Research paper

Processing of blood samples influences PBMC viability and outcome of cell-mediated immune responses in antiretroviral therapy-naïve HIV-1-infected patients

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ABSTRACT

Intracellular cytokine staining (ICS) assay is increasingly used in vaccine clinical trials to measure antigen-specific T-cell mediated immune (CMI) responses in cryopreserved peripheral blood mononuclear cells (PBMCs) and whole blood. However, recent observations indicate that several parameters involved in blood processing can impact PBMC viability and CMI responses, especially in antiretroviral therapy (ART)-naïve HIV-1-infected individuals.

In this phase I study (NCT01610427), we collected blood samples from 22 ART-naïve HIV-1-infected adults. PBMCs were isolated and processed for ICS assay. The individual and combined effects of the following parameters were investigated: time between blood collection and PBMC processing (time-to-process: 2, 7 or 24 h); time between PBMC thawing and initiation of in vitro stimulation with HIV-1 antigens (resting-time: 0, 2, 6 and 18 h); and duration of antigen-stimulation in PBMC cultures (stimulation-time: 6 h or overnight). The cell recovery after thawing, cell viability after ICS and magnitude of HIV-specific CD8+ T-cell responses were considered to determine the optimal combination of process conditions. The impact of time-to-process (2 or 4 h) on HIV-specific CD8+ T-cell responses was also assessed in a whole blood ICS assay.

A higher quality of cells in terms of recovery and viability (up to 81% and >80% respectively) was obtained with shorter time-to-process (less than 7 h) and resting-time (less than 2 h) intervals. Longer (overnight) rather than shorter (6 h) stimulation-time intervals increased the frequency of CD8+-specific T-cell responses using ICS in PBMCs without change of the functionality. The CD8+ specific T-cell responses detected using fresh whole blood showed a good correlation with the responses detected using frozen PBMCs.

Our results support the need of standardized procedures for the evaluation of CMI responses, especially in HIV-1-infected, ART-naïve patients.

1. Introduction

Monitoring antigen-specific T-cell immunity is central in clinical trials aiming to develop innovative preventative and therapeutic vaccines (Seder et al., 2008). In order to compare the immunogenicity of different vaccine candidates between multiple clinical trials, the standardization of the procedures used for blood collection, processing, preservation and blood cell analysis is of utmost importance (Maecker et al., 2005; Britten et al., 2008; Mallone et al., 2011).

Intracellular cytokine staining (ICS) is a flow cytometry-based assay increasingly used to identify, quantify and qualify antigen-specific T-cell mediated immune (CMI) responses in vaccine clinical trials (Kierstead et al., 2007; Boaz et al., 2009; Olemukan et al., 2010; Kutscher et al., 2013). The ICS assay allows rapid quantitation of a large panel of cytokine-producing cells, while the multi-parameter capability of flow cytometry allows determination of cell-specific production of individual cytokines. The ICS assay can be performed using cryopreserved peripheral blood mononuclear cells (PBMCs) (Horton et al., 2007) or fresh whole blood (Hanekom et al., 2004; Meddows-Taylor et al., 2007). The reliable evaluation of CMI responses requires cell samples that have been properly prepared. That implies cell samples of good quality, regularly assessed for the proportion of viable lymphocytes in the sample before flow cytometry analysis. Previously, it was shown that the length of time from venipuncture to cryopreservation was the most important parameter influencing T-cell performance in cellular immune assays, affecting subsequent cell recovery and function (Bull et al., 2007). Recent observations indicate that several other parameters involved in blood processing as well as antigen-stimulation can impact cell viability and the measured T-cell responses (Owen et al., 2007; Jeurink et al., 2008; McKenna et al., 2009; Weinberg et al., 2009; Afonso et al., 2010; Mallone et al., 2011; Kutscher et al., 2013). Moreover, the sensitivity of whole blood versus PBMC assays is still under debate, with different studies reaching opposite conclusions (Suni et al., 1998; Hoffmeister et al., 2003).

In recent HIV-1 vaccine trials, HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses were evaluated by ICS following in vitro stimulation with p17, p24, reverse transcriptase (RT) and Nef peptide pools to assess the expression of interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and CD40-ligand (CD40L), using PBMCs isolated from venous blood (Van Braeckel et al., 2011; Harrer et al., 2014). By compiling previous evaluations, we observed a lower PBMC viability after ICS in antiretroviral therapy (ART)-naïve HIV-1-infected patients (ART^- HIV^+) compared to ART-experienced HIV-1-infected patients (ART^+ HIV^+) (samples from trial published in Harrer et al., 2014) or uninfected volunteers (HIV^-) (samples from trial published in Denny et al., 2013) (Fig. 1).

To investigate this further, blood samples were collected from ART^- HIV^+ patients and the following parameters were investigated: (i) time between blood collection and processing or cryopreservation of PBMCs ("time-to-process"); (ii) time between PBMC thawing and initiation of the *in vitro* stimulation ("resting-time"); and (iii) duration of antigen-stimulation in PBMC cultures ("stimulation-time"). The total cell recovery, cell viability and the magnitude or quality of HIV-specific T-cell responses were assessed to determine the optimal combination of process conditions. Additionally, the influence of the "time-to-process" parameter was evaluated following ICS on fresh whole blood samples.

2. Material and methods

2.1. Study design

This was a phase I, self-contained clinical trial conducted at the Center for Vaccinology, Ghent University Hospital, Ghent, Belgium, between June and October 2012. Blood samples were collected from 22 ART^- HIV^+ adult participants.

A Design-of-experiments (DoE) statistical approach was used to evaluate the individual and combined effects of the quantitative parameters "time-to-process" (TTP) and "resting-time" (RsT) on the quality of CMI analyses in a response surface design. Cell recovery and viability were measured in blood samples during the CMI protocol using the following combination of experimental conditions: TTP (2, 7 or 24 h) and RsT (none, 2, 6 or 18 h). These measurements were used as input in a polynomial prediction model, to further calculate optimal combinations for these experimental conditions on cell viability. The same approach was used for cell recovery and measurements of CMI responses.

The study was conducted in accordance with the Good Clinical Practice Guidelines and the Declaration of Helsinki. Written informed consent was obtained from each participant prior to the performance of any study-specific procedures. This study has been registered at www.clinicaltrials.gov (NCT01610427). A summary of the protocol is available at http://www.gsk-clinicalstudyregister.com (GSK study 116329).

2.2. Study participants

Participants were ART^- HIV^+ eligible adults between 18 and 55 years of age at the time of enrollment, who were not eligible for ART treatment as per established guidelines. Participants had to have an HIV-1 RNA viral load (VL) level between and including 2000 and 100,000 copies/mL and a $CD4^+$ T-cell count >500 cells/μL at screening. Participants who at screening had any clinically relevant medical condition or grade 3 or 4 abnormalities as defined by Division of Acquired Immunodeficiency Syndrome (DAIDS) grading were not enrolled. No planned hematotoxic, investigational or non-registered product, nor vaccine not foreseen in the protocol was allowed during the study period. No pregnant or lactating women were included in the study.
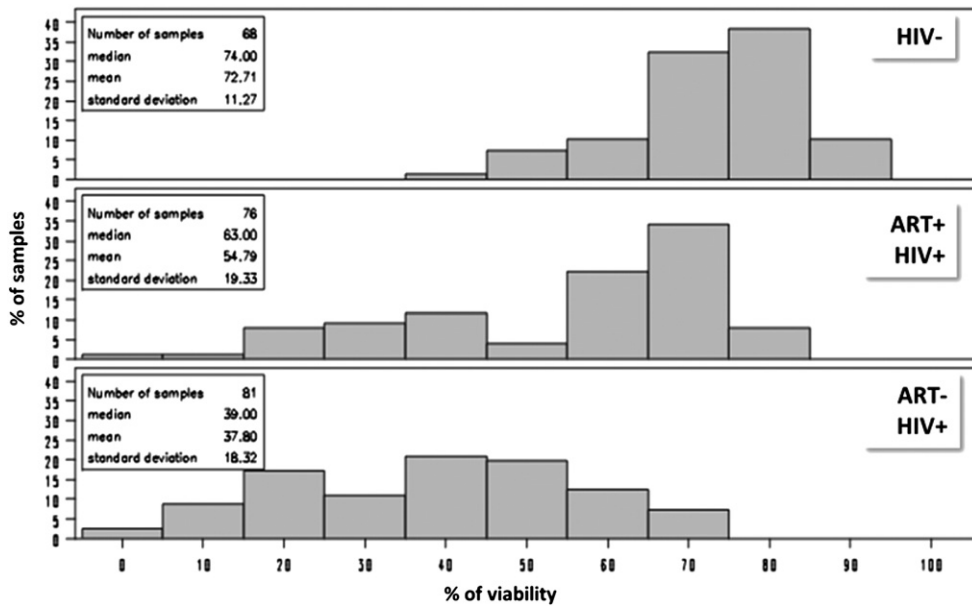
Fig. 1. Distribution of PBMC viability after ICS in ART$^-$ HIV$^+$, ART$^+$ HIV$^+$ and HIV$^-$ individuals. % of samples = viability of lymphocytes expressed as a percentage; ART$^-$ HIV$^+$ = antiretroviral therapy (ART)-naïve HIV positive individuals; ART$^+$ HIV$^+$ = ART-experienced HIV positive individuals; HIV$^-$ = HIV negative individuals.

2.3. Study objectives

The primary objective of this study was to model lymphocyte viability according to TTP and RsT conditions and to select the best combination of these two parameters with the aim to maximize the post-ICS viability in PBMC samples collected from ART$^-$ HIV$^+$ individuals.

The secondary objectives were: (i) to describe the impact of absence or presence of the resting step before ICS on the proportion of viable lymphocytes and on the CMI responses in PBMC samples, and (ii) to describe the proportion of viable lymphocytes and the magnitude of the CMI responses following 6 h (as compared to overnight) antigen stimulation before ICS. The impact of TTP and RsT on the total cell recovery has been evaluated as a post-hoc analysis.

The exploratory objectives were: (i) to describe the relationship between HIV parameters (HIV-1 VL, CD4$^+$ and CD8$^+$ T-cell counts), and cell viability and CMI responses for conditions nearest to the selected best combination of TTP and RsT; (ii) to describe the relationship between inflammatory markers (IL-6, D-dimer), and cell viability and CMI responses for conditions nearest to the selected best combination of TTP and RsT; and (iii) to evaluate the impact of TTP (2 h vs 4 h) on the whole blood ICS assay.
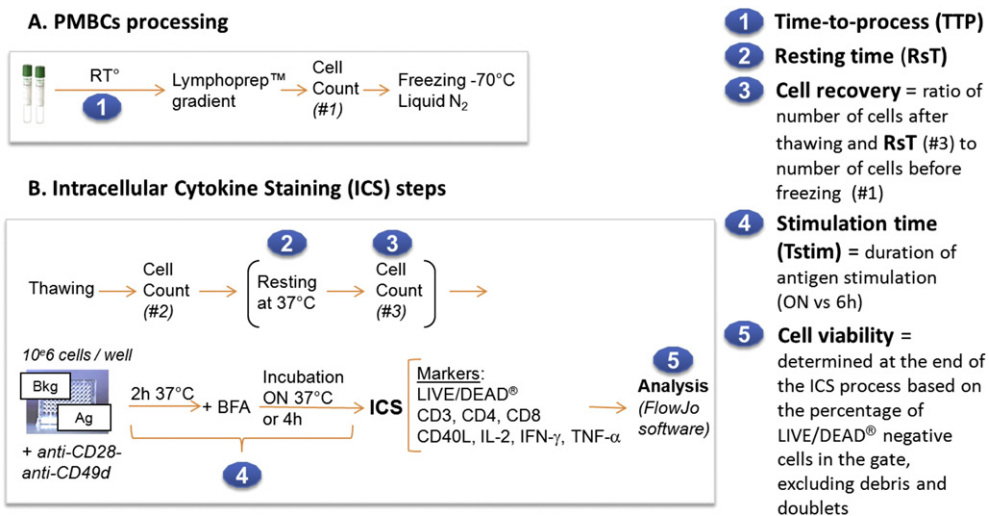

Fig. 2. PBMC processing (A), ICS steps and investigated parameters (B). Ag = antigen; BFA = brefeldin A; Bkg = background; ICS = intracellular cytokine staining; liquid N$_2$ = liquid nitrogen; ON = overnight; RT = room temperature.

2.4. PBMC processing methods

Blood samples were collected by venipuncture in lithium-heparin tubes. The tubes were kept at room temperature (20–25 °C) and shipped immediately to a designated laboratory for separation of PBMCs within 2, 7 or 24 h (TTP), in accordance with current operating procedures (Fig. 2). PBMCs were separated on Lymphoprep™ gradients, washed, counted by flow cytometry, frozen and further stored in liquid nitrogen for 9 to 23 weeks. This limited timeframe was not evaluated as a key parameter in our analysis. HIV-specific $CD4^+$ and $CD8^+$ T-cell responses were evaluated by ICS (Fig. 2B), as previously described (Van Braeckel et al., 2011; Harrer et al., 2014).

Briefly, cryopreserved PBMCs were rapidly thawed and counted by flow cytometry using propidium iodide to identify dead cells. Cells were used after thawing without resting or with a 2, 6 or 18 h RsT, prior to stimulation. Cell recovery was calculated as the ratio of number of viable cells after thawing and RsT to number of cells before freezing (cell counts). One million PBMCs were stimulated in vitro with HIV peptide pools (15-mer peptides overlapping by 11 amino acids, covering the sequences of p17, p24, RT or Nef antigens) or medium only in the presence of anti-CD28/CD49d antibodies (used as co-stimulatory molecules). After 2 h of stimulation at 37 °C, brefeldin A was added to inhibit signal molecule secretion during an additional 4-hour or overnight (16 h) incubation (Tstim). Cells were harvested, stained for surface markers ($CD4^+$ and $CD8^+$) in combination with the LIVE/DEAD® marker, fixed, permeabilized, and stained with labeled antibodies to CD3, CD40L, IL-2, IFN-γ, and TNF-α. Cell viability was calculated at the end of the ICS process, based on the percentage of LIVE/DEAD® negative cells in the gate (viable cells), excluding debris and doublets. The LIVE/DEAD® marker is based on the reaction of a fluorescent reactive dye with cellular amines whose expression differs between viable and necrotic cells.

Acquisition was performed on a BD LSRII (Becton Dickinson) flow cytometer and data were analyzed using the FlowJo software v.9.5.2 (Tree Star, Inc.).

2.5. Whole blood processing methods

The impact of TTP on an ICS assay on whole blood was evaluated as a tertiary objective (Fig. 3). Briefly, within 2 or 4 h TTP after collection, 350 μL of whole blood was stimulated in vitro using the abovementioned HIV peptide pools (and phosphate buffered saline [PBS] or Staphylococcus enterotoxin B [SEB] as negative and positive controls, respectively), in the presence of anti-CD28 and anti-CD49d antibodies. Following a 2-hour stimulation at 37 °C, brefeldin A was added to inhibit signal molecule secretion during an additional overnight incubation. After EDTA treatment to unstick adherent cells, red blood cells were then eliminated by fluorescence-activated cell sorting (FACS) lysing solution and cells were frozen at −70 °C. Subsequently, samples were thawed and cells were stained using the ICS procedure (except for the LIVE/DEAD® marker which was not used in the staining panel), analyzed by flow cytometry and FlowJo software as described previously for the ICS assay using PBMCs.

2.6. Statistical methods

The target size was 20 ART⁻ HIV⁺ adult participants based on feasibility considerations and power computations. This sample size allowed concluding on the primary objective with a power of at least 95% assuming an increase of percentage of viable lymphocytes of 25%, based on either a regression model with quantitative factors or a 3-way Analysis of Variance (ANOVA) mixed model with qualitative factors. The analyses were performed on the according-to-protocol (ATP) cohort.

To predict the percentage of viable lymphocytes in the CMI samples, a mixed model for repeated measurements was used, with TTP and RsT being considered as quantitative factors in a
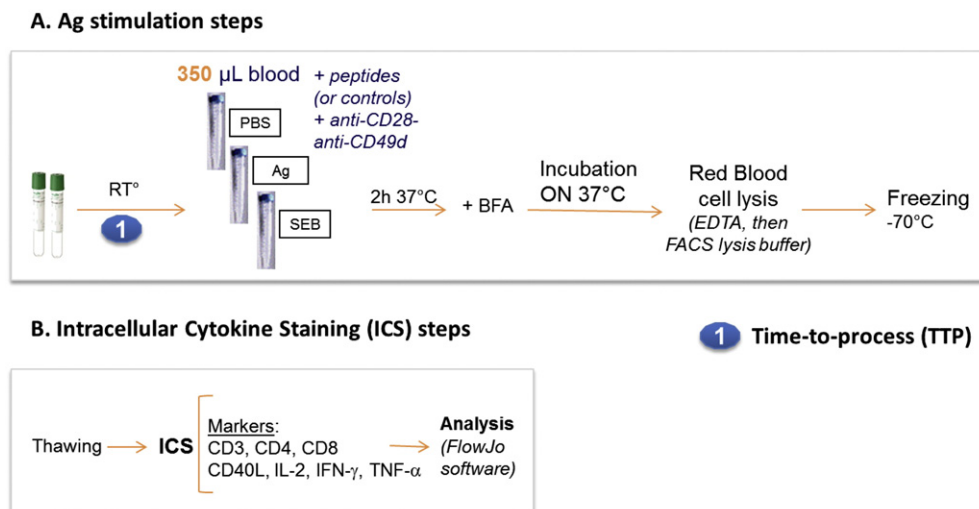

Fig. 3. Whole blood processing and Ag stimulation steps (A), ICS steps and investigated parameters (B). Ag = antigen; BFA = brefeldin A; EDTA = ethylenediaminetetraacetic acid; FACS = fluorescence-activated cell sorting; ICS = intracellular cytokine staining; ON = overnight; PBS = phosphate buffered saline; RT = room temperature; SEB = Staphylococcus enterotoxin B.

polynomial model. The exact prediction model and associated variance–covariance matrix were determined by maximizing the prediction efficiency (based on Information Criteria) while respecting the model hierarchy and preserving all fixed effect having <10% p-value.

The prediction model was used to display graphically the predicted impacts of TTP and RsT on cell recovery and viability, and to calculate their predicted optimal combinations (in order to maximize the percentage of viable lymphocytes).

For the combination of parameters nearest to the selected best combination, regression analysis was used to explore the relationship between HIV-1 VL, the CD4$^+$ and CD8$^+$ counts, the inflammatory markers (IL-6, D-dimer) and the cell recovery/viability or the magnitude of the CMI response.

The whole blood data were analyzed with an ANOVA with 1 factor (TTP: 2 h vs 4 h) using a heterogeneous variance model, i.e. identical variances were not assumed for the different levels of the factor. Estimates of the geometric mean ratios (GMRs) between groups and their 95% confidence intervals (CIs) were obtained using back-transformation on log$_{10}$ values for CD40L$^+$ CD4$^+$ and CD8$^+$ T cells expressing at least one cytokine. The criteria used to demonstrate equivalence were defined *a posteriori* as the 95% CI for the GMR had to be included in the predefined equivalence limit of [0.3–3].

The ICS results were expressed as the percentage of the total CD40L$^+$ CD4$^+$ and CD8$^+$ T cells expressing the different combinations of IL-2 and/or IFN-γ and/or TNF-α in response to stimulation with p17, p24, RT or Nef antigens minus the response measured upon in vitro stimulation with medium only.

A Pearson correlation coefficient (r) was used to compare CD8$^+$ responses of PMBCs vs whole blood.

The statistical analyses were performed using the Statistical Analysis Systems (SAS) version 9.2 on Windows and StatXact-8.1 procedure on SAS.

3. Results

3.1. Demographics

A total of 31 participants were screened in this study. Of these, 22 (71%) participants were included in the ATP cohort and completed the study. In the ATP cohort, the mean age of the participants was 36.8 ± 9.1 years, 20 (90.9%) participants were males and the majority of participants were White-Caucasian (90.9%).

3.2. ICS using frozen PBMCs

3.2.1. Cell recovery and cell viability

PBMC recovery before ICS was weakly affected by varying TTP, but declined sharply (cell recovery <50%) after an RsT of >2 h (Fig. 4A). The predicted optimum of the DoE analysis was reached for a TTP of 2 h and no RsT, with a predicted mean cell recovery of 81.5%. A slight increase was observed with a TTP of 24 h or an RsT of 18 h. Further analysis of physical parameters with Forward and Side Scatter (FSC/SCC) did not show any differences in proportion of granulocytes or large mononuclear cells (Supplementary Figure S1) that could have explained these increases. Additional cell markers should be assessed to

better characterize the cell phenotypes in these different conditions.

Lower limit of 95% CI of cell viability >80% was obtained with a TTP of <7 h and an RsT of <13 h (Fig. 4B). The optimal predicted response of the DoE analysis in terms of cell viability (87.5%) was reached for a TTP of 2 h and an RsT of 6.5 h. For a TTP of 7 h and no RsT, mean cell viability estimated by the model was 82.9% (95% CI: 80.4%; 85.1%).

3.2.2. HIV-specific T-cell responses

In this study, the magnitude of the RT-specific response of CD8$^+$ T cells expressing at least one of the tested cytokines (IL-2, IFN-γ and TNF-α) was independent of TTP and RsT parameters, but was higher after overnight compared to 6 h Tstim (Fig. 5). The increase resulted from a higher antigen specific response without a change in the background response. Similar observations were made for the 3 other antigens (Nef, p24 and p17; lower sample sizes), although the magnitude of the responses varied (Nef > RT > p24 > p17) (data not shown). A 2 fold decrease was observed between RsT 18 h and 0 h, however acceptable taking into account the variability of the assay and improvement of quality of cells at RsT 0 vs 18 h.

The HIV-specific CD8$^+$ T-cell cytokine profile was comparable after the overnight or the 6-hour antigen stimulation (Tstim) (Fig. 6). The percentages of HIV-specific CD8$^+$ T-cell responses at a TTP of 7 h differed between cytokines (IFN-γ > IFN-γ + TNF-α > TNF-α > IL-2), independently of the RsT and Tstim parameters.

HIV-specific responses of CD40L$^+$ CD4$^+$ T cells expressing at least one cytokine were very low compared to CD8$^+$ T cells and no conclusion could be drawn from the data obtained (data not shown).

#### 3.2.3. Correlation between HIV parameters/inflammatory markers and viability/CMI

No significant correlations (r between −0.6 and 0.55) could be observed between the HIV-1 VL, the CD4$^+$ and CD8$^+$ T-cell counts, the inflammatory markers and the cell recovery/viability or the magnitude of the CMI response for the specific combination TTP/RsT that is optimal (data not shown).

3.3. ICS using fresh whole blood

High HIV-specific CD8$^+$ T-cell responses in ART$^-$ HIV$^+$ participants could be detected using whole blood ICS. No significant differences could be highlighted for the HIV-specific CD8$^+$ T-cell responses between 2 and 4 h of TTP (Table 1). Equivalence (a posteriori defined as 95% CI of GMR included in [0.33–3]) was observed for antigens p17, p24 and RT. A GMR (4 h vs 2 h) of 1.46 [95% CI: 0.46–4.66] was calculated for antigen Nef. Similar to PBMCs, HIV-specific responses of CD40L$^+$ CD4$^+$ T cells expressing at least one cytokine were low and no conclusion could be drawn from the data obtained (Supplementary Table 1).

Good correlations (correlation coefficient, r > 0.8) for CD8$^+$ T-cell responses against all antigens could be observed between whole blood (a TTP of 2 h) and PBMCs (RsT 0 h and a TTP of 2 h or 7 h), except for p17 in the PBMC assay with a TTP of 2 h, due to the lower response to antigen p17 (Fig. 7, Supplementary Figure S2).
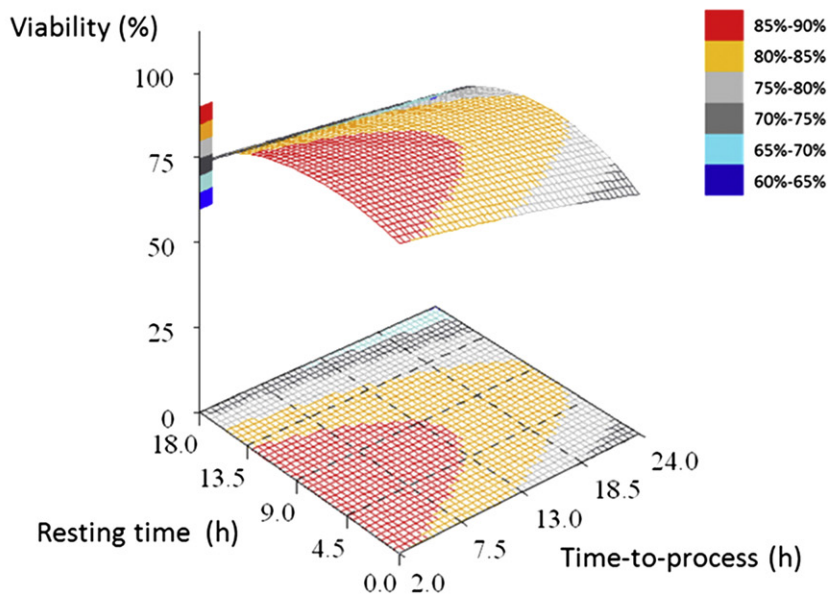
A. Cell recovery
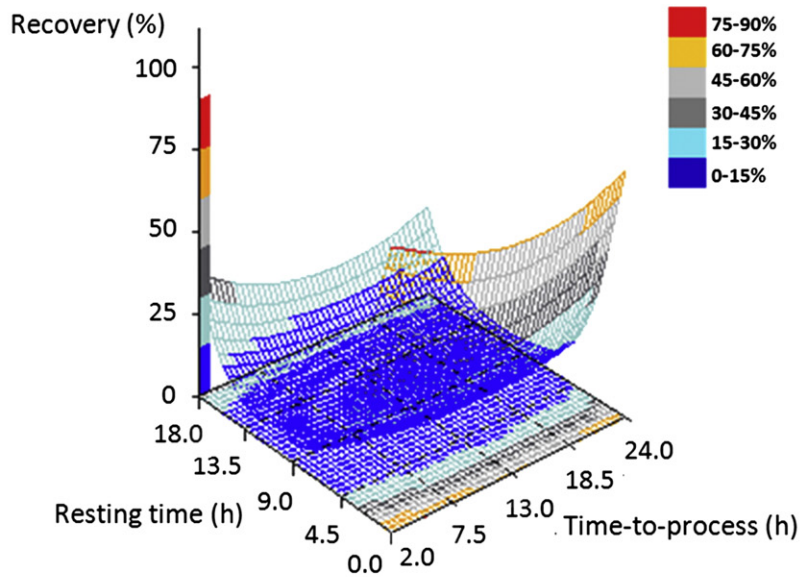
B. Cell viability

Fig. 4. Effect of time-to-process (TTP) and resting-time (RsT) parameters on PBMC recovery (A) and viability (B).

4. Discussion

The present study was designed to evaluate the effect of several parameters in blood processing and impacting on PBMC viability and T-cell responses measured by ICS in samples collected from ART-naïve HIV-1-infected participants. The selected assessed parameters were: time between blood collection and PBMC processing/cryopreservation (TTP), time between PBMC thawing and initiation of the _in vitro_ stimulation (RsT), and duration of antigen-stimulation in PBMC cultures (Tstim). The total cell recovery, viability, and the magnitude of HIV-specific T-cell responses were assessed to determine the optimal combination of these parameters. The CMI response using PBMCs was compared to the one using whole blood, which could be perceived as an ex vivo evaluation of the CMI response.

In our study, cell recovery and viability values were higher for shorter time intervals between phlebotomy and PBMC cryopreservation (TTP < 7 h) than for longer time intervals. With these shorter time intervals, the estimated PBMC viability
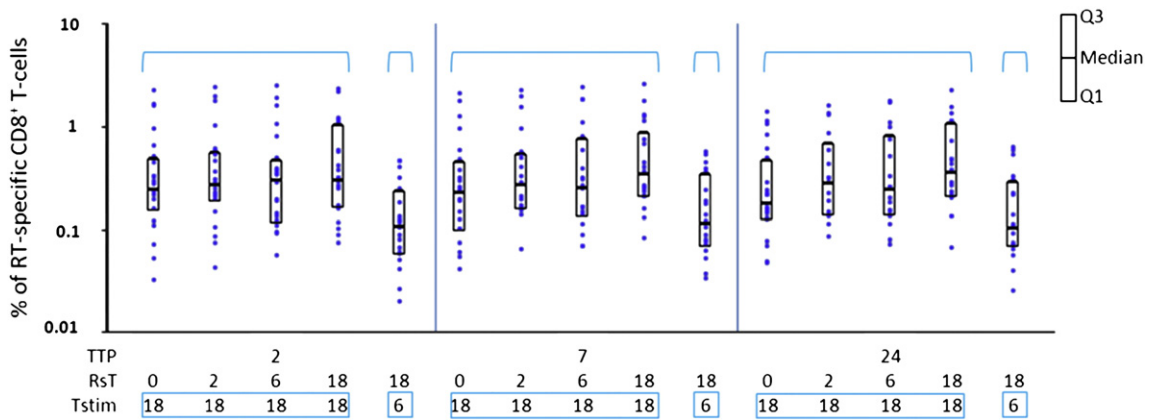
Fig. 5. Effect of time-to-process (TTP), resting-time (RsT), and duration of antigen stimulation (Tstim) parameters on the response magnitude of HIV-1 RT-specific CD8$^+$ T cells expressing at least one cytokine among IL-2, IFN-γ and TNF-α.

in ART-naïve HIV-1-infected participants was significantly improved, from 40% to more than 80%, corresponding to similar levels observed in healthy HIV-1 negative and ART-experienced HIV-1 infected participants (Fig. 1). Similar findings have already been reported in the literature (Bull et al., 2007; Kierstead et al., 2007). When comparing blood from healthy volunteers processed at 8 h vs 24 h (TTP) after venipuncture in a multi-center study, Bull et al. observed a modest reduction in PBMC viability when TTP increased, an important loss in cell recovery (~32%), and a loss in viral peptide-reactive T-cell frequency (IFN-γ ELISPOT) (36–56%) (Bull et al., 2007). Similar results were obtained in an HIV-vaccine trial, in which processing of blood samples within 12 h compared to longer time intervals, led to three-fold higher T-cell responses (Kierstead et al., 2007). Granulocyte contamination in blood stored for prolonged periods at room temperature has been shown not only to reduce the relative number of T cells present in PBMCs, but also to inhibit T-cell proliferation following stimulation in ~75% of samples (McKenna et al., 2009) and to inhibit IFN-γ ELISPOT responses to CD8$^+$ T-cell viral epitope peptides (Afonso et al., 2010).

In our study, better cell recovery and viability were predicted for shorter RsT intervals (<13 h for viability and <2 h for

recovery), but the HIV-specific CD8$^+$ T-cell cytokine profile was independent of the RsT parameter. However, several reports have shown better antigen-specific T-cell responses by IFN-γ ELISPOT using cryopreserved PBMCs when cells are rested (overnight) prior to performing the assay (Kierstead et al., 2007; Britten et al., 2008; Janetzki et al., 2008; Boaz et al., 2009; Kutscher et al., 2013). It has been suggested that this improvement was due to the fact that a fraction of cells undergoing death is eliminated with resting, thus allowing more accurate counts of viable cells used for the assay and eliminating interference by non-viable cells, resulting in decreased background noise. On the other hand, a recent study argues that resting is not a universally applicable approach to improve the performance of cryopreserved PBMC for detection of low frequency T-cell responses (Kuerten et al., 2012), which underlines the importance of establishing whether resting could benefit immune monitoring in a specific read-out.

In our case, overnight antigen stimulation (Tstim) compared to the standard 6 h improved the magnitude of the RT-specific CD8$^+$ T-cell response following ICS on PBMCs, without affecting the cytokine profile. A previous study has shown that a PBMC Tstim of 6 h gave the highest values for intracellular cytokine production (Horton et al., 2007). Another study using
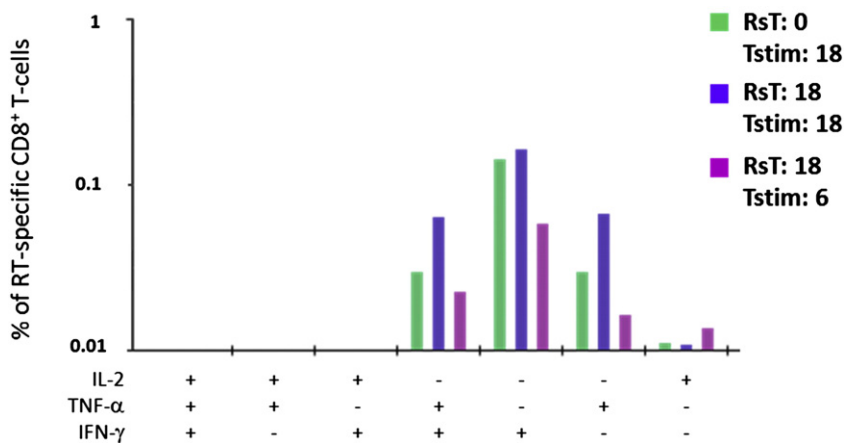

Fig. 6. Effect of resting-time (RsT) and duration of antigen stimulation (Tstim) parameters on the functionality of HIV-1 RT-specific CD8$^+$ T-cell responses for a time-to-process of 7 h.

Table 1
Effect of time-to-process (TTP; 2 h vs 4 h) on HIV-1 RT-specific CD8$^+$ T-cell response (ICS on whole blood).

Antigen	Time-to-process (h)	Estimate (GM)[a]	95% CI lower limit	95% CI upper limit
Nef	2	4747	2062	10,931
	4	6950	2836	17,032
GMR[b] (4 h vs 2 h)		1.464	0.459	4.665
p17	2	2912	1488	5697
	4	2385	1094	5201
GMR (4 h vs 2 h)		0.819	0.310	2.164
p24	2	5764	3398	9775
	4	5351	3022	9474
GMR (4 h vs 2 h)		0.928	0.445	1.937
RT	2	3933	2031	7614
	4	3786	2268	6321
GMR (4 h vs 2 h)		0.963	0.433	2.142

GM = geometric mean; GMR = geometric mean ratio; CI = confidence interval.
[a] Number of CD8$^+$ T cells expressing at least one cytokine/10^6 CD8 cells.
[b] Ratio between the values at 4 h and 2 h.

ICS on whole blood has found that the optimal time for antigen stimulation was 18 h (Hanekom et al., 2004).

The ICS assay on whole blood was previously developed and used in several clinical trials (Agnandji et al., 2011; Day et al., 2013; Harenberg et al., 2013). An advantage of using whole blood instead of PBMCs is the smaller sample volumes needed when blood is used without preliminary PBMC purification, making this type of assay particularly attractive in children, in

settings where testing at frequent time intervals is planned or for trials conducted in areas with high HIV endemicity, where access to liquid nitrogen might be problematic (Agnandji et al., 2011; Mallone et al., 2011; Day et al., 2013; Harenberg et al., 2013).

Given the good correlations for CD8$^+$ T-cell responses between ICS performed on whole blood compared to frozen PBMCs, we have demonstrated that the whole blood ICS assay is a valid tool to study HIV-1-infected individuals. The performance of PBMC vs whole blood T-cell assays has been previously assessed, with contradictory findings. Suni et al. showed that the whole blood method consistently yielded higher T-cell frequencies than the method using purified PBMCs (Suni et al., 1998). However, Hoffmeister et al. obtained higher T-cell frequencies with PBMCs than with whole blood, and the responses were sometimes detected only with PBMCs (Hoffmeister et al., 2003).

Our study revealed no significant differences for HIV-specific CD8$^+$ T-cell response between 2 and 4 h of TTP using whole blood, although in the case of ICS, TTP has been shown to affect assay performance critically, already at 1 h after drawing, with a marked effect on IFN-γ (Petrovsky and Harrison, 1995).

This study is limited by the fact that the effect of the ICS parameters on CD4$^+$ T-cell responses was not interpretable since these responses were low and masked by the CD8$^+$ T-cell responses, regardless of using frozen PBMCs or fresh whole blood. This is not surprising since the participants in the current study were HIV-1 infected and not vaccinated against HIV-1
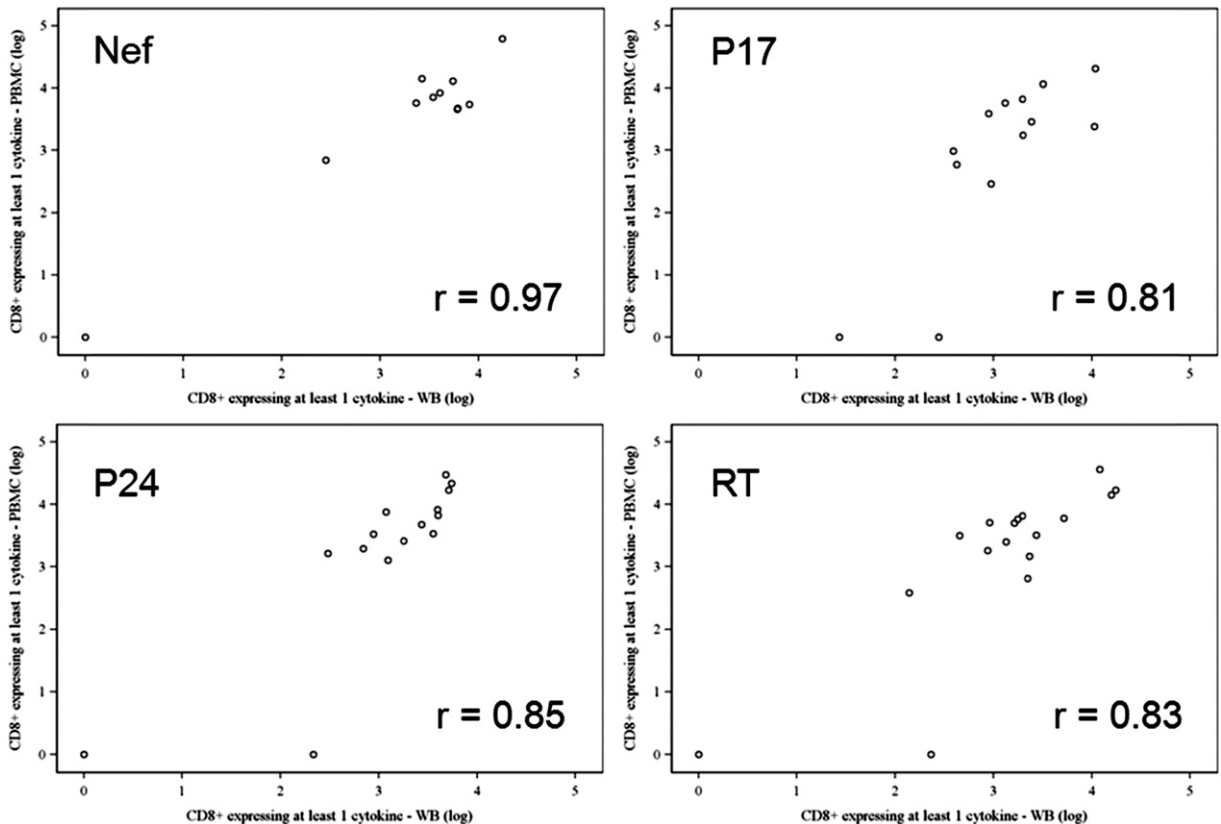

Fig. 7. Correlation between CD8$^+$ responses of PBMCs (a resting-time of 0 h and a time-to-process of 7 h) vs whole blood (a time-to-process of 2 h).

(Harrer et al., 2014). Also, the conclusions of this study are restricted to non-vaccinated ART⁻ HIV⁺ participants where the PBMC viability was shown to be the lowest.

5. Conclusions

In samples collected from HIV⁺ ART⁻ participants, a higher quality of cells in terms of viability and recovery was observed when shorter time intervals between phlebotomy and PBMC cryopreservation (less than 7 h), and between PBMC thawing and antigen-stimulation (less than 2 h) were used to assess antigen-specific T-cell responses using ICS. The peak response of the DoE analysis in terms of cell viability (87.5%) was reached for a TTP of 2 h and an RsT of 6.5 h. Longer (overnight) rather than shorter (6 h) duration of antigen-stimulation increased the observed frequencies of specific T-cell responses without changing the functionality.

High HIV-1 specific CD8⁺ T-cell responses were detected with ICS using fresh whole blood, with a good correlation with the CMI responses detected using PBMCs. The current whole blood ICS method could be applied in cases of HIV-1 infection. This could potentially be of interest for trials conducted in resource-limited settings (no liquid nitrogen required) or in infants (small blood volumes).

Our results support the need to use standardized procedures for the evaluation of CMI responses in the field of vaccine development (and particularly for HIV vaccine development), and describe an alternative whole blood assay when liquid nitrogen is not easily available and blood volumes are small.

6. Competing interests

Funding source

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2014.09.001.

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