Large-Scale Prospective T Cell Function Assays in Shipped, Unfrozen Blood Samples: Experiences from the Multicenter TRIGR Trial

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Broad consensus assigns T lymphocytes fundamental roles in inflammatory, infectious, and autoimmune diseases. However, clinical investigations have lacked fully characterized and validated procedures, equivalent to those of widely practiced biochemical tests with established clinical roles, for measuring core T cell functions. The Trial to Reduce Insulin-dependent diabetes mellitus in the Genetically at Risk (TRIGR) type 1 diabetes prevention trial used consecutive measurements of T cell proliferative responses in prospectively collected fresh heparinized blood samples shipped by courier within North America. In this article, we report on the quality control implications of this simple and pragmatic shipping practice and the interpretation of positive- and negative-control analytes in our assay. We used polyclonal and postvaccination responses in 4,919 samples to analyze the development of T cell immunocompetence. We have found that the vast majority of the samples were viable up to 3 days from the blood draw, yet meaningful responses were found in a proportion of those with longer travel times. Furthermore, the shipping time of uncooled samples significantly decreased both the viabilities of the samples and the unstimulated cell counts in the viable samples. Also, subject age was significantly associated with the number of unstimulated cells and T cell proliferation to ping time of uncooled samples significantly decreased both the viabilities of the samples and the unstimulated cell counts in the viable samples. Also, subject age was significantly associated with the number of unstimulated cells and T cell proliferation to positive activators. Finally, we observed a pattern of statistically significant increases in T cell responses to tetanus toxin around the timing of infant vaccinations. This assay platform and shipping protocol satisfy the criteria for robust and reproducible long-term measurements of human T cell function, comparable to those of established blood biochemical tests. We present a stable technology for prospective disease-relevant T cell analysis in immunological diseases, vaccination medicine, and measurement of herd immunity.

T cell function lies at the core of host resistance. However, the development of assays that reliably assess T cell immunocompetence in humans has been a challenge with few successful attempts at long-term validation efforts typical for biochemical markers of health- or disease-associated conditions (1, 2). Exceptions include flow cytometric measurements of T cell phenotypes and subset distributions with rapid evolution of the flow cytometric procedures developed to address pressing clinical needs such as the HIV epidemic (3). However, until now, there has been a dearth of widely applied well-understood T cell function assays, which would allow the evaluation of the intact or diseased immune system in multicenter clinical trials. Our analyses characterize T cell function assays as robust scalable procedures that compare well to the biochemical tools in clinical use.

While many T cell in vitro assays have been described (1, 4), invariably based on relatively small numbers of donor blood samples, they have not reached routine clinical practice even in immunological disorders. One challenge is the common necessity to ship samples to a central laboratory specialized in such assays with the need to sustain sample viability and functionality during the shipping process. Considerable efforts have been made in NIH TrialNet studies, where the single major recommendation might best be summarized as the avoidance of cryopreservation during sample shipment (1). This sets T cell studies apart from most biochemical assays of blood samples where cryopreservation is routine and practical. For our assay development, all samples were shipped via overnight courier at room temperature, using standard heparinized collection tubes and small polystyrene shipping boxes as a simple and, in fact, the sole expedient that worked surprisingly well for as many as several days of shipping.

The T cell assay developed for the Trial to Reduce Insulin-dependent Diabetes in the Genetically at Risk (TRIGR) evolved from previous work in mouse models (5, 6) and for smaller-scale studies, which contained five groups of test antigens or “analytes” with different roles: positive controls, negative controls, and test analytes related to cows’ milk and islet and glial cells (4, 7, 8). The present initial data analysis is focused on positive- and negative-control responses, which bracket the full proliferative response range. For the positive controls, two polyclonal activators, phytohemagglutinin (PHA) and anti-CD3, were used to assess expectedly broad proliferative responses. In addition, tetanus toxoid was included in the panel to examine a cognate postvaccination response. The negative controls, to which little or no T cell response was expected, included ovalbumin and actin and no stimulation. Our aims were to evaluate the shipping method efficacy using...
these control analytes and to describe the observed T cell proliferations in this substantial cohort from infancy into childhood.

**MATERIALS AND METHODS**

**Subjects.** The Trial to Reduce Insulin-dependent Diabetes in the Genetically at Risk (TRIGR) is a type 1 diabetes (T1D) primary prevention trial (9), which controls early infant feeding practices. The intervention arm excludes exposure to foreign weaning proteins, including cows’ milk, in the first 6 to 8 months of life. Subjects with T1D in a first-degree relative were selected for T1D risk-associated HLA genotypes (n = 2,159) (9, 10) and are monitored for emergence and progression of T1D-associated autoreactivity in 77 centers on three continents. Data on childhood immunizations and developmental and lifestyle milestones are collected prospectively, and 15 blood samples were scheduled for each subject from birth until 10 years of age or diagnosis of T1D. The trial-wide protocol compliance since the first recruitment on 1 May 2002 is currently 94% in the 2,159 eligible newborns (11). The trial participants are now 6 to 10 years old (mean and median age, 8.4 years). TRIGR participants enrolled and followed in Canada and the United States were analyzed in this study, constituting the first large (n = 865) birth cohort to emerge and progression of T1D-associated autoreactivity in 77 centers on three continents. Data on childhood immunizations and developmental and lifestyle milestones are collected prospectively, and 15 blood samples were scheduled for each subject from birth until 10 years of age or diagnosis of T1D. The trial-wide protocol compliance since the first recruitment on 1 May 2002 is currently 94% in the 2,159 eligible newborns (11). The trial participants are now 6 to 10 years old (mean and median age, 8.4 years). TRIGR participants enrolled and followed in Canada and the United States were analyzed in this study, constituting the first large (n = 865) birth cohort to ambulatory infants and children.

**Sample collection and preparation.** Heparinized whole-blood samples were routinely collected in parallel to serum samples during scheduled follow-up visits according to the study protocol (9). The total volume of blood to be drawn increased with age, from 3 ml at 3, 6, and 9 months of age to 4 ml by 12 months and to 5 ml by 18 and 24 months, with annual sampling thereafter. The subjects are followed until a diagnosis of T1D has been made or the last enrolled subject reaches 10 years of age.

To blind the laboratory, the plastic heparinized sample tubes were identified solely with barcode labels generated online by the TRIGR data management unit, printed, and affixed by the clinical centers for each blood draw. The tube size was taken into consideration so that small volumes were shipped in small-volume tubes. Boxes made from expanded polystyrene were used to ship the samples at room temperature. The box size was also considered, such that the container was small enough to facilitate compact packing of the tube(s). The clinical centers were required to immediately ship blood samples by courier to the T cell laboratory in Toronto.

**Laboratory reagents and methods.** Upon arrival at the laboratory, anonymized samples were excluded from further analysis if there was obvious hemolysis from a visual inspection (darker color and increased opacity). This was the first stage of laboratory quality control (QC). Samples that appeared to have anything less than extreme hemolysis, including questionable amounts of hemolysis, were processed immediately at the time of receipt, by first diluting the blood sample with an equal volume of serum- and protein-free hybridoma medium (catalog no. S2897; Sigma), extensively tested during assay development (5, 6). A 5- to 10-ml layer of the diluted blood was then placed on a Ficoll-Paque gradient in a 5-ml or 15-ml Falcon tube. Following centrifugation (30 min at 2,000 rpm and 20°C), the peripheral blood mononuclear cells (PBMCs) were removed from the interface, washed (10 min at 2,000 rpm) three times, and resuspended in 5 ml of serum-free medium.

To measure cell counts, the second stage of laboratory QC, 50 µl of the PBMC suspension was mixed with 50 µl of trypan blue (0.4%). Viable (unstained) cells were counted manually in a hemocytometer, and samples were adjusted to contain 1.5 × 10^6 viable cells/ml. If a sample did not contain a sufficient number of viable cells to complete the assay, this sample was reported as having failed laboratory QC and was not processed any further.

The T cell cultures used 96-well plates, and in serum-free medium, duplicate analytes (Table 1) were preloaded and frozen (−20°C) until thawed to room temperature for use. Ten units of interleukin-2 (IL-2), previously shown to improve reproducibility and signal in replicate samples without generating spurious signal in the negative-control cultures (12), were added to each well. Then 1.5 × 10^5 PBMCs/well were suspended in 200 µl of serum-free complete medium (catalog no. S2897; Sigma). The assay was incubated at 37°C in 5% CO_2 for 6 days. Eighteen hours before termination, 1 µCi of [3H]thymidine ([3H]TdR) was added. The plates were often stored frozen at this stage for batch processing until harvesting onto fiberglass mats with a robotic cell harvester and scintillation counter for DNA-incorporated isotope that reported isotope content (cpm/individual culture well) to a computer. The assay was tested regularly using control samples from different healthy donors.

**Statistical analysis.** To compare different samples, the stimulation index (SI) for analytes was automatically calculated by dividing the proliferative well response to an analyte by that of cells alone. For the positive polyclonal or cognate control analytes, the expected response was an SI of ≥4 or ≥10 in the case of PHA. Based on experience during assay development, the cutoff responses in negative controls were set to an SI of ≤1.5.

Statistical analyses were performed using SAS software, version 9.2 for Unix (SAS Institute, Inc., Cary, NC). Log_{10} transformation was used for analysis of SIs, as these ratios naturally form a skewed distribution. Non-parametric tests for trend used the Cuzick command written by Rick Wicklin, based on Cuzick’s extension of the Wilcoxon signed-rank test (13). Generalized estimating equations (GEEs) were implemented by proc genmod with the outcome as log_{10}-transformed cell count poststimulation, using an autoregressive correlation structure. Since GEEs require multiple data points per subject, 0.06% of the data points, which came from 29 subjects having only one data point available, were not used. The dataset used in this article, which consisted of results from 865 subjects (4,676 viable samples generating 56,112 replicate proliferation sets for the 6 analytes), was frozen as of 31 October 2012. Because of the staggered recruitment and accrual, the median number of samples per subject was 3, with a range of 1 to 9, and the final target was 15 per subject.

The figures presented show raw data, which include all factors influencing the outcomes, as the reader might observe in practice. In contrast,
the GEE models include adjustment for known and discovered covariates to test for differences.

**RESULTS**

The proliferative responses to each analyte were averaged across the duplicate wells, and the mean responses (cpm) were divided by those in unstimulated cultures (cells alone). This ratio is referred to as the stimulation index (SI), which proved to be a robust measure of in vitro responsiveness to mitogenic as well as antigen- and autoantigen-specific (D. Hadley, R. Cheung, and H.-M. Dosch, unpublished data) activation. The sample viability refers to the binary determination of whether or not a blood sample passed the laboratory QC measures.

**Effect of transit time on samples.** Of the 4,919 samples processed, 95.1% passed the laboratory QC criteria. Most samples were sent on the day of the blood draw by courier (FedEx), resulting in 83.7% of samples arriving at the Toronto laboratory within 2 days of the blood draw. The proportion of samples passing lab QC, which includes PBMC viability, remained high after the third day in transit (94%) and declined to 77% by the fourth day in transit (Fig. 1). As expected, the longer a sample was in transit, the more likely it was to fail the QC procedure. Surprisingly, many late samples could still be processed and generated meaningful reproducible data up to 9 days after blood draw.

Figure 2 demonstrates decreasing median counts (cpm [*H*]TdR, cells only) of sample PBMCs as the transit time increases. This decrease was statistically significant (Cuzick non-parametric test for trend, *P* = 0.005), but the results also show that samples which pass laboratory QC after longer transit times still have a median DNA incorporation of approximately 100 cpm above the low cutoff range across all samples in Fig. 2.

**Aliquot reproducibility.** As part of the assay design, each sample was split into two aliquots per analyte so that each reaction was performed twice and an average SI was recorded, allowing concordance to be checked. The cpm results in the two replicate cultures of cells alone, without any analyte added, indicated strong concordance and produced a correlation coefficient (*r*) of 0.95 (Fig. 3). When using data from a positive-control analyte (PHA), i.e., expecting a positive proliferative response, we found that the concordance between the two aliquots was similarly stable (Fig. 3), with a correlation coefficient (*r*) of 0.94. Thus, the culture environment offers little stress, and in stimulated cultures, it allowed the complex chain of lymphocyte activation events and broad, polyclonal proliferative expansion over the 6-day culture period and the highly coordinated, terminal [*H*]TdR incorporation.

**Time, age, and climate effects on samples.** A benefit of having a prospective cohort is that we can analyze changes, such as those due to both the older age of the subjects and the year of the study, over time. As expected, we found that the number of cells increased with age; the reasons include larger blood draws in older children. Less expected was a pattern of increasing cell counts and reduced variability by year of the study (Fig. 4). Using a generalized estimating equation to account for within-subject correlation, with the count of cells alone assumed to have a Poisson distribution and the highly coordinated, terminal [*H*]TdR incorporation, we found that the year of the study categorical effects on the cell count were statistically significant, as were subject age (*P* < 0.0001) and, as discussed above, transit time (*P* = 0.003).

An analysis of the center effects on sample viability, which examined geographic latitude as a proxy for the ambient tempera-
ture differences at the various centers, was conducted. Using a generalized estimating equation (GEE) to account for within-subject effects, we observed a significant association of increasing viability with the collection site’s latitude in degrees from the equator (odds ratio, 1.027; 95% confidence interval [CI], 1.011 to 1.044; \( P < 0.001 \)), having adjusted for transit time, year of the study, and age of the subject. The indicator variables for the four seasons during which the samples were collected were explored but did not show significant differences.

Validation using control analytes. We sought to determine aspects of postnatal immune system development in our large birth cohort. The positive control analytes (tetanus toxoid [TT], PHA, and anti-CD3) were examined first. The vast majority of proliferative responses had stimulation indices at or above the expected levels across all of the time points (94% for PHA, 83% for anti-CD3, and 81% for TT). There were 175 samples (3.7%) where all three of the positive-control reactions did not generate an SI ≥4 or ≥10 for PHA. Those samples were examined to see if there was a pattern of underresponse for other analytes. There were only 24 subjects with two or more such results over time, and, in general, there was some tendency for the underresponse to occur at an earlier age: 43% of the 175 samples occurred in the 3-month sample, mainly related to a slight delay in the development of TT responses. Figure 5 shows the proportion of samples which underresponded compared to the total number of samples for a study visit. The 3-month sample also has the highest proportion of nonresponders, reflecting younger ages; this observation maps the ontogeny of the responses: PHA before anti-CD3 before TT.

Looking again at the effects of shipping time, we found that there was a trend of decreasing proliferation to PHA as shipping time increased (Fig. 6). While samples longer than 3 days in shipping may pass laboratory QC criteria, the proportion of subnormal responses in positive-control cultures increased significantly with longer transit times (Cuzick nonparametric test for trend, \( P = 0.0015 \)). However, relatively few samples were ≥4 days in transit, and, for those, a data QC step of removing samples with low stimulation indices for both PHA and anti-CD3 might be appropriate. However, some samples in a clinical setting may be of unique importance, and we can also conclude that measuring responses in samples after prolonged transit times may still provide meaningful results, in particular when the relative strength of positive signals in the three positive-control analytes is sustained even if small.

Analyzing the negative controls, ovalbumin and actin, as a measure of baseline T cell turnover at the time of sampling, we found that the results were highly correlated with those for the unstimulated culture as expected and were consistent over time. Of the 4,676 viable samples tested, only 1% had a proliferation SI of >1.5 to ovalbumin and 1.3% to actin. There were only 9/4,676 samples (0.2%) with greater than expected proliferative responses to both actin and ovalbumin. Such samples should be flagged and might be removed from analysis as part of future data QC practice.

Control analyte responses over time. PHA and anti-CD3 showed little changes in absolute levels between 3 months and 3 years, consistent with the prenatal and early postnatal development of T cell competence (15). However, there was a trend for increasing median proliferative responses over time (Fig. 7) and decreasing variations with age. The substantially larger amplitudes of PHA versus anti-CD3 responses are reflective of the concomitant T cell-dependent B cell stimulation by PHA (16). Using a generalized estimating equation (GEE) to account for within-subject effects, we found that both the log-transformed PHA and anti-CD3 had statistically significant associations with continuous

FIG 2 Decreasing median counts of unstimulated cells as shipping time increases, changes which were statistically significant using the Cuzick nonparametric test for trend (\( P = 0.005 \)).
variables for subject age, transit time, and log$_{10}$-transformed number of unstimulated cells and its square (each $P < 0.0001$) in a multivariate model. The year of the study, as a categorical variable, was also included to account for fluctuations during the study. The age, number of unstimulated cells, and year of the study covariates were associated with increased proliferative responses to the positive controls. The square of the logged number of cells alone had a negative influence, as did shipping time. The finding for age was reflected in the proportion of samples that have low or no reaction to PHA between the ages of 3 and 9 months, which declined nearly 3-fold, from 16.2% to 5.7%. A similar pattern was observed for anti-CD3, where 30.3% of samples had an SI of about 4 at 3 months, which dropped to 23.3% by 9 months of age.

The schedule of vaccinations for infants in the United States and Canada recommends that immunization to tetanus be administered at 2, 4, 6, and 18 months of age, with a booster between 4 and 6 years of age (17, 18). The TT responses follow this pattern (Fig. 8), with the largest increase in median responses between ages 3 and 6 months and a smaller, but sizable, increase between 6 and 9 months. A GEE similar to that applied earlier was used, adjusting for the time in transit, year of the study, and log$_{10}$-transformed unstimulated cell count and its square. This analysis found that the increases from 3 to 6 and from 6 to 9 months were both significant ($P = 4 \times 10^{-50}$ and $9 \times 10^{-8}$, respectively). There was a nonsignificant 1.5% increase between 9 and 12 months (median SI of 6.26 and 6.36, respectively), when no tetanus immunization is scheduled, with the second largest relative increase of 9.3% between 12 and 18 months ($P = 0.03$). Thus, this cognate T cell response quantitatively mirrors the immunization practices. Limiting of dilution analysis and antigen dose responses is needed for definitive conclusions, but the effects observed here likely represent a combination of growing pool size and T cell receptor (TCR) affinities among circulating TT-specific T cells. This conclusion will be revisited for the analysis of autoimmune responses in the near future (our unpublished data).

**DISCUSSION**

We have analyzed and validated a practical, reproducible method for quantifying the T cell responses from large numbers of samples of PBMCs from shipped 3- to 10-ml fresh blood samples. Of par-

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**FIG 3** Replicate values for [$^3$H]thymidine incorporation in unstimulated and stimulated microcultures, presented on the log$_{10}$ scale (cpm), show high concordance between wells. The Pearson correlation coefficients were 0.95 for cells alone, 0.94 for PHA, 0.91 for tetanus toxin, and 0.89 for ovalbumin (OVA). Each of the four correlations are significantly different from a rho of 0 ($P < 0.0001$).
ticular interest was the surprisingly stable viability and competence for complex biological in vitro functions of shipped, fresh blood samples, with adequate responsiveness after several days of transit. The laboratory QC procedures removed a relatively small proportion (~5%) of samples from further analysis. While samples may appear to contain a sufficient number of T cells, we have found that longer shipping times, beyond a 4-day shipping period, result in losses of competent T cells. However, surprising respon-

![FIG 4](https://example.com/4.png) Counts of cells alone (cpm), split by the year of the sample collection and age of subject (follow-up visit), with interquartile ranges, means, and medians. The graph excludes outliers and the relatively small number of results from 2002 to 2003 and 2008 to improve resolution of the other years. Counts of unstimulated PBMCs increase with subject age, and variability is reduced over the course of the study.

![FIG 5](https://example.com/5.png) Proportion of samples which did not proliferate to the expected level for all three positive-control analytes falls during infancy, suggesting that an appropriate T cell response develops rapidly between 3 and 6 months of age.
siveness was observed occasionally after shipping times of as many as 9 days; we do not know what sustains viability in such samples. We recommend a 2-day shipping period, as detrimental effects appear in the samples from 3 days onward. However, a key finding of this work is that samples arriving outside the 2-day window should still be considered for investigation, with shipping time taken into account in the analyses.

We have not examined deleterious shipping effects on short-lived platelets and neutrophils. Post-blood draw separations, such as removal of platelets and neutrophils in the clinical centers, were tried in preliminary experiments and generated unacceptably large variations, delays, and contamination issues. The presented data demonstrated meaningful results in the vast majority of samples, validating the room temperature shipping protocol. This finding is concordant with a trial of shipping protocols using thermal packs by Olson et al. (19), where the advice was to avoid extremes of temperature. We extend this by showing that T cell function can be preserved in fresh samples using only insulating
packaging, across a range of climates and seasons. This was the case even for the sites with the most extreme latitude, with 91.2% of samples from Ponce, Puerto Rico (18° latitude), and 99.5% of the samples from the other extreme, Edmonton, Canada (53.53° latitude), passing laboratory QC for up to 3 days of transit.

During the more recent years of the study data, there has been a distinct tightening of the interquartile ranges for subjects at all ages (Fig. 4). We believe this reflects systematic improvements in sample handling in the clinical centers, where fewer, but experienced, personnel carry out sample procurement and shipping. The assay personnel in the Toronto lab did not change throughout the study. Additionally, as subjects age, there is an observed reduction in the variance in response to positive controls (Fig. 5 and 6). We believe that this is indicative of the development of immunocompetence: extremely large or small responses to the positive controls tend to disappear with age.

The use of several positive controls in the assay provided a tool to distinguish rare samples with reduced functionality from those with lost functionality. The observation of cognate, i.e., antigen-specific, T cell reactivities developing in parallel to the tetanus immunization schedules added considerable confidence that this assay methodology is suited to probe cognate immune responses and immune responsiveness in general, a relevant prerequisite for additional utility in other diseases or conditions with immunological elements. Further investigation using a new stimulation index for the response to tetanus, where log10-transformed absolute counts from anti-CD3 response cpm rather than those from unstimulated cultures were used as the reference, was conducted. This GEEs still showed the significant rising responses, corresponding to the immunization schedule, at 3 to 6 months and 6 to 9 months ($P = 2 \times 10^{-48}$ and $0.0001$, respectively), which accounted for the effects of a developing immune system with rising responsiveness to anti-CD3 and PHA during infancy.

We expect to report an analysis of T1D disease analytes for the scheduled TRIGR interim analysis in 2014. In the interpretation of future results for test analytes, it is worth considering the control reactions too, as we did observe an age dependence for the PHA responses. Unusual results for control reactions may describe an immune system in flux, immune incompetence, or immune abnormalities related to development of a given disease.

T cell analyses, such as those presented here, can be a useful part of the routine collections in long-term prospective multicenter studies, since the validation characteristics suggest they are robust and comparable to other medical biochemical tests. We have quantified the effect of transit time on viability, which is useful for planning future studies of fresh blood T cells. The data identified a practical time window for sample shipment within one continent. Furthermore, we describe a pattern of human immune ontogeny, contrasting responsiveness to PHA, anti-CD3, and tetanus immunization. These data provide a stable and promising platform for measuring evolution of prediabetic autoimmunity and characteristics of abnormal cows’ milk immunity in a large, long-term prospective pediatric trial.

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