Flow Cytometric Detection of Intracellular Th1/Th2 Cytokines Using Whole Blood: Validation of Immunologic Biomarker for Use in Epidemiologic Studies

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Abstract

Few biological markers of immune function have been thoroughly validated for use in epidemiologic studies that involve delayed sample processing and analysis. Here, we report our validation results for flow cytometric detection of intracellular T-helper 1/T-helper 2 (Th1/Th2) cytokines using 500 μL of whole blood obtained from children and adults. The detection of Th1/Th2 cytokine profiles by flow cytometry is a practical and mechanistically relevant assay because dysregulated cytokine production has been observed in many immune-mediated disorders, including cancer. We evaluated the intraassay and intraindividual and interindividual variability and the effects of a 24- to 72-hour delayed analysis on Th1 and Th2 end points. We compared the distributions of %CD4 lymphocytes, %Th1, and %Th2 in young children (age 1 year, $n = 50$) and adults (age 25–52 years, $n = 16$). Subjects sampled monthly for up to 1 year showed minimal variation in CD4, Th1, and Th2 end points. Delayed analysis of samples (up to 24 hours) resulted in no significant differences in the expression of CD4, Th1, and Th2; however, at 48 and 72 hours, all end points differed significantly from baseline ($P < 0.01$). A random effects model confirmed that interindividual variability was much greater than intraindividual variability for CD4 and Th1. Compared with adults, children had marginally higher %CD4, similar %Th2, but significantly lower %Th1 ($P < 0.01$). These results show that flow cytometric detection of CD4, Th1, and Th2 markers using whole blood is reproducible and that these biomarkers can be effectively used in human population studies that involve transported samples, delayed processing and analysis, and limited blood volumes. (Cancer Epidemiol Biomarkers Prev 2004; 13(9):1452–8)

Introduction

Cytokines are important protein mediators of the immune response (1). Type 1 cytokines [IFN-γ, interleukin (IL)-12, and tumor necrosis factor-β] promote pro-inflammatory immune responses, whereas type 2 cytokines (IL-4, IL-5, IL-10, and IL-13) promote anti-inflammatory, antibody-dependent immune responses (2). Dysregulated type 1/type 2 cytokine production and skewed development of memory T-helper 1 (Th1) or T-helper 2 (Th2) subsets, which secrete type 1/type 2 cytokines, respectively, have been implicated in the progression of multiple immune disorders including asthma (3, 4), leukemia (5), and other cancers (6). As a result, there is a great interest in using type 1 and type 2 cytokines as markers of human immune function.

Numerous cytokine assays are available including single and multiplexed ELISAs (Luminex and LINCOplex), reverse transcription-PCR, Taqman real-time PCR, and immunohistochemistry (7-9). The ELISA method is most commonly employed in epidemiologic studies because of its relative ease; however, the rapid uptake of released cytokines by nearby immune cells requires that samples be collected and processed at appropriate times to account for cytokine absorption kinetics. One limitation of ELISAs is that they cannot identify the cellular source of the cytokines secreted into plasma or serum (8). The recently optimized method of intracellular cytokine detection in specific cells by flow cytometry circumvents these limitations (10) and has great potential for biomonitoring of immune function in human populations (11).

Flow cytometric detection of intracellular cytokines is a functional assay that measures the ability of specific immune cells to express type 1 and type 2 cytokines after polyclonal stimulation with mitogens (8, 10, 12). Two functionally distinct T-helper lymphocyte subsets are distinguished by their signature cytokines: IFN-γ for Th1 lymphocytes and IL-4 for Th2 lymphocytes (2). Jung et al. (12) reported that this method was sensitive enough to detect IL-4 secretion in human T-cell clones when cytokine secretion inhibitors were used. Other investigators have improved on this method. For example, Schuerwegh et al. (13) showed that brefeldin A is a more potent, effective, and less toxic inhibitor of cytokine secretion than monensin. Rostaing et al. (14) showed

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that a 4-hour incubation period is optimal to observe simultaneous peak expressions of IFN-γ and IL-4. Using this assay, Zhang et al. (5) reported that children with leukemia, when compared with healthy controls, had reduced percentages of Th1 and elevated percentages of Th2 cells, suggesting that altered cytokine expression by T-helper cells plays an important role in the development of leukemia.

Application of flow cytometric detection of Th1/Th2 cells by intracellular cytokine staining to epidemiologic studies is relatively new and still requires thorough validation. A recently modified version of this method uses <500 μL of whole blood (15), instead of isolated lymphocytes, making it more practical for studies that have access to limited blood volumes. Another important advantage of this method is that sample processing

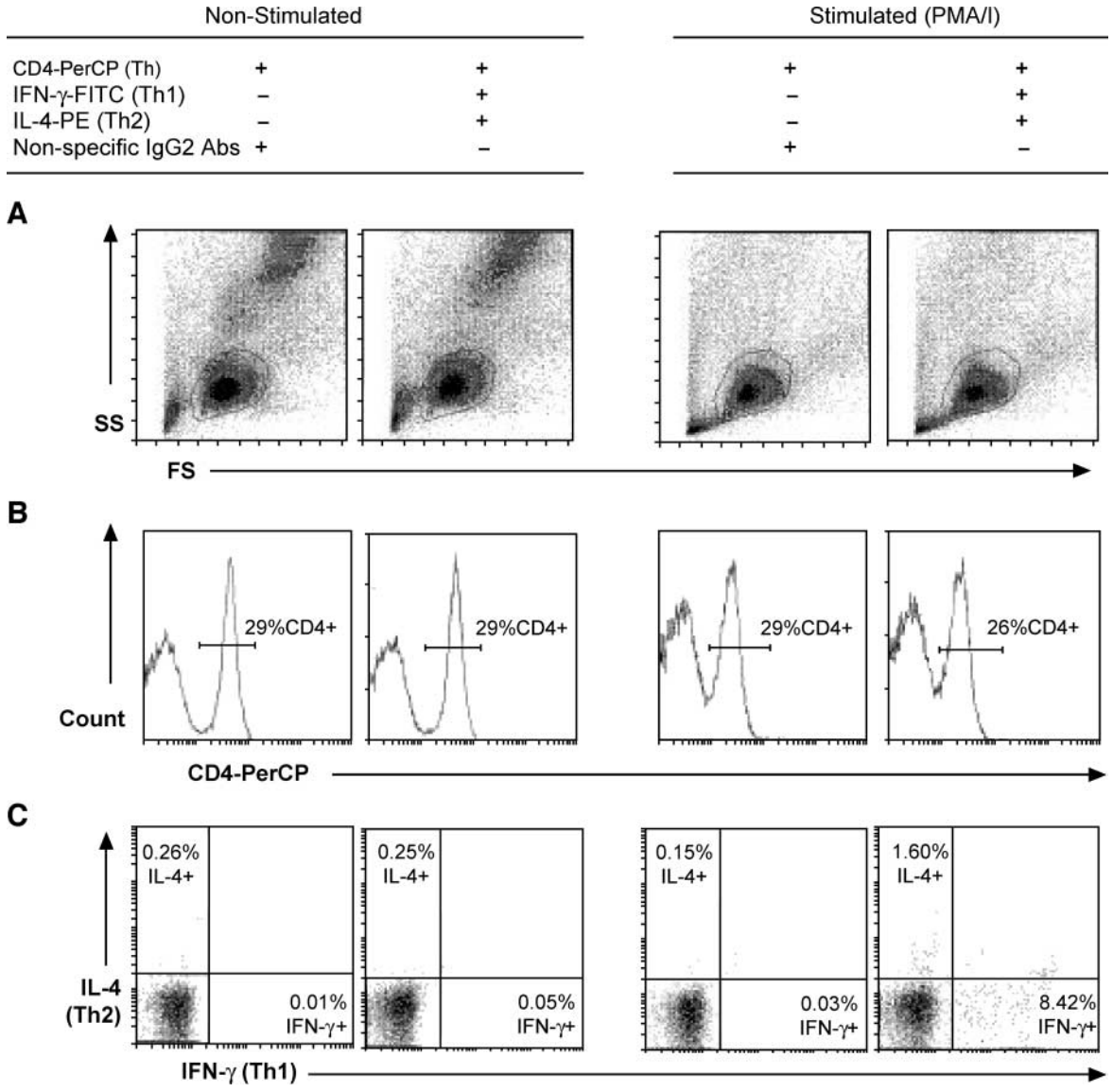

Figure 1. Flow cytometric detection of intracellular Th1/Th2 cytokines in whole blood. Parallel whole blood cultures were set to compare Th1/Th2 cytokine expression in response to stimulation with PMA/I to those without stimulation. Whole blood aliquots from each culture were stained with either cytokine-specific antibodies (IFN-γ-FITC for Th1 cells and IL-4-PE for Th2 cells) or their fluorochrome-equivalent IgG2 isotype control antibodies to detect nonspecific antibody binding. In the scatter plot of all cells (**A**), a circular gate was placed around the live lymphocyte population based on size [forward scatter (*FS*)] and granularity [side scatter (*SS*)]. This live lymphocyte population is acquired to **B**. The percentages for CD4 antigen (of the total lymphocytes) are *above each linear gate*. **C.** The CD4-positive subgroup is examined for expression of IFN-γ and IL-4 cytokines. Cells that stained positive for IFN-γ only (*lower right quadrant*) were classified as Th1 cells and those that stained positive for IL-4 only (*upper left quadrant*) were classified as Th2 cells. Baseline IL-4 expression is higher than IFN-γ; however, unlike IL-4, IFN-γ can only be detected with PMA/I stimulation. The percentages of Th1 and Th2 were calculated as the number of cells positively stained IFN-γ and IL-4, respectively, out of the total CD4+ cell population.

Table 1. Intraassay variability of flow cytometric detection of Th1/Th2 using whole blood

Outcome	Adult (n = 1, Age 28 y)				Child (n = 1, Age 1 y)			
	1	2	3	CV*	1	2	3	CV*
%CD4†	30.8	32.1	30.5	2.7	34.8	33.3	32.0	4.2
%Th1‡	15.5	14.9	16.7	5.9	5.5	5.8	6.0	4.9
%Th2‡	0.23	0.23	0.25	5.5	1.7	1.9	2.1	9.0

*The intraassay variability, expressed as CV (SD/mean), was calculated based on three separate whole blood cultures (1, 2, and 3) set for each donor (n = 2). Three staining replicates for CD4/IFN-/IL-4 were done for each culture. The means for each culture are reported here and these means were used to calculate the CV.

†%CD4 was determined by dividing the number of cells that stained positive for CD4 antibody by total number of lymphocytes. A minimum of 5,000 CD4 cells was collected for each data point.

‡%Th1 and %Th2 were calculated as the number of positively stained IFN-γ and IL-4 out of the total CD4+ cell population.

takes less time, because isolation of lymphocytes is no longer required. Moreover, the whole blood method retains T-helper lymphocytes in a microenvironment most similar to the one *in vivo* and is thus more suitable for studies that involve transportation of blood samples from the field to the laboratory (8, 10). Here, we present the results of our biomarker validation efforts to evaluate the effects of (*1*) intraassay variability, (*2*) intraindividual and interindividual variability, and (*3*) a 24- to 72-hour delay in blood sample processing.

Materials and Methods

Subjects. Venous blood samples were collected from 50 children (age 1 year) and 16 adults (age 25–52 years) directly into Vacutainer containing sodium heparin anticoagulant. Blood samples were stored at room temperature (22°C) in the dark. Informed consent was obtained from all subjects or their guardians prior to blood collection. Participants were considered healthy if they did not show any symptoms of asthma exacerbation or respiratory infection.

Cell Culture and Activation. Blood samples collected from participants were cultured at 2, 24, 48, and 72 hours following collection. Whole blood (500 μL) was diluted 1:1 with RPMI 1640 in 12 × 75 mm fluorescence-activated cell sorting tubes and activated with phorbol 12-myristate 13-acetate (PMA; 2.5 ng/mL, Sigma Chemical Co., St. Louis, MO) and ionomycin (1 μg/mL, Sigma Chemical). These cultures were incubated for 4 hours at 37°C and 5% CO_2 in the presence of brefeldin A (10 μg/mL, Sigma Chemical), a transport inhibitor that prevents cytokine release from cells. Samples incubated with brefeldin A alone served as nonstimulated controls.

Fluorescent Labeling and Flow Cytometry. Whole blood (200 μL) was pipetted directly into a 12 × 75 mm fluorescence-activated cell sorting tube containing 20 μL of monoclonal antibodies for the T-helper surface antigen CD4 (CD4-PerCP, Becton Dickinson, San Diego, CA) and incubated at room temperature in the dark for 10 minutes. Then, 1% paraformaldehyde (0.5 mL) was added for 8 minutes to stabilize the monoclonal antibody-surface antigen complex. RBCs were lysed using 3 mL of 1× fluorescence-activated cell sorting lysing solution (Becton Dickinson) for 8 minutes. After centrifugation at 1,930 rpm for 5 minutes, the supernatant was aspirated and 1× permeabilizing solution (500 μL, Becton Dickinson) was added into the pellet and incubated for 10 minutes at room temperature in the dark. After washing with 3 mL buffer (1% bovine serum albumin, 0.1% NaN_3, 1× PBS), cytokine-specific antibodies (20 μL, IFN-γ-FITC, IL-4-PE, Becton Dickinson) were added to the cells and incubated for 30 minutes at room temperature in the dark. After one final wash, cells were resuspended in 1% paraformaldehyde (500 μL) and stored at 4°C until flow cytometry analysis. Cells were acquired using a Beckman-Coulter EPICS XL flow cytometer (Miami, FL) and data were analyzed using CellQuest software. Percentages of Th1 and Th2 cytokine-producing cells were identified as the number of IFN-γ-positive and IL-4-positive cells present, respectively, in the total population of CD4+ T-helper cells. A minimum of 5,000 CD4+ cells was counted from each sample.

Isotype Control. Fluorochrome-equivalent IgG2 isotype controls (Becton Dickinson) were used to detect nonspecific binding. Compensation for dual-fluorochrome spectral overlap was made using cells individually stained with FITC-only and PE-only antibodies.

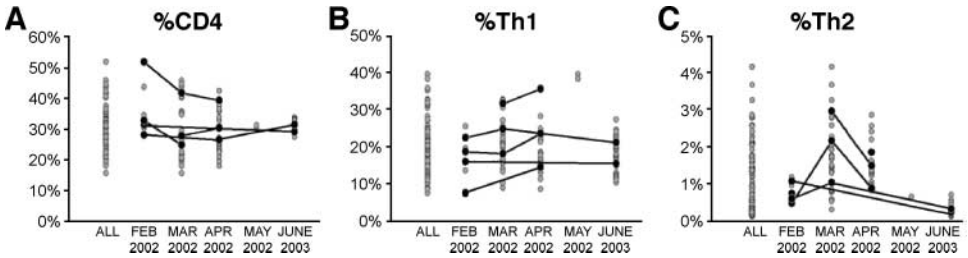

Figure 2. Intraindividual and interindividual variability in %CD4 (**A**), %Th1 (**B**), and %Th2 (**C**) measurements. Blood samples were collected from 16 individuals during the period of February 2002 to June 2003; five individuals were repeatedly sampled, with each sampling event separated by at least 1 month. *Black circles,* means for each of the five individuals connected by lines to illustrate the changes in these end points over time. *Light circles,* raw data with the means showing the extent of intraindividual versus interindividual variability. *First column in each graph,* overall range of measurements and all the data points acquired (n = 16, observed = 419).

Table 2. Variance components for CD4, Th1, and Th2 end points (n = 16, observed = 419)

Outcome	Mean	Variance between subjects (%)	Variance within subjects (%)	Residual variance (%)
%CD4	30.3	6.9 (71)	4.0 (24)	1.9 (5)
%Th1	19.3	7.3 (83)	2.0 (6)	2.6 (11)
%Th2	0.8	0.3 (15)	0.6 (67)	0.3 (16)

NOTE: A linear mixed, random effects model was used to evaluate sources of variability for CD4, Th1, and Th2.

Statistical Analysis. Data analyses were done using Stata. Descriptive statistics (raw means and percentages) stratified by age were used to determine differences in the distribution of CD4, Th1, and Th2 cells between children and adults. Coefficient of variance (CV; SD/mean) was calculated to determine intraassay variability. For statistical analysis, the data for Th1 and Th2 were log transformed to normalize skewed distributions. A linear mixed (random effects) model (16) was used to evaluate the sources of variability that contributed to differences in the measurements over time (specifically Th1, Th2, and CD4 measured longitudinally in adults). In all analyses involving repeated measurements on the same individual, generalized estimate equations and robust SEs were calculated to adjust for correlations that can result from repeated sampling of the same individual (17).

Results

Optimization of Flow Cytometric Detection of Intracellular Th1/Th2 Cytokines. To confirm that PMA/ionomycin (PMA/I) stimulation did not significantly down-regulate surface expression of the CD4 receptor and that antibodies used to detect IFN-γ and IL-4 did not show a high degree of nonspecific binding, we set up parallel whole blood cultures, one stimulated with PMA/I and one not stimulated with PMA/I. Cells from each culture were stained using either cytokine-specific antibodies or nonspecific, isotype control antibodies. The results of our flow acquisition are depicted in Fig. 1 for both PMA/I-stimulated and nonstimulated whole blood cultures. In the scatter plots of all immune cells (Fig. 1A), an electronic, circular gate was used to select the live lymphocyte population based on cellular size (forward scatter) and granularity (side scatter). The right peaks in Fig. 1B represent the lymphocytes that stained positive for the CD4 surface antigen. This subpopulation was further examined (Fig. 1C) for expression of IFN-γ and IL-4, the signature cytokines of Th1 and Th2, respectively. As expected, spontaneous T-helper cell cytokine production was not observed in whole blood cultures not stimulated with PMA/I cytokines. Stimulation with PMA/I caused some down-regulation of CD4 surface levels but did not have an effect on the gating of CD4+ cells. Use of isotype controls confirmed that there was minimal nonspecific binding of monoclonal antibodies (<0.5% for all variants).

Intraassay Variability for Children and Adult Samples. Whole blood samples were obtained from one adult and one child and three replicate cultures were set and stimulated with PMA/I. From each culture, three replicates were stained with the CD4-PerCP/IFN-γ-FITC/IL-4-PE monoclonal antibody combination and three end points were determined by flow cytometry: %CD4, %Th1, and %Th2. A minimum of 5,000 CD4+ lymphocytes was counted each time. The means for both child and adult samples are summarized in Table 1 together with the CV (%CV = SD/mean × 100), which was calculated to assess the intraassay variability for each end point. The CVs for %CD4, %Th1, and %Th2 were 2.7%, 5.9%, and 5.5%, respectively, for the adult samples, and 4.2%, 4.9%, and 9.0%, respectively, in the child samples. All end point CVs were <10%, indicating high reproducibility; however, measurements could be further improved by collecting more cells in cases wherein low percentages are being measured (e.g., IL-4 Th2 cells).

Intraindividual and Interindividual Variability in Adults. To evaluate the within-subjects (intraindividual) and between-subjects (interindividual) variability, we collected blood samples from 16 adults during the period of February 2002 to June 2003. To measure variability within the same subjects over time, we collected multiple blood samples from 5 of these 16 adults for up to 1 year; each repeated sampling event was separated by at least 1 month. These results are depicted in Fig. 2. The first columns of graphs A to C represent the overall ranges for %CD4 (15.72–52.06%), %Th1 (7.20–39.43%), and %Th2 (0.11–4.17%), respectively. The means for each of the five individuals followed longitudinally are represented by filled black circles, which are connected by lines to illustrate the changes over time. The %CD4, %Th1, and %Th2 markers remained stable in individuals assayed in consecutive months for up to 1 year. A linear

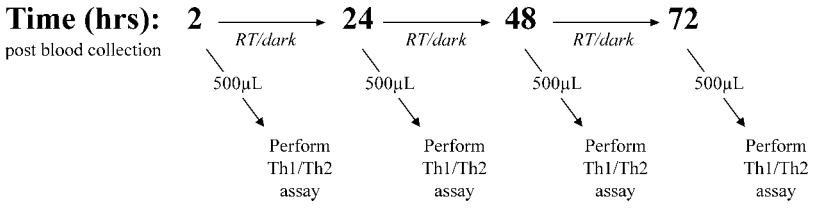

Figure 3. Protocol for evaluating the effects of delay and storage on CD4, Th1, and Th2 measurements. Whole blood samples collected from adults (n = 7) were stored at room temperature and in the dark (*RT/dark*). At 2, 24, 48, and 72 hours post–blood collection, an aliquot (500 μL) was removed from the heparin Vacutainer, cultured, stimulated with PMA/I for 4 hours, and stained for IFN-γ and IL-4 cytokines, the signature Th1 and Th2 cytokines.

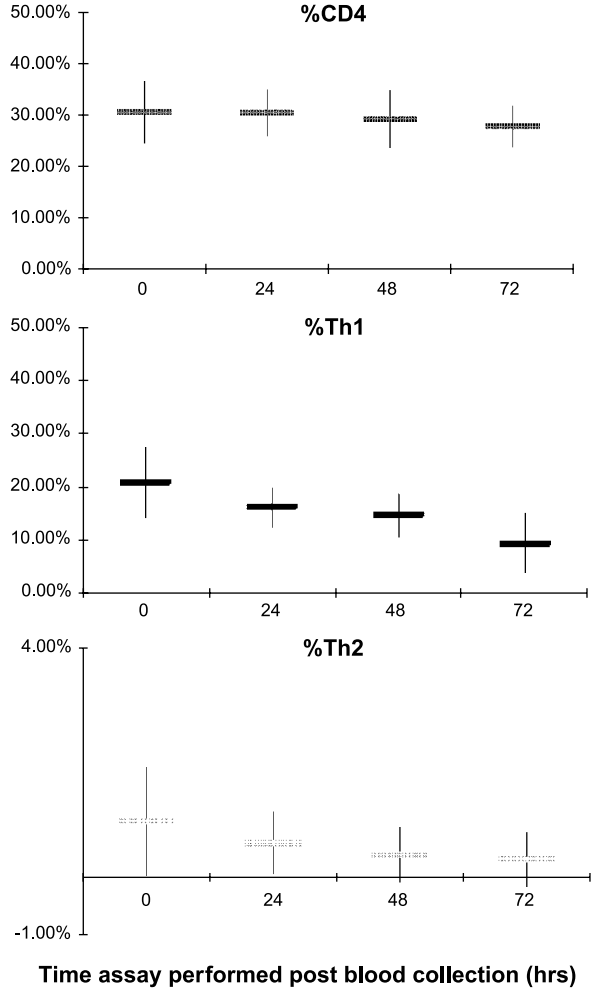

Figure 4. Effect of delayed analyses and room temperature storage on CD4, Th1, and Th2 expression. Whole blood samples (n = 7) were stored at room temperature in the dark. Aliquots were analyzed using the same flow cytometric protocol at 2, 24, 48, and 72 hours post–blood collection. Using a linear mixed model, generalized estimate equations and robust SEs were calculated to adjust for correlations that can result from repeated sampling of the same individual. No significant differences in expression of CD4, Th1, and Th2 were found between 2 and 24 hours; however, after 48 and 72 hours, the differences were statistically significant ($P < 0.01$).

mixed (random effects) model was used to separate the contributions of different sources of variability, including intraindividual variability (i.e., variation within the same individual assessed in different months throughout 1 year), interindividual variability, and residual (random) variation. These results are summarized in Table 2. Intraindividual variability of %Th2 was greater than interindividual variability, which is suggestive of a temporal dependence for Th2 expression. For both %CD4 and %Th1, the interindividual variation was 3- to 10-fold higher than both intraindividual variability and residual (random) variability.

Effect of Storage Conditions and Delayed Analysis. To evaluate the effect of delayed analysis and storage temperature on the CD4, Th1, and Th2 markers, adult samples (n = 7) were collected and stored at room temperature and in the dark prior to culture and analysis of Th1/Th2 cytokine production. Aliquots were removed at 2, 24, 48, and 72 hours post–blood collection (see Fig. 3), cultured, stimulated with PMA/I, and examined for Th1/Th2 cytokine expression according to the protocol described in Materials and Methods. The results of delayed analysis are presented in Fig. 4. Although there is a downward trend for both %Th1 and %Th2 over time, there was no significant difference between 2-hour (baseline) and 24-hour delayed analysis for all three end points (%CD4, %Th1, and %Th2; P = 0.51, 0.22, and 0.51, respectively). However, after 48- and 72-hour processing delays, Th1 and Th2 expression was significantly decreased ($P < 0.01$). These results show that flow cytometric detection of %CD4, %Th1, and %Th2 using whole blood is reproducible for up to 24 hours post–blood collection.

Th1/Th2 Subsets in Children versus Adults. Next, we used this optimized method to quantify the percentages of Th1 and Th2 cells in 50 healthy children (age 1) and 16 healthy adults (age 25–52 years). Cells positive for IFN-γ were categorized as Th1 cells, whereas cells positive for IL-4 were categorized as Th2 cells. The mean, median, minimum, and maximum percentages of CD4, Th1, and Th2 are summarized in Table 3 and their distributions are illustrated in Fig. 5. For statistical analysis, Th1 and Th2 data were log transformed to normalize skewed distributions. Children had slightly higher CD4+ cells than adults, whereas adults have higher percentages of Th1 cells ($P < 0.01$). These results indicate that flow cytometric detection of Th1/Th2 subsets can be used to detect age-related differences over time.

Discussion

In this study, we assessed the intraindividual and interindividual variability of three immune markers (%CD4, %Th1, and %Th2) in young children and adults. We also evaluated the effects of collection and transportation variables, including 24-, 48-, and 72-hour delays and storage conditions on Th1/Th2 cytokine outcomes. We found that the whole blood protocol for analyzing intracellular cytokine expression was highly reproducible for samples stored at room temperature for up to 24 hours. However, significant changes in CD4, Th1 (IFN-γ), and Th2 (IL-4) cytokine expression were detected using blood samples stored at room temperature for 48 and 72 hours.

We next established reference ranges for Th1 and Th2 in young children (age 1 year) and compared them with those of healthy adults (age 25–52 years). To date, there are limited data that characterize infant and early childhood immune development. Here, we report that 1-year-old children had a 5-fold lower percentage of IFN-γ-producing Th1 cells than adults (age 25–52 years) but similar percentages of IL-4-producing Th2 cells. McNerlan, et al. (15) reported that elderly subjects had significantly higher numbers of Th1 cells compared with young subjects. However, the young age category in that

Table 3. Descriptive statistics of CD4, Th1, and Th2 in adults and children

Outcome	Adults (n = 16)				Children (n = 50)			
	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum
%CD4*	30.8	30.8	18.1	52.1	32.7	32.9	31.1	34.8
%Th1†	20.1	20.1	7.2	39.4	4.7	4.5	0.9	10.5
%Th2‡	0.88	0.55	0.14	4.2	1.1	0.9	0.13	3.4

NOTE: For adults, the absolute cell count ranges were 360–1,970 (Th1) and 7–210 (Th2). For children, the absolute cell count ranges were 45–525 (Th1) and 6–170 (Th2). These absolute cell counts were based on a minimum of 5,000 CD4 cells.
*%CD4 was determined by dividing the number of cells that stained positive for CD4 antibody by total number of lymphocytes.
† %Th1 was calculated by dividing the number of IFN-γ-positive cells by the total number of CD4+ lymphocytes acquired.
‡ %Th2 was calculated by dividing the number of IL-4-positive cells by the total number of CD4+ lymphocytes acquired.

study included 20- to 29-year-old subjects, which is significantly older than the young age category in our study. In addition, two different cytokine markers, tumor necrosis factor-α and IL-2, as opposed to IFN-γ were used to identify Th1 cells and the Th2 variable was not examined in their study. Buck et al. (18) recently reported that, between age 2 and 6 months, there is little change in the Th1 and Th2 status in newborns but that significant changes occur during age 7 to 12 months. Together with our results, these findings suggest that development of memory Th1 and Th2 cells occurs rapidly during the early years of life.

Despite the recognition that early childhood represents a critical period of immune development, most of the data available for infants and children have been acquired from pediatric populations afflicted with either acute or chronic health conditions or they have been extrapolated from adult studies. However, these approaches are limited for two reasons: (*1*) children's immune systems are not equivalent to adults as they are less developed and thus potentially more susceptible to environmental exposures (19) and (*2*), without baseline or ''normal'' infant data, it is difficult to interpret data obtained from the ''at risk'' populations. To contribute to current efforts to understand infant immune development, we have validated a useful marker of immune function that can be practically used in pediatric studies.

Biological markers are important tools for molecular epidemiology and human biomonitoring studies. By employing immunologic markers, we can better understand the mechanisms underlying the associations between environmental exposures and immune-mediated disorders, including cancer. The results of our study show that flow cytometric detection of intracellular Th1/Th2 cytokines in whole blood is both advantageous and convenient for human biomonitoring studies and will prove most valuable to projects that involve delayed sample analysis and those that have access to limited blood volumes. We are currently using this Th1/Th2 method in multiple epidemiologic studies to identify the mechanisms of childhood leukemia and the effects of pesticide and air pollution exposures on children's immune function and development.

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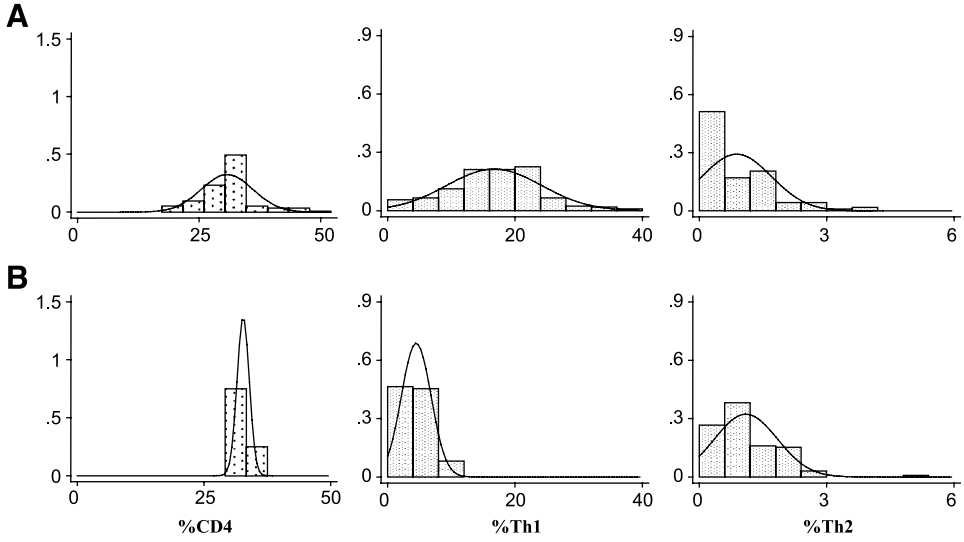

Figure 5. Distributions of CD4, Th1, and Th2 in adults (n = 16; **A**) and children (n = 50; **B**). Whole blood samples collected from adults and children were stimulated with PMA and ionomycin in the presence of brefeldin A for 4 hours at 37°C and stained with monoclonal antibodies specific for the CD4 surface antigen and intracellular IFN-γ/IL-4 cytokines, with IFN-γ and IL-4 serving as the signature cytokines of Th1 and Th2 cells, respectively.

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