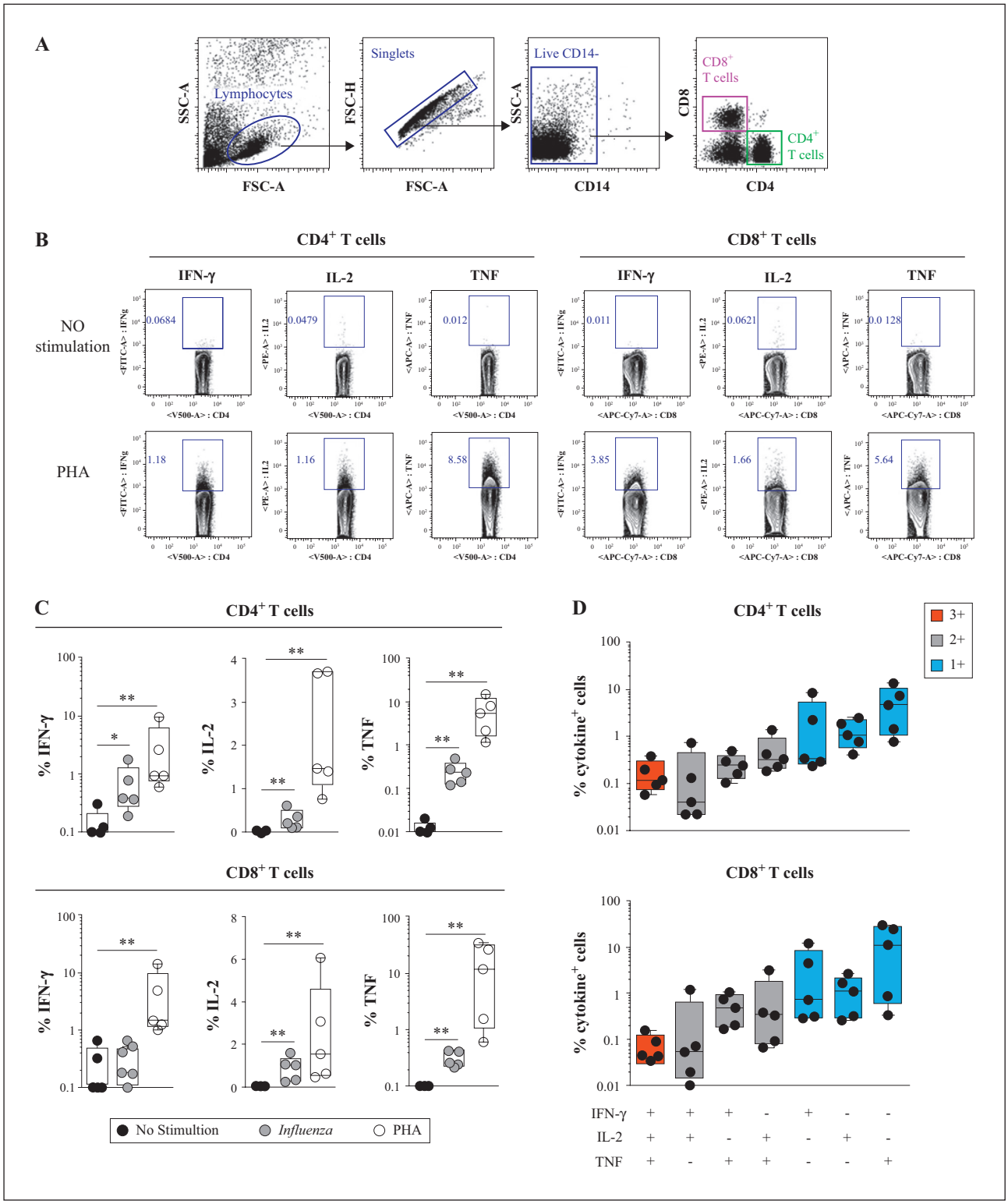

Figure 2

Detection of IFN-γ, IL-2 and TNF production by T cells using the three-colour, polyfunctional cytokine secretion assay. Cytokine secretion was detected on whole blood samples after *in vitro* stimulation with PHA (10 μg/mL) or a pool of synthetic influenza peptides (5 μg/mL each) to induce cytokine production. IFN-γ-, IL-2- and TNF-secreting cells were detected using the three-colour, polyfunctional cytokine secretion assay. **(A)** Gating strategy to identify viable $CD4^+$ and $CD8^+$ T cells. **(B)** Representative staining for IFN-γ, IL-2 and TNF production by $CD4^+$ T and $CD8^+$ T cells in unstimulated and stimulated samples. **(C)** Frequencies of total IFN-γ-, IL-2- and TNF-producing $CD4^+$ T cells and $CD8^+$ T cells in unstimulated and stimulated samples. **(D)** Frequencies of triple-positive, double-positive and single-positive $CD4^+$ T cells and $CD8^+$ T cells in PHA-stimulated samples. Whole blood samples were collected from five healthy volunteers (PHA stimulation) and five healthy volunteers five weeks post-influenza vaccination (influenza peptides stimulation). Graphs show combined data from five volunteers from two independent experiments; * $p<0.05$, ** $p<0.01$ (Mann-Whitney test, GraphPad Prism V6).

detection and isolation of viable polyfunctional T cells from whole blood samples. This assay could be used in a wide range of downstream applications in the laboratory and the clinic, including studies aimed at understanding the biology and functional significance of polyfunctional T cells, identifying correlates of immune control in infectious diseases, and facilitating the development of vaccines which induce polyfunctional T cell responses.

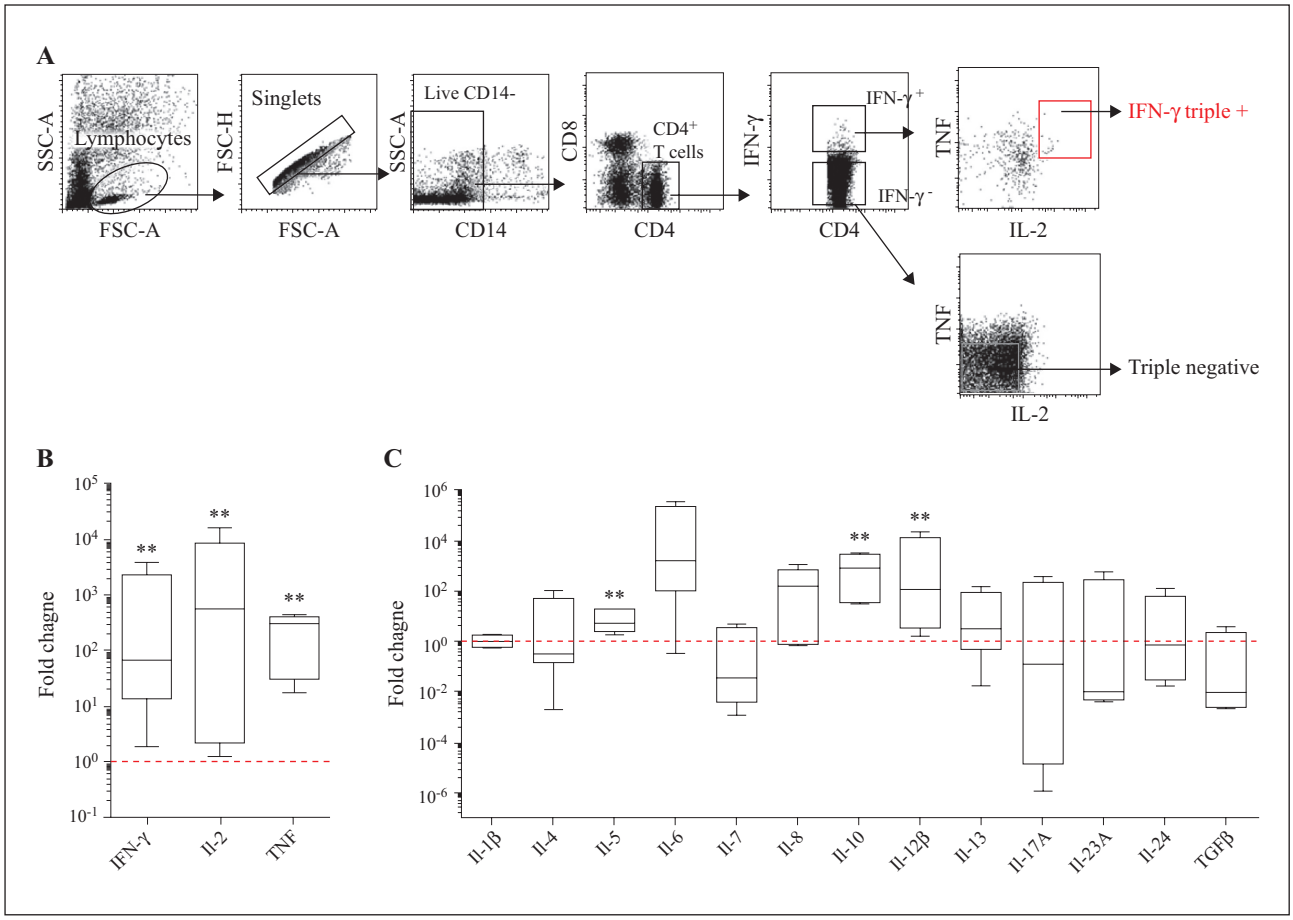

Figure 3

Isolation and cytokine gene expression profiling in CD4$^+$ T cells expressing IFN-γ, IL-2 and TNF production using the three-colour, polyfunctional cytokine secretion assay. Whole blood samples were stimulated overnight with 10 μg/mL PHA, to induce cytokine production, and cytokine-secreting CD4$^+$ T cells sorted using the three-colour, polyfunctional cytokine secretion assay. **(A)** Gating strategy to isolate triple-positive and triple-negative CD4$^+$ T cells. Plots show concatenated data from three volunteers. **(B)** Cytokine mRNA expression in IFN-γ triple-positive sorted CD4$^+$ T cells measured by RT-qPCR. Fold-changes were calculated with the ddCt method using triple-negative CD4$^+$ T cells as baseline. Graph shows combined data from five volunteers from two independent experiments, ** $p<0.01$ (Mann-Whitney test, GraphPad Prism V6).

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