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ARTICLE

Achieving intracellular cytokine staining assay concordance on two continents to assess HIV vaccine-induced T-cell responses

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Summary sentence: Rigorous quality control of reagents and techniques as well as ongoing monitoring are critical to achieving interlaboratory concordance of the ICS assay.

Summary sentence: Interlaboratory concordance of the intracellular cytokine assay can be achieved and maintained over time by rigorous control of shared reagents, operator technique, and on-going testing.

Abstract

The HIV Vaccine Trials Network (HVTN) conducts clinical trials on 4 continents in pursuit of a safe and effective HIV vaccine. Cellular immune responses to vaccination that define vaccine immunogenicity and/or immune correlates of protection can be measured using multiparameter intracellular cytokine staining (ICS) assays. The HVTN cellular immunology laboratory, located in Seattle, WA, conducts ICS assays for vaccine trials according to Good Clinical Laboratory Practices (GCLP). In 2013, the HVTN established a second GCLP compliant cellular immunology laboratory in Cape Town, South Africa to assess vaccine immunogenicity for HVTN trials conducted on the African continent. To ensure ICS readouts in the 2 laboratories were directly comparable, we conducted concordance testing using PBMC from healthy controls and vaccine trial participants. Despite standardized procedures and instrumentation, shared quality control measures and quality assurance oversight, several factors impacted our ability to obtain close agreement in T-cell responses measured in the 2 laboratories. One of these was the type of fetal bovine serum (FBS) used in the assay, which impacted lymphocyte cell viability and background responses. In addition, the differences in supernatant removal technique also significantly affected our ability to detect positive responses to vaccine antigens. Standardization of these factors allowed us to achieve and maintain ICS assay concordance across the 2 laboratories over multiple years, accelerating our efforts to evaluate HIV vaccines. The insights gained in this process are valuable for assay transfer efforts by groups of investigators that need to directly compare data generated in different laboratories around the globe.

KEYWORDS
cellular, equivalence, flow cytometry, interlaboratory, standardization

Abbreviations: AViD, Aqua Viability Dye; CCC, concordance correlation coefficient; CI, confidence interval; ENV, envelope; FBS, fetal bovine serum; Gag, GROUP antigen; GCLP, Good Clinical Laboratory Practices; HVTN, HIV Vaccine Trials Network; ICS, intracellular cytokine staining; LC, laboratory center; Pol, polymerase; PTE, potential T-cell epitopes; SOP, Standard Operating Procedures; FBS, fetal bovine serum.

1 | INTRODUCTION

A vaccine to prevent infection by HIV remains a top global health priority. The HIV Vaccine Trials Network (HVTN) is the world's largest publicly funded organization working to discover an effective vaccine to prevent HIV/AIDS. The HVTN conducts all phases of clinical trials, from evaluating experimental first-in-human vaccines for safety and immunogenicity to testing vaccine efficacy in large phase III trials. Many pivotal HVTN studies are conducted on the African continent, where HIV/AIDS has hit hardest, and include the recent efficacy trials testing a clade C adapted version of the RV144 pox-protein regimen[1,2] and an adenoviral-vectored prime/boost regimen designed to provide protection against different clades of HIV (HVTN 705/HPX2008, ClinicalTrials.gov Identifier: NCT03060629).

Evaluation of vaccine immunogenicity is critical for understanding whether a given regimen can induce a protective and durable immune response and enable comparisons across vaccine regimens tested in different clinical trials. Cellular immune responses to the different vaccine regimens have been evaluated through the HVTN Laboratory Center's laboratory in Seattle since the network's inception over 20 years ago. Assays to assess vaccine immunogenicity are conducted by the HVTN in specialized immunology laboratories that operate according to Good Clinical Laboratory Practice (GCLP) guidelines to ensure data integrity, reproducibility, and comparability across trials.[3] In 2013, due to the increasing number of trials being conducted on the African continent, the HVTN Laboratory Center decided to establish a second cellular immunology laboratory in Cape Town, South Africa.

Flow cytometry-based assays are employed by many laboratories around the world to evaluate cellular immune responses to HIV, malaria, tuberculosis, SARS-CoV-2, and other infectious diseases. The HVTN Laboratory Center has developed a validated flow cytometry-based intracellular cytokine staining (ICS) assay to evaluate cellular immune responses in cryopreserved PBMC isolated from study participants.[4–7] This validated ICS assay is primarily used to measure CD4+ and CD8+ T-cell responses to vaccine candidates, allowing the determination of both the number and frequency of antigen-specific T cells induced as well as the proportion of individuals in a vaccine trial who mount a response to the vaccine. Data from the HVTN's ICS assay have been used to inform go/no go decisions to advance vaccine regimens to further testing.[8,9]

The ICS assay is complex and although it was validated in our Seattle laboratory, transfer to the Cape Town laboratory posed a challenge. The assay requires sourcing of more than 20 different reagents and the flow cytometer instruments used to measure responses are notoriously difficult to standardize.[10] Many opportunities for variability to be introduced arise in the 3 days required to perform the assay: cryopreserved PBMC are thawed on day 1, incubated overnight, stimulated on day 2 with vaccine-matched antigens, labeled with fluorescent antibodies on day 3 and analyzed for marker expression. Although the External Quality Assurance Program Oversight Laboratory (EQAPOL) has developed a program to perform comparison testing in ICS assays across NIH-funded laboratories[11] and some published studies have compared ICS assays across different laboratories,[12] few groups performing the ICS assay have established rigorous standardized procedures with acceptable high specificity and sensitivity for consistent use in blinded analyses that enable head-to-head comparisons of data across laboratories.

In this study, we describe an experimental and statistical framework to establish ICS assay concordance between laboratories and demonstrate how this framework assisted us in identifying procedural differences between laboratories that affected overall assay concordance. We highlight the role of standardized critical reagents to achieve assay concordance and demonstrate that on-going concordance testing can be used to successfully maintain concordance over 5 years. This study provides a roadmap to laboratories that need to establish concordance of cellular assays across centers.

2 | MATERIALS AND METHODS

2.1 | Establishment of the ICS assay in Cape Town

GCLP guidelines stipulate the use of Standard Operating Procedures (SOPs) in the HVTN Laboratory Center (HVTN LC).[3] ICS assay-related SOPs were developed in the Seattle HVTN laboratory, including SOPs for the ICS procedure and all related equipment, a competency assessment for all research technicians performing the assay, and a standardized data analysis SOP. The Seattle Quality Assurance Unit oversees all laboratory work, performing internal audits and ensuring strict adherence to all SOPs. When the Cape Town HVTN laboratory opened, shared SOPs were developed to allow both laboratories to utilize the same SOPs relevant to the ICS assay. Staff from the Seattle laboratory trained Cape Town laboratory staff and control samples were shared between the laboratories. Flow cytometers underwent extensive standardization between the laboratories according to rigorous procedures published by other groups[13–17] and FlowJo analysis templates were also shared to eliminate biases introduced in the data analysis stage, as documented by other groups.[18]

2.2 | ICS procedure

Cryopreserved PBMC were thawed, incubated overnight in a 37°C incubator maintained with 5% CO_2, and then stimulated for 6 h with antigenic peptide pools (15-mer peptides overlapping by 11 amino acids; Bio-Synthesis Inc., Lewisville, TX, USA), DMSO (0.5%; Sigma–Aldrich, St. Louis, MO, USA; negative control) or Staphylococcus Enterotoxin B (0.25 μg/ml; Sigma–Aldrich; positive control) in the presence of costimulatory antibodies (CD28 and CD49d, 1 μg/ml; BD Biosciences, San Jose, CA, USA) and brefeldin A (10 μg/ml; Sigma–Aldrich).[4,5] Cells were incubated overnight at 4°C with EDTA (2 mM; Life Technologies, Carlsbad, CA, USA), then stained with a 17-color antibody staining panel,[19] acquired on a BD LSRFortessa flow cytome-

ter (BD Biosciences), and analyzed using FlowJo version 9.9.4 (BD Biosciences).

2.3 | Peptide pools

Vaccine matched and potential T-cell epitope (PTE) peptide pools were used to evaluate T-cell responses. Synthesized peptides consisted of 15-mer peptides overlapping by 11 amino acids spanning the entire protein sequence of interest (Bio-Synthesis Inc.). The following peptide pools were used: Cytomegalovirus (CMV) pp65, Hepatitis B surface antigen, HIV-1 group antigen (Gag) B, Gag ZM96, Envelope (Env) gp120 ZM96, Env 92TH023, Env gp120 1086, Env gp120 TV1, Env-1-PTE, Env-2-PTE, Env-3-PTE, Gag-1-PTE, DNA polymerase (Pol)—1-PTE, and Pol-2-PTE. Peptide pools were all reconstituted at the HVTN LC laboratory in Seattle and shipped to Cape Town to ensure both laboratories used the same batch. Peptide pools were used at a final concentration of 1 μg/ml.

2.4 | FBS

Fetal bovine serum (FBS) was used to prepare cell culture medium at a concentration of 10% FBS in RPMI. Two lots of FBS were tested initially: (1) non-irradiated FBS (Gemini Bio, West Sacramento, CA, USA; catalog no. 100–106, lot A63C00C) and (2) irradiated FBS (Gemini Bio; catalog no. 100–201, lot A71E22F). The non-irradiated A71E22F lot of FBS was extensively tested in the Seattle laboratory and bridged to previous lots, then irradiated to meet the requirements for import into South Africa and shipped to Cape Town for testing. The culture medium made with the irradiated FBS was filtered using a 0.2 μm stericup filter (Merck Millipore; cat no. S2GPU02RE); due to extensive particulates in the FBS, we were only able to filter approximately 100 ml of FBS through each filter. Non-irradiated FBS of Australian origin suitable for use in Cape Town was acquired from Gemini Bio (catalog no. 100–700, lot no. A96D22E) and shipped to Cape Town after extensive bridging testing in the Seattle laboratory against the lot of FBS in use in Seattle (Gemini Bio, catalog no. 100–106, lot no. A46D03D). Both laboratories then used the same FBS for the concordance assays (Gemini Bio, catalog no. 100–106, lot no. A96D22E).

2.5 | Design of the ICS concordance studies

The statistical analysis plan, which included positivity criteria and acceptance criteria for equivalence and concordance, was prespecified prior to the running of assays to ensure objectivity.[10] Two co-primary objectives were set for the concordance studies: (1) to assess equivalence of the proportions of positive responders between the 2 assays and (2) to assess concordance of positivity calls on the same set of specimens between the 2 assays. For the first co-primary objective, equivalence was established if the 95% confidence interval (95% CI) for the difference in response rates was entirely contained in the interval (−15%, 15%). For the second co-primary objective, concordance was established if the lower bound of the 95% CI for the proportion of concordant positivity calls was greater than or equal to 70%. For both objectives, the proportion of CD4+ or CD8+ T cells expressing IFN-γ and/or IL-2 after stimulation by HIV antigens and CMV were evaluated separately.

To evaluate the co-primary objectives, equivalence tests for the difference in response rates were performed based on the adjusted Wald interval for the difference in proportions for matched pairs. The 95% CI for the proportion of concordant positivity calls (both calls were positive or both were negative) was calculated using the score method. Positivity for a peptide pool was based on comparing the percentage of T cells with positive staining for IFN-γ and/or IL-2 between the experimental and negative control wells using a one-sided Fisher's exact test with a p-value cut-off for positivity of 10^{-5}. Positivity calls to CMV and HIV antigens were adjusted for multiple comparisons with a Bonferroni–Holm multiplicity adjustment.[20]

Table S1 presents the statistical power to meet both pre-specified co-primary equivalence criteria between 2 laboratories given a sample size of 120, assuming a true response rate of 40, 50, and 60% in 1 of the 2 laboratories, for CD4+ and CD8+ T-cell responses. Statistical power is estimated by the percentage of datasets that satisfy both equivalence criteria using 10,000 simulated datasets. With a sample size of 120, when the true response rates from the 2 laboratories are the same, there is 79% (or greater) power to establish equivalence if the proportion of concordance in the response calls between the 2 laboratories is 85% (or 90%) and the threshold for the upper/lower bound for the 95% CI of the response rate difference is +/−15% or higher (Table S1).

For the secondary objective, the concordance correlation coefficient (CCC) for the positive responders in both laboratories was calculated to assess the agreement between the net responses from the 2 assays and to identify sources of disagreement. The CCC is a combined measure of precision and accuracy that measures, respectively, how well the paired data fit to a simple linear regression line and how closely the fitted line is to the 45° identity line. A concordance coefficient with value of 1 indicates a perfect agreement, −1 indicates a perfect disagreement, and 0 indicates no agreement. The level of concordance between the 2 laboratories was deemed acceptable if the lower bound of the 95% CI of estimated CCC was at least 0.75.

Samples were excluded from analyses if after thawing and overnight incubation the PBMC viability was <66%. If the cell viability was not at least 66%, a new specimen for that participant was thawed for testing. If the PBMC viability of the second thawed aliquot was also below this threshold, the ICS assay was not performed, and no data were reported for that participant's sample. For the negative control acceptance criteria, if the average cytokine response (expression of IFN-γ and/or IL-2) for the negative control wells was above 0.1% for either the CD4+ or CD8+ T-cell subset, the sample was retested. If the retested results were above 0.1%, the data were excluded from analysis; otherwise, the retest data were used.

The total number of CD4+ and CD8+ T cells retrieved after acquisition on the flow cytometers was also a criterion for inclusion in the

analyses. If the number of CD4+ or CD8+ T cells was less than 5,000 for any of the HIV-1 peptide pools tested or for one of the negative control replicates for a particular sample, data for that stimulation were not included in the analyses. If both negative control replicates contained <5000 cells, the sample was retested. If upon retesting, 1 negative control replicate contained <5000 cells, the negative control replicate with at least 5000 cells was used. If both negative control replicates from the retest for a T-cell subset contained <5000 cells, then data for that T-cell subset were not included in the analysis.

2.6 | Peripheral blood mononuclear cells

2.6.1 | Control cohort specimens

Previously cryopreserved PBMC collected from healthy volunteers enrolled in the Seattle Assay Control cohort (enrolled at the Seattle Vaccine Trials Unit) and the Cape Town Assay control cohorts (enrolled at the Groote Schuur Clinical Research Site) were used for FBS testing and to determine the source of the cell count differences in the ICS assay. Volunteers in these control cohorts were healthy men and women ≥18 years old who had not been previously enrolled in an HIV vaccine trial, were HIV-seronegative or -seropositive and provided informed written consent prior to enrolment. Both protocols were approved by the relevant Institutional Review Board, Fred Hutchinson Cancer Research Center in Seattle or the University Cape Town.

2.6.2 | Vaccine participant specimens

Previously cryopreserved PBMC collected from volunteers enrolled in 4 HVTN vaccine trials were used for ICS concordance testing in the Seattle and Cape Town laboratories. Study participants were healthy, HIV-1-uninfected adults enrolled in the HVTN clinical research sites in the United States of America or in sub-Saharan Africa. Each protocol enrolled men and women ≥18 years old. Protocols participants provided informed written consent prior to enrolment, and all studies were approved by the relevant Institutional Review Boards for the HVTN clinical research sites.

2.7 | Specimen selection for assays

Based on our power calculations, approximately 120 samples were needed for each concordance study. We selected samples from vaccine recipients in the 4 vaccine trials; a small number of samples for each concordance study were chosen from placebo recipients to maintain blinding. These samples covered a range of response magnitudes for both CD4+ and CD8+ T cells to CMV and the HIV vaccine-matched peptide pool antigens, based on data from prior testing. In addition, samples were chosen based on the availability of a sufficient number of PBMC vials from the same blood draw to allow samples to be assayed in both laboratories.

3 | RESULTS

3.1 | Impact of shared reagents: irradiated FBS reduced cell viability

For most reagents specified in the HVTN Laboratory Center ICS SOP, we were able to source identical or similar products in the USA and South Africa. However, we encountered an obstacle with FBS. FBS is used in the ICS assay to supplement cell culture medium and provides undefined growth factors critical to the maintenance of healthy cells in culture.[21–24] We routinely perform bridging experiments when we switch to a new lot of FBS since we have observed lot-to-lot variability that can affect background responses of unstimulated cells in the assay. When the Cape Town laboratory was started, we imported the same lot of FBS as used in Seattle. However, in 2016, the South African government restricted the import of FBS into the country, requiring that all FBS be irradiated due to concerns over prion contamination.[25–27] Consequently, we had to test irradiated FBS in our ICS assay.

As we were unable to irradiate FBS ourselves, we compared our initial lot of non-irradiated FBS with a different lot of irradiated FBS from the same supplier. The ICS assay was performed on 5 healthy donors to explore the impact of irradiated FBS on assay performance. Use of the irradiated FBS did not significantly affect cell viability percentages after stimulation with Env or Hepatitis B peptide pools, but showed a concerning pattern of decreased cell viability in the negative control condition (Figure 1(A)). An examination of the Aqua Viability Dye (AViD) viability dye staining pattern, however, revealed dramatic alterations in the profiles observed when irradiated FBS was used, particularly in the stimulated conditions. The AViD dim population (middle population in the dot plots) showed brighter staining in the simulated conditions with the irradiated FBS, indicating that there were more cells taking up the live/dead dye and were likely on their way to death (Figure 1(B)). In addition, when we looked at cytokine responses from CD4+ T cells in the irradiated FBS samples (Figure 1(C)), we noted increased aggregates in the antigen stimulated conditions (Figure 1(C)) when looking at the T cell response. Together, these effects of the irradiated FBS had the potential to greatly impact our ability to measure antigen-specific T-cell responses in our assay.

We performed further testing in 7 samples to explore whether filtering of the FBS to remove the particulate matter would improve its performance in the ICS assay. Filtering the irradiated FBS almost completely restored lymphocyte viability after stimulation with the Env peptide pool, but this was not observed for the hepatitis B surface antigen peptide pool; viabilities were only partially restored (Figure 1(B)). Filtering also improved the T cell population profiles for the Env and Hepatitis stimulated conditions and removed the aggregates observed prior to filtering (Figure 1(C)). Due to the decreased cell viability observed and concerns about the impact of aggregates on our ability to accurately assess cytokine expression in the assay, we sought an alternate solution to source non-irradiated FBS.

We applied to the South African government for an exemption to import non-irradiated FBS of specific origin (from Australia and New

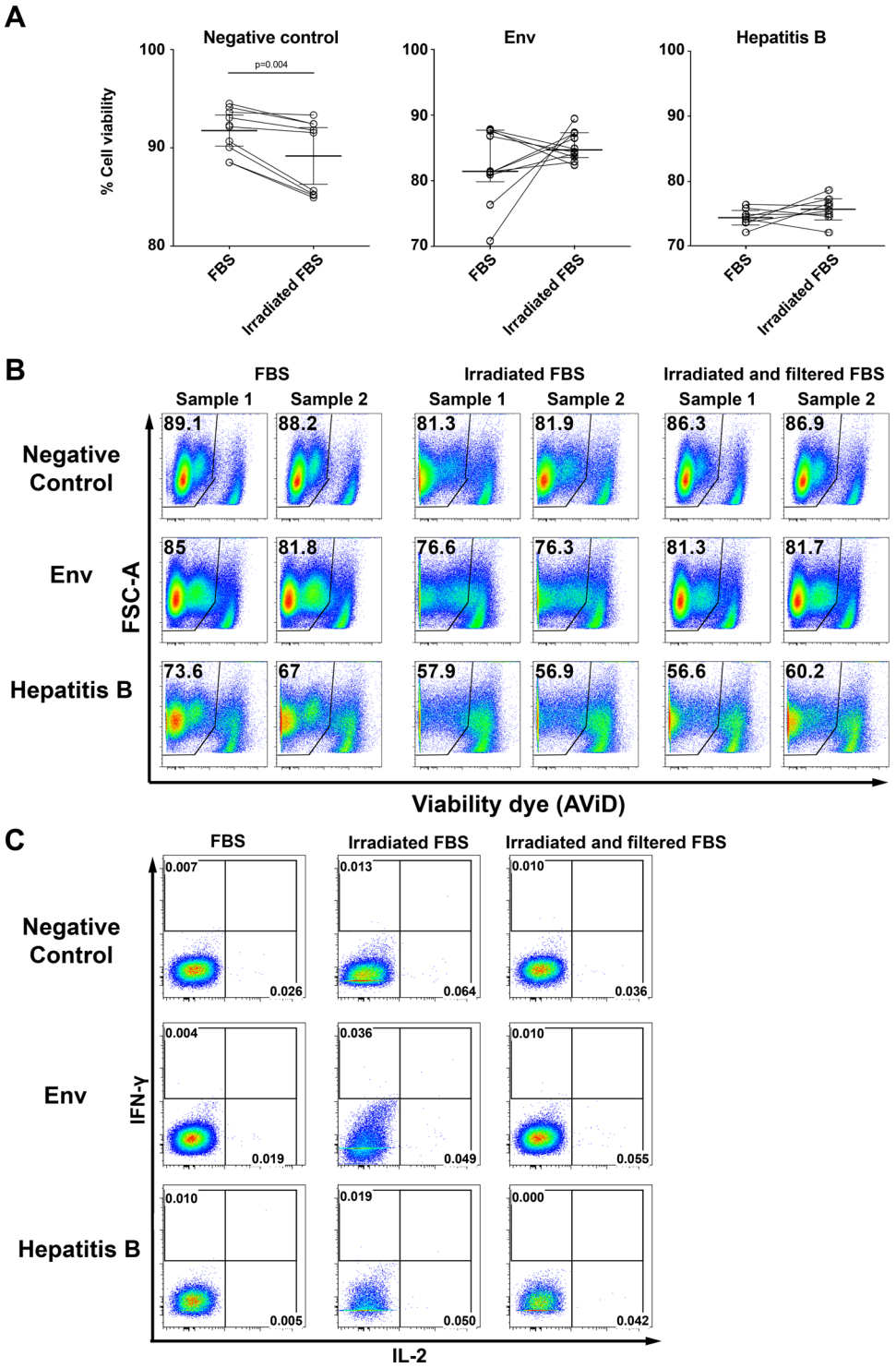

FIGURE 1 Irradiation of FBS causes toxicity in the ICS assay. (A) Scatterplots showing the median cell viability plots as measured by the uptake of the live/dead dye (AViD) for the negative control, envelope and Hepatitis B stimulated conditions after cryopreserved cells were thawed, plated in duplicate, stimulated and run in the ICS assay in non-irradiated and irradiated FBS ($n = 5$). (B) Flow cytometry dot plots from a representative experiment conducted on PBMC from 2 control cohort participants. Forward scatter (FSC-A) and cell viability (measured by the AViD viability dye) measured after stimulation with DMSO or the Clade C 92TH023-Env and Hepatitis B peptide pools in the intracellular cytokine staining assay ($n = 2$) are shown. Three cell culture media (RPMI supplemented with 10% FBS) were tested- standard non-irradiated FBS, irradiated FBS of the same lot or irradiated FBS that had been filtered to remove particulates in an attempt to improve cell viability. (C) Flow cytometry dot plots from a representative experiment conducted on PBMC from a single donor, using non-filtered and filtered irradiated FBS after stimulation in the ICS, showing the observed aggregates in the IFN-g channel, which may potentially confound the ability to detect antigen-specific cytokine producing cells

Zealand, where Bovine Spongiform Encephalopathy was not a concern) into South Africa and were fortunately granted the exemption due to our work on clinical trials. Going forward, we used the same carefully tested lots of non-irradiated FBS in all our subsequent assays in both the Seattle and Cape Town laboratories.

3.2 | First concordance study

Having successfully sourced acceptable non-irradiated FBS, we then designed an ICS concordance study. One primary readout of our ICS assay is the response rate—the proportion of participants who exhibit a positive CD4+ or CD8+ T-cell response to a vaccine antigen, as determined by the Fisher's Exact statistical test.[28] As described in the *Materials and Methods*, we preset 2 co-primary objectives for the concordance study: (1) to assess equivalence of the proportions of positive responders between the 2 assays and (2) to assess concordance of positivity calls on the same set of specimens between the 2 assays. The first co-primary objective was assessed by determining the difference in response rate between the 2 laboratories for CD4+ or CD8+ T-cell responses to the CMV pp65 or HIV-1 Gag antigens; this testing did not distinguish whether the same samples were called positive or negative in the 2 laboratories. The second co-primary objective was assessed by determining the percentage of concordant calls on the same samples (see *Materials and Methods*).

Vials of cryopreserved PBMC collected from the same participants in several HIV vaccine trials were shipped to each laboratory for ICS testing. We found that the CD4+ T-cell response rate to the CMV peptide pool measured in the Seattle laboratory was 61.7% and in the Cape Town laboratory was 57.7%. CD8+ T-cell response rates to CMV were also very similar in the 2 laboratories: 68.4% in Seattle and 67.9% in Cape Town (Table 1). CD4+ T-cell response rates to the HIV-1 Gag peptide pool were not as close in the 2 laboratories: 55.7% in Seattle and 43.0% in Cape Town. CD8+ T-cell response rates, however, were more similar at 27.2% in Seattle and 25.9% in Cape Town. Based on these results, both the CD4+ and CD8+ T cell CMV-specific responses as well as the CD8+ Gag-specific responses passed the acceptance criteria for the 2 co-primary objectives. The CD4+ Gag-specific responses, however, failed the first co-primary acceptance criterion since the upper bound of the 95% CI was 21.0% for the difference in response rates (Table 1). CD4+ Gag-specific responses passed the second co-primary objective with 95% CI lower bound of 72.8%

In addition, the background-subtracted responses (% of T cells expressing cytokine in the stimulated condition minus that in the unstimulated condition) from the 2 laboratories were in high degree of agreement with CCC > 0.9 and 95% LCI > 0.8 for all stimulation conditions (Figure 2). All the parameters assessed passed the criterion set out with a high degree of precision and accuracy (Table 2). It was clear from these analyses that the magnitude of the responses was in close agreement in both laboratories; however, the discordance in the positivity calls needed further investigation.

TABLE 1 Summary of the response rates, response rate differences, and the concordance rates for assays run in the Seattle and Cape Town laboratories

Antigen	T-cell	Response rates				Difference		Concordance	
		Seattle (*n*)	95% CI	Cape Town (*n*)	95% CI	Rate (*n*)	95% CI	Rate (*n*)	95% CI
CMV	CD4+	61.7% (71/115)	(52.6%, 70.1%)	57.7% (64/111)	(48.4%, 66.4%)	6.4% (7/110)	(0.8%, 11.7%)	91.8% (101/110)	(85.2%, 95.6%)
	CD8+	68.4% (78/114)	(59.4%, 76.2%)	67.9% (74/109)	(58.6%, 75.9%)	1.8% (2/109)	(−2.9%, 6.5%)	94.5% (103/109)	(88.5%, 97.5%)
Gag	CD4+	55.7% (64/115)	(46.5%, 64.4%)	43.0% (49/114)	(34.3%, 52.2%)	13.5% (15/111)	(5.5%, **21.0%**)	68.1% (90/111)	(72.8%, 87.3%)
	CD8+	27.2% (31/114)	(19.9%, 36.0%)	25.9% (29/112)	(18.7%, 34.77%)	1.8% (2/111)	(−2.1%, 5.6%)	96.4% (107/111)	(91.1%, 98.6%)

The bolding is to show the result that did not pass the pre-defined criterion.

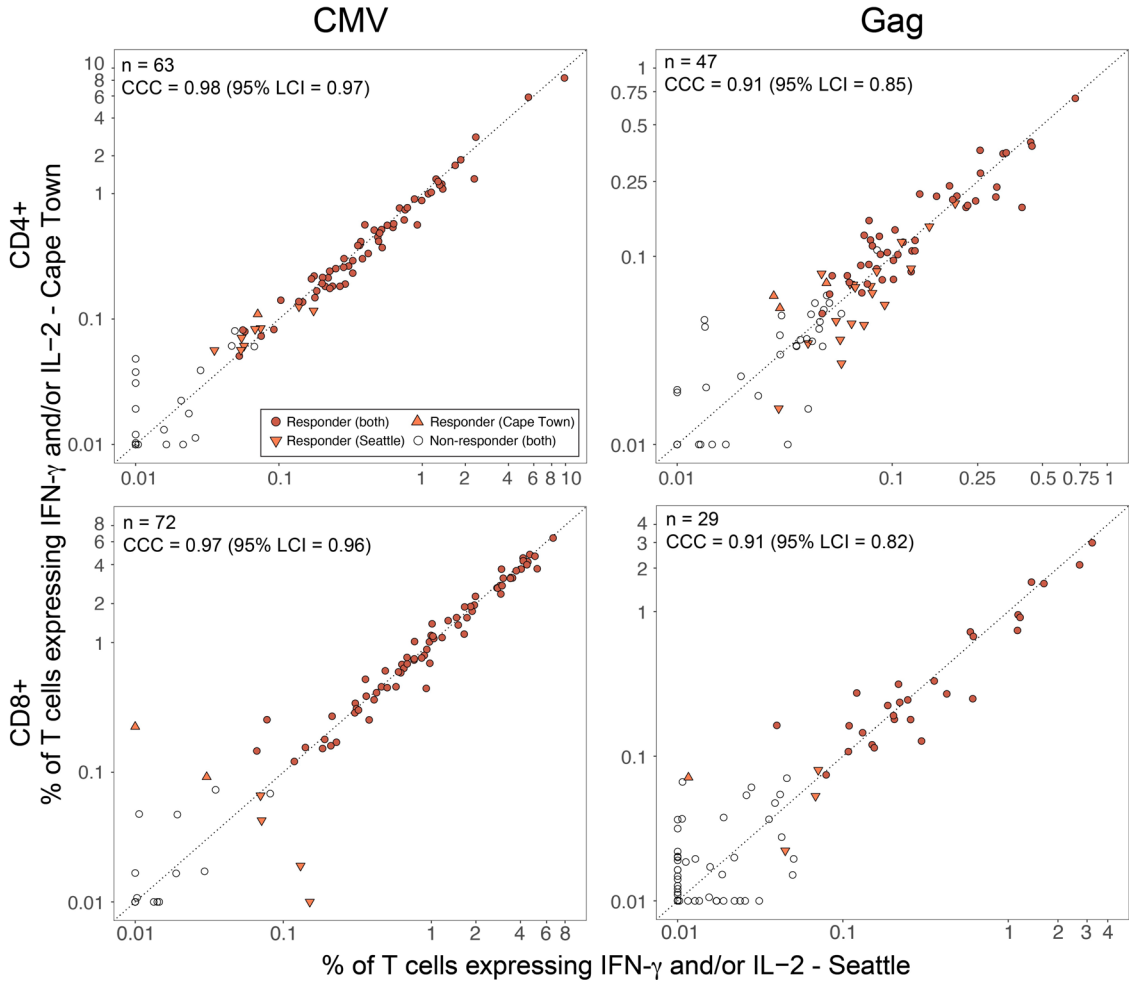

FIGURE 2 Concordance of T-cell response magnitudes achieved in the first concordance study. Scatterplots showing the background-subtracted proportion of either CD4+ or CD8+ T cells expressing IFN-γ and/or IL-2 after stimulation with a CMV or an HIV-Gag (clade B vaccine-matched) peptide pool. Cryopreserved PBMC collected from the same vaccine trial participants at the same time points were tested in the ICS assay in either Seattle (*x*-axis) and Cape Town (*y*-axis) laboratories. Points are colored according to response call—positive response measured in both laboratories (filled circle), positive response measured only in Cape Town (vertical triangle), positive response measured only in Seattle (inverted triangle), or negative response in both laboratories (open circle). The concordance correlation coefficient (CCC), calculated for positive responders, is shown in the upper-left hand corner of each graph, along with the 95% lower confidence interval (LCI). The number of positive responders used to calculate the CCC is also shown in the upper left hand corner of the graph

TABLE 2 Concordance of net responses for positive responders only

Peptide pool	T-cell subset	Cytokine	*N*	CCC responders (95% LCI)	Accuracy (95% LCI)	Precision (95% LCI)
CMV	CD4+	IFN-γ and/or IL-2	63	0.98 (0.97)	1 (1.0)	0.98 (0.97)
	CD8+	IFN-γ and/or IL-2	72	0.97 (0.96)	1 (1.0)	0.98 (0.96)
Gag	CD4+	IFN-γ and/or IL-2	47	0.91 (0.85)	0.99 (0.99)	0.92 (0.86)
	CD8+	IFN-γ and/or IL-2	29	0.91 (0.82)	0.91 (0.82)	0.91 (0.82)

3.3 | Cell loss attributed to technique

The Fisher's exact test compares the number of cytokine-expressing cells among the total number of CD4+ or CD8+ T cells in the stimulated versus unstimulated condition to determine positivity. Therefore, because the sensitivity of the Fisher's exact test is influenced by the number of T cells, we investigated potential differences between the 2 laboratories and the impact of the number of T cells

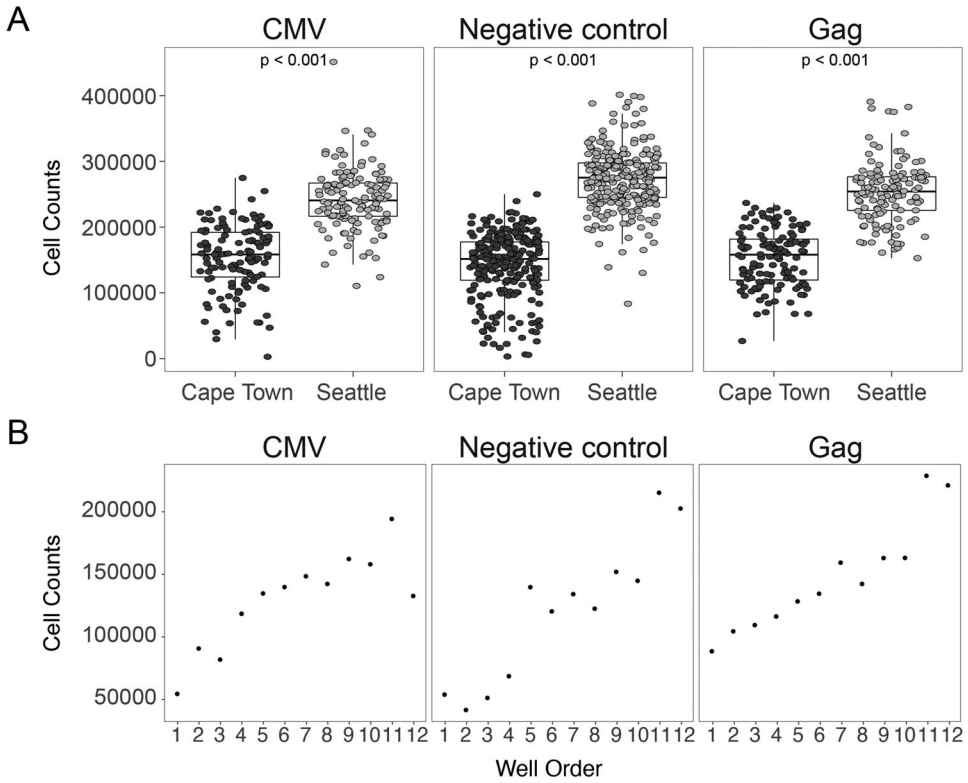

FIGURE 3 Fewer lymphocytes collected at the end of the ICS assay in the Cape Town laboratory. (A) Lymphocyte cell counts as measured on the flow cytometer in the Cape Town and Seattle laboratories. Each dot represents the lymphocyte count from 1 stimulation condition (well) for the data shown in Figure 2. The Wilcoxon signed rank test was used to compare data from the 2 laboratories. (B) Number of lymphocytes measured on the flow cytometer for each well in 1 row of a 96-well plate (representative data from 1 experiment). *X*-axis shows the well number—each well contained PBMC from a different vaccine trial participant and different rows were stimulated with either CMV (left panel), Gag (middle panel), or negative control (DMSO, right panel)

on the positivity calls. Figure 3(A) shows the number of total lymphocytes collected on the cytometer for the CMV, HIV-1 Gag, and negative control conditions in Seattle and Cape Town. Although the same number of cells are plated for each condition in both laboratories, cell counts collected in Cape Town were significantly lower than in Seattle, indicating that cells were being disproportionately lost during the assay.

We investigated several possible explanations, including incorrect cell counting, increased cell death, and cell loss during sample acquisition on the flow cytometer in Cape Town; however, none of these revealed the problem. We then examined the cell counts by well (the ICS assay is conducted in 96-well plates to increase throughput) and found a surprising pattern. The number of cells collected by the cytometer were low in the first well (well #1) and then increased as it progressed to subsequent wells across the plate (through well #12). Figure 3(B) shows a consistent pattern for a representative sample and demonstrates the pattern was not stimulation dependent. Further investigations revealed that the numerous cell washing steps performed on day 3 of the assay during labeling of the cells with fluorescent antibodies were affecting the cell recovery in this consistent pattern. The ICS wash steps are performed by adding wash buffer, mixing with the cells, and centrifuging the plate. The supernatant is then removed by swiftly inverting the plate- the wash buffer is ejected and the cell pellet remains in the well. Research technologists in Cape Town were inverting the plate in a manner that involved an arc-like motion of the forearm, causing increased loss of cells from wells at the top of the plate, due to the increased force. When the research technologists in Cape Town were retrained to invert the plates by rotating only the wrist as was done by the research technicians in Seattle a consistent and higher number of cells was collected in all wells across the plates (data not shown).

3.4 | Second concordance study

A second ICS concordance study was then performed to determine whether the standardization of cell counts between the laboratories would improve the concordance. The assay was performed on PBMC samples from 136 vaccine trial participants in both laboratories. Table 3 shows the response rates in the Seattle and Cape Town laboratories for CD4+ and CD8+ T-cell responses to the CMV and Gag peptide pools. The 95% CI of the difference in the response rates between the laboratories all fell within the (−15%, 15%) interval, thereby passing the acceptance criteria for the first co-primary objective. In addition, the acceptance criteria for the second co-primary objective to assess the concordance of the positivity calls on the same samples in the 2

TABLE 3 Summary of the response rates, response rate differences, and the concordance rates for the second round of assays run in the Seattle and Cape Town laboratories

Antigen	T-cell	Response rate				Difference		Concordance	
		Seattle (*n*)	95% CI	Cape Town (*n*)	95% CI	Rate (*n*)	95% CI	Rate (*n*)	95% CI
CMV	CD4+	59.6% (81/136)	(51.2%, 67.4%)	59.6% (81/136)	(51.2%, 67.4%)	0.0% (0/136)	(−4.3%, 4.3%)	94.1% (128/136)	(88.8%, 97.0%)
	CD8+	63% (85/135)	(54.6%, 70.6%)	62.7% (84/135)	(54.3%, 70.4%)	0.7% (1/134)	(−2.1%, 3.6%)	97.8% (131/134)	(93.6%, 99.2%)
Gag	CD4+	52.9% (72/136)	(44.6%, 61.1%)	55.1% (75/136)	(46.8%, 63.2%)	2.2% (3/136)	(−4.5%, 8.8%)	84.6% (115/136)	(77.5%, 89.7%)
	CD8+	20.7% (28/135)	(14.8%, 28.3%)	20.9% (28/134)	(14.9%, 28.5%)	0.0% (0/134)	(−4.8%, 4.8%)	92.5% (115/136)	(86.8%, 95.9%)

laboratories was also met—the concordance rates were all above 84% and the lower bound of 95% CI was greater than 70% (Table 3).

Figure 4 shows scatterplots of the magnitudes of responses measured in the Seattle and Cape Town laboratories. CD4+ and CD8+ T-cell responses to the CMV peptide pool passed the pre-specified criteria: the 95% lower CI of the CCCs for CD4+ was 0.90 and for CD8+ was 0.93 (Table 4). CD4+ T-cell responses to Gag were also highly concordant, with a CCC of 0.91 and 95% lower CI of 0.86. CD8+ T-cell responses to Gag had a high CCC at 0.82; however, the 95% lower CI was 0.62, below the prespecified limit of 0.75. On closer inspection, the CD8+ T-cell response rates to the Gag peptide pool were only around 20%, resulting in the CCC being calculated using responses from only 23 data points and leading to a much wider CI. Based on this result, CD8+ T-cell responses to HIV antigens should be further evaluated in the future, with a pre-requisite number of positive responders to provide sufficient precision in the estimate of CCC.

3.5 | Maintenance of on-going concordance

Although concordance was achieved between the laboratories, our initial FBS testing in combination with our first 2 concordance studies demonstrated that minor differences in reagents and operator technique between the Seattle and Cape Town laboratories could result in divergent measurement of T-cell responses to vaccination in the 2 laboratories. Despite shared SOPs, careful tracking of control samples with each assay batch and excellent quality assurance oversight, it was still possible that divergent practices could arise in the laboratories. We therefore began a program to monitor concordance on an on-going basis by performing more frequent smaller sets of concordance testing between the laboratories that could be informally compared to identify potential problems. Upon testing a minimum of 120 samples in both laboratories, a formal concordance analysis would be performed to ensure all criteria were met.

Between 2018 and 2020, the Seattle and Cape Town laboratories performed on-going concordance testing using approximately 30 samples in each study. In these smaller studies, informal analyses of response rates and magnitudes and cell count comparisons across laboratories were performed. These smaller studies showed no trends of concern (data not shown), and that response magnitudes and rates were comparable between the laboratories.

When 134 samples in total had been assayed, a formal concordance analysis was performed. The difference in the response rates as well as the overall proportion of concordant calls passed the 2 co-primary concordance criteria for both CD4+ and CD8+ T-cell responses to CMV and to the HIV antigens tested (Table 5). The CCC and its 95% CI were then calculated to assess the agreement between the net responses (Figure 5). The results for the formal CCC analyses for responders are summarized in Table 6. All the parameters assessed passed the preset criterion. In addition, the cell counts between the 2 laboratories were similar, with cell counts for the CMV stimulations slightly higher in the Cape Town laboratory on this round of testing (Figure 6(A)).

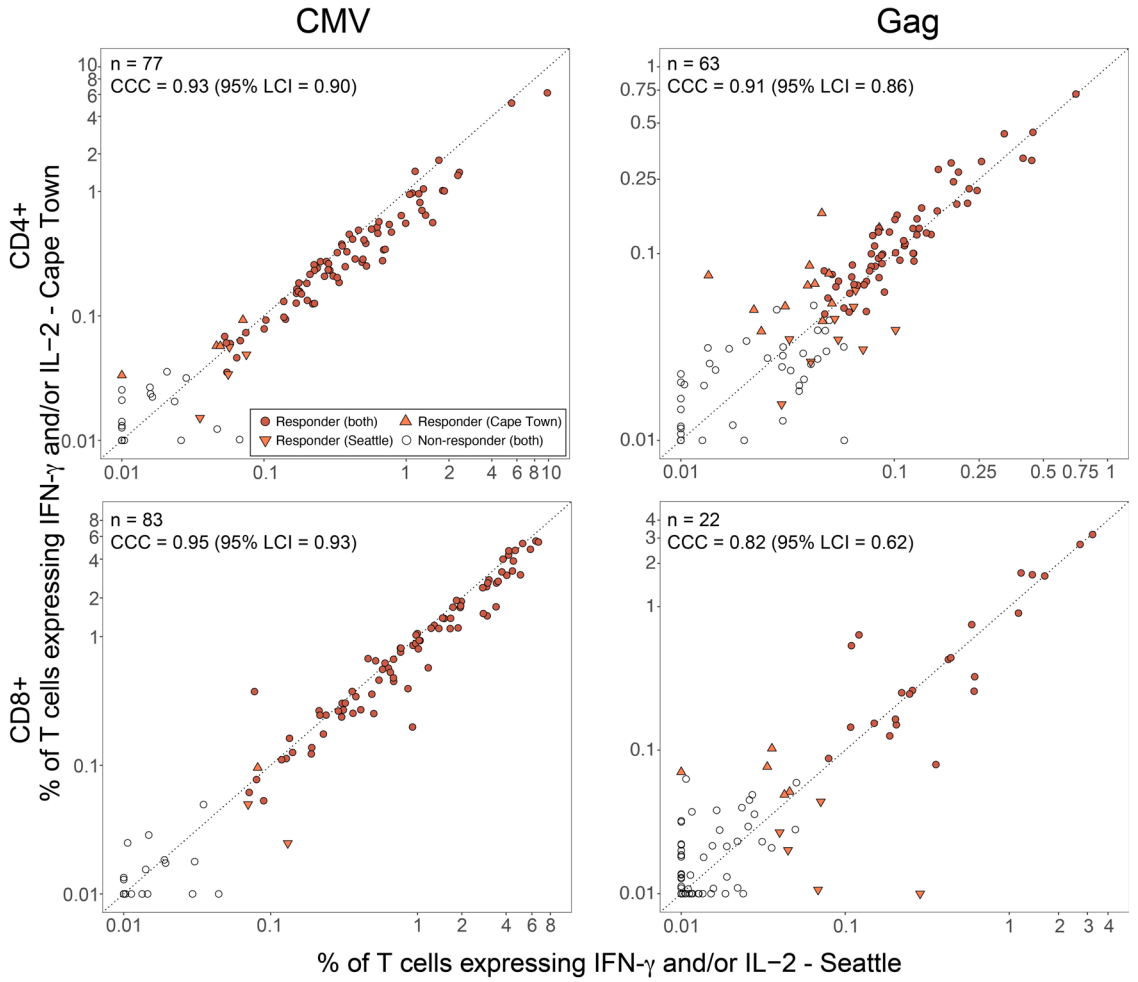

FIGURE 4 Concordance of T-cell response magnitudes achieved after correction of supernatant removal technique. Scatterplots showing the background-subtracted proportion of either CD4+ or CD8+ T cells expressing IFN-γ and/or IL-2 after stimulation with a CMV or an HIV-1 Gag (clade B vaccine-matched) peptide pool. Cryopreserved PBMC collected from the same vaccine trial participants at the same time points were tested in the ICS assay in either Seattle (*x*-axis) and Cape Town (*y*-axis) laboratories. Points are colored according to response call—positive response measured in both laboratories (filled circle), positive response measured only in Cape Town (vertical triangle), positive response measured only in Seattle (inverted triangle), or negative response in both laboratories (open circle). The concordance correlation coefficient (CCC), calculated for positive responders, is shown in the upper-left hand corner of each graph, along with the 95% lower confidence interval (LCI). The number of positive responders used to calculate the CCC is also shown in the upper left hand corner of the graph. Note, the 95% LCI for CD8+ T-cell responses to Gag did not pass the prespecified criteria at 0.62 but $n = 23$ was very low for calculating the CCC

An exploratory analysis was also performed to look at background response levels in the negative control stimulation conditions across the laboratories. The median background for CD4+ T-cell responses was 0.044% in Cape Town and 0.033% in Seattle (Figure 6(B)). Although this difference was statistically significant, the magnitude of the difference had little biologic relevance and did not impact assay concordance, so was not concerning (Figure 6(B)). CD8+ T-cell background response median magnitudes were extremely low and very similar as well—0.0095% in Cape Town and 0.0064% in Seattle (Figure 6(B)).

As part of the selection of samples for the last set of on-going concordance testing, PBMC collected from a trial that had previously been assayed twice in the Seattle laboratory were tested. The Seattle laboratory had performed the ICS assay in 2015 using a 16-color panel and again in 2017 using an updated 17-color ICS panel on these samples. We compared the CD4+ T-cell expression of IFN-γ and/or IL-2 to Env from the assays conducted over this 5-year timeframe. We found very similar response rates: 64.3% in Seattle in 2015, 70.9% in Seattle in 2017, and 66.7% in Cape Town in 2020 (Figure 6(C)). The response magnitudes across the 3 datasets were not significantly different (all $p > 0.10$) with CD4+ T-cell response medians of 0.174%, 0.170%, and 0.184%, respectively (Figure 6(C)).

4 | DISCUSSION

A complex functional assay such as ICS poses many challenges when considering standardization across laboratories. Although other less complex cellular immune response assays such as the IFN-γ

TABLE 4 Concordance of net responses for positive responders only for the second round of assays

Peptide pool	T-cell subset	Cytokine	*N*	CCC responders (95% LCI)	Accuracy (95% LCI)	Precision (95% LCI)
CMV	CD4+	IFN-γ and/or IL-2	77	0.93 (0.90)	0.97 (0.94)	0.97 (0.95)
	CD8+	IFN-γ and/or IL-2	83	0.95 (0.93)	0.99 (0.98)	0.96 (0.94)
Gag	CD4+	IFN-γ and/or IL-2	63	0.91 (0.86)	0.99 (0.96)	0.92 (0.87)
	CD8+	IFN-γ and/or IL-2	22	0.82 (**0.62**)	1 (0.0)	0.82 (0.61)

The bolding is to show the result that did not pass the pre-defined criterion.

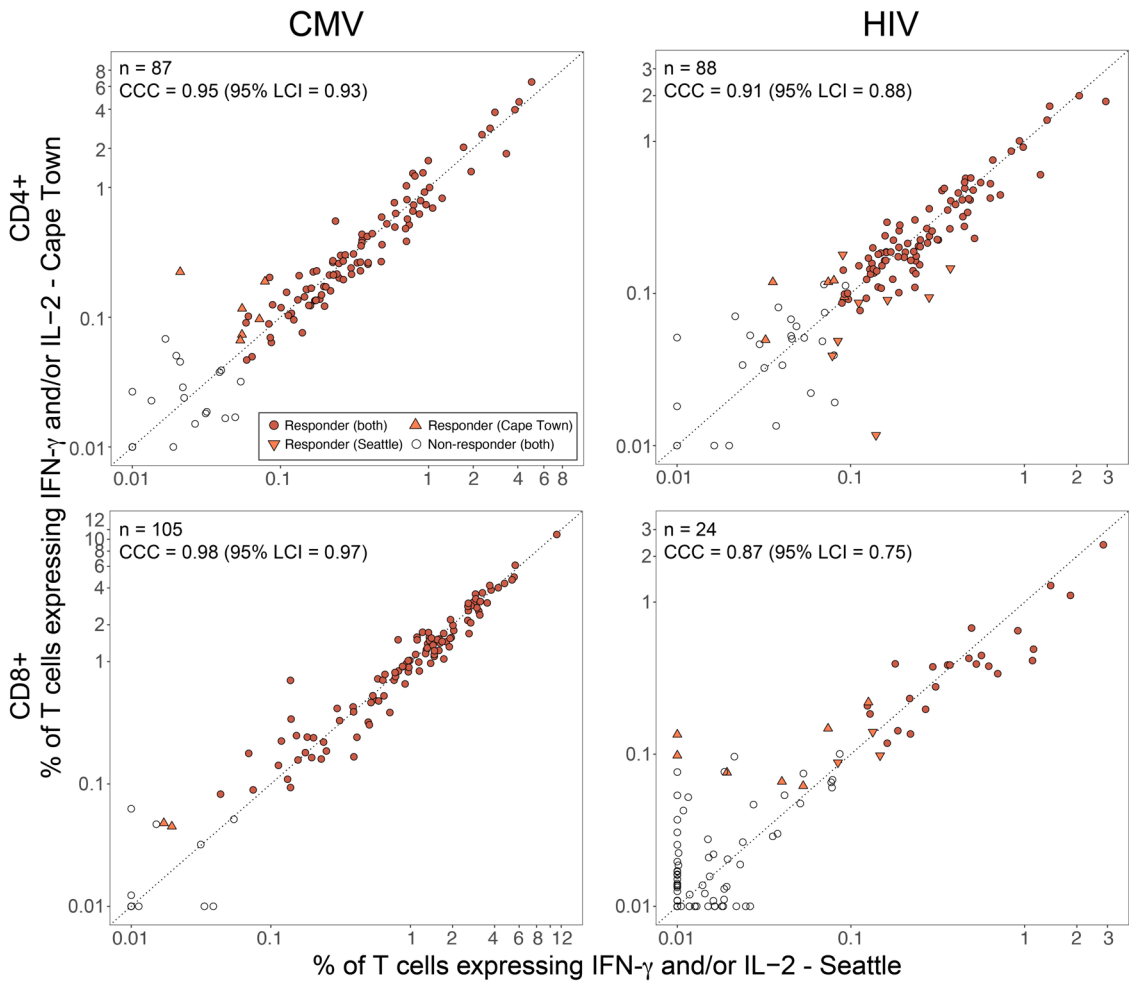

FIGURE 5 Concordance of T-cell response magnitudes achieved in the third concordance study. Scatterplots showing the background-subtracted proportion of either CD4+ or CD8+ T cells expressing IFN-γ and/or IL-2 after stimulation with a CMV or the sum of the vaccine matched antigen (ANY HIV) peptide pool. Cryopreserved PBMC collected from the same vaccine trial participants at the same time points were tested in the ICS assay in either Seattle (*x*-axis) and Cape Town (*y*-axis) laboratories. Points are colored according to response call—positive response measured in both laboratories (filled circle), positive response measured only in Cape Town (vertical triangle), positive response measured only in Seattle (inverted triangle), or negative response in both laboratories (open circle). The concordance correlation coefficient (CCC), calculated for positive responders, is shown in the upper-left hand corner of each graph, along with the 95% lower confidence interval (LCI). The number of positive responders used to calculate the CCC is shown in the upper left hand corner of the graph

ELISpot[29] and stimulated whole blood/PBMC ELISA assays may be more amenable to implementation across laboratories, the ICS assay uniquely provides multiparameter single-cell characterization, yielding much more information about the character of the immune response.

In these concordance studies, antigen-specific CD4+ or CD8+ T-cell expression of IFN-γ and/or IL-2 was used as the primary readout. These 2 cytokines are central to T-cell responses to vaccination—IFN-γ is characteristic of Th1-type immune responses and enhances killing

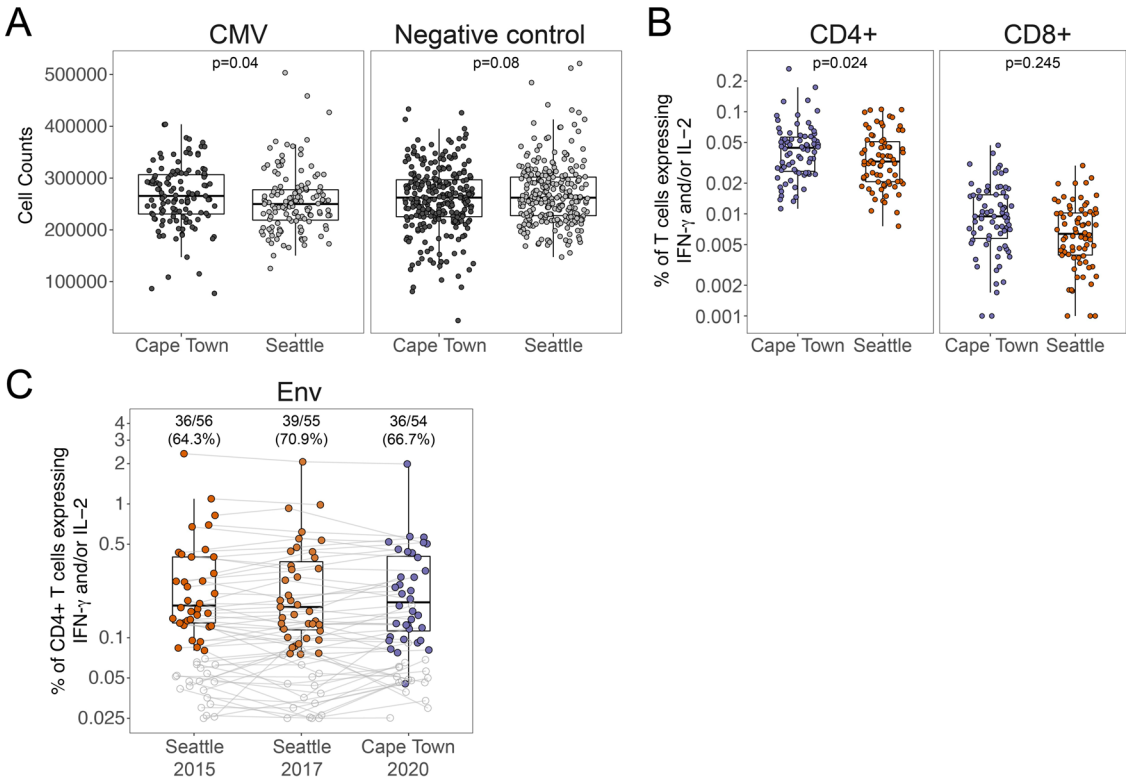

FIGURE 6 ICS assay concordance is maintained over a 5-year period. (A) Lymphocyte cell counts as measured in the Cape Town and Seattle laboratories. Each dot represents the lymphocyte count from 1 stimulation condition for the data shown in Figure 5 (the present figure includes the negative control conditions, resulting in a larger *n*). (B) Proportion of either CD4+ or CD8+ T cells expressing IFN-γ and/or IL-2 in the negative control (DMSO-stimulated) wells in Seattle or Cape Town for one of the vaccine trials tested ($n = 72$). (C) Proportion of CD4+ T cells expressing IFN-γ and/or IL-2 in the vaccine matched Env peptide pool stimulated wells in Seattle or Cape Town for one of the vaccine trials tested ($n = 57$). The Wilcoxon signed rank test was used to compare datasets from the 2 laboratories

of HIV-infected cells; IL-2 is an important auto and paracrine growth factor for T cells. Measuring cells that express one or both of these cytokines gives a good indication of the vaccine-induced T-cell immune response, but many more markers are relevant for determining the memory subsets and phenotype of the cellular response. The HVTN originally validated expression of IFN-γ and/or IL-2 in the ICS assay using an 8-color panel[4] but now employs ICS assays that profile up to 28 parameters on each cell. As we develop new panels incorporating new markers (e.g., Refs. [5-7,30,31]), they are cross-validated to the previous panels using IFN-γ and/or IL-2 as the primary readout. We have yet to formally evaluate the concordance of other markers, but these markers could be of high interest for other laboratories. Of note, in our experience, some markers are more consistent and easier to standardize, while others can be quite variable and not appropriate to validate.

For flow cytometric assays, a key issue to consider is standardization across instruments and therefore we installed similar instruments with nearly identical optical configurations in the Seattle and Cape Town laboratories (Table S2). We carefully characterized each instrument and followed published protocols for standardization.[16] To avoid drifting of settings over time, standardization is repeated on a regular basis. In addition, for any flow cytometric assay the first steps in the data analysis of the raw data (referred to as gating the populations of interest) can lead to divergent results.[12,32] Therefore, gating is carefully standardized and the same gating template is shared between the laboratories (and between different instruments in either laboratory). This is only possible because of the instrument standardization that results in consistent fluorescent intensities for each marker.

The performance of the assay was standardized with an SOP shared between laboratories. Even with this detailed SOP, there were some subtle operator-dependent procedural aspects that differed between the laboratories, as exemplified by the removal of the supernatants resulting in lower cell counts described here. To maintain standard procedures over time, we conduct a periodic exchange of research technicians between laboratories and regularly conduct much more frequent individual level communications between technical staff.

To achieve concordance, critical reagents were identified and procedures established for either providing the same lot or for assessing comparability of different lots. The effect of irradiation of FBS noted here is a special concern and highlights unexpected challenges in working in different countries with different regulations. Our approach to filtering the FBS was empirical and alleviated some, but not all, of the viability issues we observed. Fortunately, we found a way to import non-irradiated FBS from select countries, but this may not be an option for other laboratories so additional mitigation measures may need to be considered.

TABLE 5 Summary of the response rate differences and the concordance rates for the ongoing concordance after implementation of measures to standardize assay performance

Antigen	T-cell	Response rate				Difference		Concordance	
		Seattle	95% CI	Cape Town	95% CI	Rate	95% CI	Rate	95% CI
ANY HIV	CD4+	75.8% (94/124)	(67.6%, 82.5%)	74.2% (92/124)	(65.8%, 81.1%)	1.6% (2/124)	(−3.6%, 6.7%)	91.9% (114/124)	(85.8%, 95.6%)
	CD8+	20.1% (27/134)	(14.2%, 27.7%)	23.1% (31/134)	(16.8%, 31.0%)	3.0% (4/134)	(−7.7%, 1.8%)	92.5% (124/134)	(86.8%, 95.9%)
CMV	CD4+	77.0% (87/113)	(68.4%, 83.8%)	82.3% (93/113)	(74.2%, 88.2%)	5.3% (6/113)	(−9.5%, −0.8%)	94.7% (107/113)	(88.9%, 97.5%)
	CD8+	85.4% (105/123)	(78.1%, 90.5%)	87.0% (107/123)	(79.9%, 91.8%)	1.6% (2/123)	(−4.3%, 1.1%)	98.4% (121/123)	(94.3%, 99.6%)

Our approach to concordance is very stringent to allow data to be considered comparable between laboratories. Both the Seattle and Cape Town HVTN laboratories also participate in an NIH-funded program to monitor ICS performance across multiple laboratories referred to as EQAPOL (External Quality Assurance Program Oversight Laboratory). Although not intended to establish concordance, it is a useful program to track performance over time and to identify potential issues of concern in individual laboratories.

One of the essential needs for concordance testing is access to a large repository of high-quality PBMC samples with responses to the antigens of interest, most appropriately the antigens assessed in vaccine or infection studies. The isolation and processing of PBMC to ensure high cell recovery and viability as well as careful cold chain management of these samples is critical to maintenance of cell integrity and compromise of cell quality impacts immunoassay outcomes. The HVTN LC has a well-established PBMC QA program to monitor and ensure PBMC sample integrity. Our power calculations indicated a need for over 100 samples with the majority of these required to have a positive response to the antigens of interest. It is most appropriate to test samples that span the range of response magnitudes expected from the clinical samples tested within the laboratories. In a multiparametric assay such as ICS, as discussed above, there are also many different types of responses that can be assessed at the single-cell level. We focused on the combination of IFN-γ and/or IL-2 expression from CD4+ and CD8+ T cells, and even in that case, for some specific antigen stimulations, there were too few positive responses in our samples to assess concordance. When considering other cytokines and other cell types, it will be more difficult to select samples with enough positive responders. Thus, full concordance testing for all functions and cell types of interest may not be feasible in some cases.

In sum, the studies presented here illustrate the complexity of achieving cross-laboratory concordance of a functional multiday assay that requires primary human cells. Despite the challenges in standardization of critical reagents and the fine detail of laboratory assay techniques, we demonstrate that on-going concordance of the ICS assay over a period of more than 5 years in 2 laboratories on different continents is achievable. In particular, our efforts highlight that ongoing investment in laboratory infrastructure and research staff on the African continent yields high returns—we have significantly increased the HVTN's capacity to characterize cellular immune responses in vaccine trials, accelerating our path toward an effective HIV vaccine.

AUTHORSHIP

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TABLE 6 Concordance of net responses for positive responders only

Peptide pool	T-cell subset	Cytokine	*N*	CCC responders (95% LCI)	Accuracy (95% LCI)	Precision (95% LCI)
ANY HIV	CD4+	IFN-γ and/or IL-2	88	0.91 (0.88)	0.98 (0.97)	0.93 (0.90)
	CD8+	IFN-γ and/or IL-2	24	0.87 (0.75)	0.93 (0.84)	0.93 (0.84)
CMV	CD4+	IFN-γ and/or IL-2	88	0.95 (0.93)	0.99 (0.98)	0.96 (0.94)
	CD8+	IFN-γ and/or IL-2	106	0.98 (0.97)	1.0 (0.99)	0.98 (0.97)

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DISCLOSURE

The authors declare no potential conflicts of interest exist.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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