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# Single-Cell Analysis of Innate Cytokine Responses to Pattern Recognition Receptor Stimulation in Children across Four Continents

Kinga K. Smolen,\* Bing Cai,<sup>†</sup> Laura Gelinas,\* Edgardo S. Fortuno, III,<sup>†</sup> Martin Larsen,<sup>‡,§</sup> David P. Speert,<sup>†</sup> Mustapha Chamekh,<sup>¶</sup> Philip J. Cooper,<sup>||, #, \*\*, 1</sup> Monika Esser,<sup>††, 1</sup> Arnaud Marchant,<sup>¶, ‡, †, 1</sup> and Tobias R. Kollmann\*,<sup>†</sup>

Innate immunity instructs adaptive immunity, and suppression of innate immunity is associated with an increased risk for infection. We showed previously that whole-blood cellular components from a cohort of South African children secreted significantly lower levels of most cytokines following stimulation of pattern recognition receptors compared with whole blood from cohorts of Ecuadorian, Belgian, or Canadian children. To begin dissecting the responsible molecular mechanisms, we set out to identify the relevant cellular source of these differences. Across the four cohorts represented in our study, we identified significant variation in the cellular composition of whole blood; however, a significant reduction in the intracellular cytokine production on the single-cell level was only detected in South African children's monocytes, conventional dendritic cells, and plasmacytoid dendritic cells. We also uncovered a marked reduction in polyfunctionality for each of these cellular compartments in South African children compared with children from the other continents. Together, our data identify differences in cell composition, as well as profoundly lower functional responses of innate cells, in our cohort of South African children. A possible link between altered innate immunity and increased risk for infection or lower response to vaccines in South African infants needs to be explored. *The Journal of Immunology*, 2014, 193: 3003–3012.

**S**usceptibility to infection and response to vaccines differs among children from different regions of the world (1). These differences in vaccine response have been attributed to variation in host genetic background and/or environmental exposures (2, 3). However, the underlying mechanism(s) remain largely unknown. This lack of understanding prevents optimization of interventions (3). Because innate immunity instructs adaptive immunity, we hypothesized that differences in innate immune responses of children from different parts of the world might contribute to variation in susceptibility to infection or response to vaccines. No worldwide comparison of cell-specific innate immune response has been conducted.

The innate immune system relies on germline-encoded pattern recognition receptors (PRRs), which recognize conserved molecular motifs in microbes known as pathogen-associated molecular patterns. Sensing of pathogen-associated molecular patterns by

PRRs triggers an effector response aimed at eliminating the potential pathogen. To allow for a global comparison, we used a variety of PRR ligands to activate the specific receptor pathways. We previously found that children from a South African cohort secreted markedly lower amounts of nearly every cytokine measured following PRR stimulation compared with children from cohorts in Belgium, Canada, or Ecuador (4). However, the cause(s) leading to the observed differences in responses to PRR stimulation could not be elucidated using the coarse measurement of cytokines secreted into culture supernatant. We reasoned that differences in cytokines detected in the supernatant of whole blood could be due to differences in the cellular composition, the response of particular cell subsets, or both. As the first step toward identifying the responsible cellular and molecular mechanisms, we used single-cell intracellular cytokine cytometry to identify the cellular compartment(s) from which the observed differences arose.

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The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional dendritic cell; LAZ, length-for-age Z-score; MDP, muramyl dipeptide; NOD, nucleotide-binding oligomerization domain; PAM, PAM3CSK4; PBSAN, PBS containing 0.5% BSA and 0.1% sodium azide; pDC, plasmacytoid dendritic cell; PGN, peptidoglycan; PI, polyfunctionality index; Poly I:C, polyinosinic-polycytidylic acid; PRR, pattern recognition receptor; WAZ, weight-for-age Z-score; WHO, World Health Organization; WLZ, weight-for-length Z-score.

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Given our eventual goal to determine the impact of innate immune variation on vaccine responses, we focused on the main APCs: conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), and monocytes. We also included granulocytes, TCR  $\alpha\beta$  T cells and  $\gamma\delta$  T cells, and B cells to allow a more complete assessment of the overall cellular composition of samples. Our findings reveal that altered cellular composition, as well as a reduced response on the single-cell level following PRR stimulation, in South African children contributed to the overall strikingly lower cytokine response. The potential upstream cause and downstream consequence of such a suppressed innate immune response to PRR stimulation in South African children needs to be assessed.

## Materials and Methods

### Ethics statement

This study was conducted according to the principles expressed in the Good Clinical Practice Guidelines and the Declaration of Helsinki and was approved by the University of British Columbia Ethics Board (protocol: H11-01423). Additionally, each site involved obtained ethics approval from their respective research center. Informed written consent from the next of kin, caregivers, or guardians on the behalf of the minors involved in this study was obtained for all study participants.

### Participant recruitment and enrollment

This study compared children 2 y of age from four sites: Vancouver, BC, Canada; Brussels, Belgium; Quininde, Ecuador; and Cape Town, South Africa. Canadian subjects were recruited from a pool of healthy children participating in other ongoing research studies at the University of British Columbia (5). Subjects in Belgium were part of a pilot study for a larger urban-based birth cohort study established at St. Pierre Hospital (Brussels, Belgium). Children from Ecuador were recruited within a rural-based population cohort study (6), and South African children had been enrolled in an urban-based birth cohort established at Stellenbosch University (7). A subject was included in the study if he/she was considered healthy based upon a history-driven health assessment. Subjects were excluded from the study if they had one or more of the following criteria: significant chronic medical condition, including HIV infection or HIV exposure during gestation, immune deficiency, immunosuppression by medication, cancer, receipt of blood products within 3 mo, bleeding disorder, major congenital malformation, or genetic disorder.

### Blood collection

All blood draws were performed in the hospital by a trained phlebotomist. Peripheral blood (3–5 ml) was drawn via sterile venipuncture into Vacutainers containing 143 U sodium-heparin (BD Biosciences, cat. no. 8019839). Blood samples were kept at room temperature and processed within <4 h of the blood draw, as described previously (8, 9).

### PRR stimulation

Given the inherent sensitivity of the innate immune system, analysis of innate immune responses via PRR stimulation is vulnerable to technical artifacts (8). To minimize technical artifacts, we used a highly standardized, stringently controlled protocol to contrast the innate immune status of children across four continents (Africa, Europe, North America, and South America). Master mixes of all reagents were made in quantities adequate for the entire study, frozen, and shipped under monitored conditions to all four sites. Premade 96-well plates contained the following specific PRR ligands with specified concentrations and specifically targeted PRR: PAM3CSK4 (PAM; TLR2/1; InvivoGen) at 1  $\mu\text{g/ml}$ ; polyinosinic-polycytidylic acid (Poly I:C; TLR3; Amersham Biosciences) at 100  $\mu\text{g/ml}$ ; LPS (TLR4; InvivoGen) at 10  $\text{ng/ml}$ ; R848 (TLR7/8; InvivoGen) at 10  $\mu\text{M}$ ; peptidoglycan (PGN; NOD1/2; InvivoGen) at 10  $\mu\text{g/ml}$ ; muramyl dipeptide (MDP; nucleotide-binding oligomerization domain [NOD]2; InvivoGen) at 0.1  $\mu\text{g/ml}$ ; and media alone. All of the wells contained brefeldin A (Sigma-Aldrich) at 10  $\mu\text{g/ml}$ . The same person (K.K.S.) performed all aspects of the experiments at all sites using our validated and quality-controlled innate immune phenotyping protocol (5, 8–11). Whole blood was diluted 1:1 with sterile prewarmed RPMI 1640, and 200  $\mu\text{l}$  of the diluted blood was added to each well of the premade plates containing the specific TLR ligands. Samples were incubated for 6 h at 37°C in 5%  $\text{CO}_2$  and then treated with a final concentration of 2 mM EDTA for 10 min at 37°C. The cells were collected and resuspended in 1.4 ml  $1\times$  BD FACS Lysing Solution, placed into fresh tubes, and stored at  $-80^\circ\text{C}$ .

### Intracellular cytokine staining and flow cytometric acquisition

Preparation of the samples for flow cytometric analysis was performed as described previously (5, 9, 12). Briefly, frozen tubes were thawed and spun, and pellets were resuspended in 200  $\mu\text{l}$  BD FACS permeabilizing solution and incubated at room temperature for 10 min. After two washes in PBS containing 0.5% BSA and 0.1% sodium azide (PBSAN), cells were stained in a final volume of 100  $\mu\text{l}$  PBSAN for 45 min at room temperature. After two additional washes with PBSAN, cells were resuspended in PBSAN and analyzed on an LSR II Flow Cytometer (BD Biosciences). Cytometer Setup and Tracking beads (BD Biosciences) were used to calibrate the machine prior to each use to ensure that machine performance remained the same. Compensation beads (CompBeads; BD Biosciences) were used to standardize voltage settings and were used as single-stain positive and negative controls, as described previously (8, 9, 13). Frozen stock of one adult whole-blood sample stimulated with R848 was used in every run to determine whether the median fluorescence intensity of cytokine populations remained the same between experiments (i.e., ensuring that the Cytometer Setup and Tracking beads-based set-up performed according to the manufacturer's specifications). A total of 500,000 uncompensated events was acquired per sample. Compensation was set in FlowJo (TreeStar), and samples were analyzed compensated. Gates were set based on the fluorescence-minus-one principle (13, 14). We positioned the unstimulated flow cytometric samples as a biological negative control; values obtained with biological negative controls were subtracted from the stimulated sample response on a per-individual basis, as described (13). Viability was not assessed directly, only indirectly via forward and side scatter appearance, as described previously (8, 9); no difference in viability was noted among samples from the four sites.

### Standardization

Given its role in rapid sensing of environmental change (15, 16), the innate immune system is prone to technical artifacts that can quickly impact the innate immune assessment (8). Thus, we implemented an experimental approach with stringent rigor and focus on quality assurance to reliably contrast samples obtained across the four continents. Master mixes of all reagents were made in quantities adequate for the entire study, frozen, and shipped under monitored and temperature-recorded conditions to each site. Materials and reagents used to draw blood or that came into contact with the blood were all sourced and tested to ensure the absence of innate immune-activating substances (8, 9). Samples postprocessing were shipped on dry ice with temperature monitors; this revealed that temperatures remained stable at  $-80^\circ\text{C}$  during all shipments. Upon arrival at the central analysis site (Vancouver, BC, Canada), samples were stored frozen in liquid nitrogen. All samples were run within 12 mo of collection. Each flow cytometric run contained randomly chosen samples from each site to avoid batch artifacts, storage, or run effects.

### Polyfunctional analysis and polyfunctionality index

Polyfunctional analysis is defined as the assessment of multiple parameters at the single-cell level (17). The polyfunctionality index (PI) numerically evaluates the degree and variation of polyfunctionality within a particular dataset, enabling comparative and correlative statistical analysis, as described (17). The polyfunctionality analysis was performed using the software "FunkyCells - Boolean Data Miner" (www.funkycells.com) developed by Dr. M. Larsen (Paris, France).

### Statistical analysis

Kruskal–Wallis analysis was performed to compare the four sites for significant variance among the median cytokine response. The Dunn posttest was used to determine which of the sites contributed to the significant differences. Statistical analysis was conducted using Prism Version 6 (GraphPad, La Jolla, CA).

### Z-score analysis

The World Health Organization (WHO) Anthropometric calculator was used to determine each participants' individual z-score (WHO Anthro version 3.2.2) (18).

## Results

### Cohort characteristics

Four populations were included in this study. The characteristics of the study population are described in Table I. To allow for the completed administration of locally recommended early childhood

vaccines, the average age at blood draw was ~24 mo. Based on clinical history, all children were healthy at the time of sample collection. Anthropometric data, such as weight, height, and mid-upper arm circumference, in vaccine studies can provide useful information about the uniformity and general health of the study population (19). In this study, we collected the weight and height to assess the overall health of our cohort populations and compare them to the WHO standards (20). Based on the WHO documentation of Child Growth Standards, the mean weight-for-age Z-score (WAZ), length-for-age Z-score (LAZ), and weight-for-length Z-score (WLZ) of the four cohorts fell within the normal range, with no more than  $\pm 2$  SD.

#### Cellular composition of the whole-blood samples

To determine the components involved in the cellular response of children from different parts of the world, we used polychromatic single-cell flow cytometry. Cell surface anchor markers were used to identify the major APC target populations in whole blood. These included monocytes (HLA-DR<sup>+</sup>, CD14<sup>+</sup>), cDCs (HLA-DR<sup>+</sup>, CD14<sup>+</sup>, CD11c<sup>+</sup>, CD123<sup>+</sup>), and pDCs (HLA-DR<sup>+</sup>, CD14<sup>+</sup>, CD11c<sup>+</sup>, CD123<sup>+</sup>). We also identified  $\alpha\beta$  T cells (CD3<sup>+</sup>,  $\gamma\delta$ TCR<sup>+</sup>),  $\gamma\delta$  T cells (CD3<sup>+</sup>,  $\gamma\delta$ TCR<sup>+</sup>), B cells (HLA-DR<sup>+</sup>, CD14<sup>+</sup>, CD11c<sup>+</sup>, CD123<sup>+</sup>), and granulocytes (HLA-DR<sup>+</sup>, CD14<sup>+</sup>) in the same sample. An example of the gating strategy used to identify cell populations and their cytokine response following PRR stimulation is shown in Fig. 1. The use of these comprehensive anchor markers allowed direct comparison of cell composition among sites (Fig. 2). This comparison identified several differences among cellular subpopulations. For example, although samples from South African children had percentages of granulocytes that were similar to the other three sites, they contained fewer monocytes, cDCs, pDCs,  $\alpha\beta$  T cells,  $\gamma\delta$  T cells, and B cells, whereas children from the Canadian cohort displayed the lowest percentage of  $\gamma\delta$  T cells.

#### Single-cytokine analysis

The expression of major innate cytokines, specifically IL-6, IL-12, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$ , was next identified at the single-cell level for each of our subpopulations of cells. We focused on these cytokines because they permit assessment of a broad range of immune functions (5, 10). An example of cytokine gating is shown in Fig. 1 and Supplemental Fig. 1.

*$\gamma\delta$  T cells,  $\alpha\beta$  T cells, B cells, and granulocytes.* Cytokine secretion following PRR stimulation was not observed above the level of unstimulated samples for granulocytes,  $\gamma\delta$  T cells,  $\alpha\beta$  T cells, and B cells. Therefore, we did not include these cell populations in the subsequent higher-level cytokine-based analysis.

*Monocytes.* Monocytes responded to stimulation with R848 (TLR7/8), LPS (TLR4), PAM (TLR1/2), PGN (TLR2 and NOD1/2), and MDP (NOD2) by producing IL-6, IL-12, IFN- $\gamma$ ,

and TNF- $\alpha$  but not IFN- $\alpha$ . No response was detected to stimulation with Poly I:C (TLR 3) (data not shown); thus, Poly I:C was not included in additional analyses. Monocyte production of cytokines in response to R848, LPS, PAM, PGN, and MDP differed significantly among groups, with subjects from South Africa harboring lower numbers of cytokine-expressing monocytes compared with the other three sites (Fig. 3, Supplemental Fig. 2A). The IL-6, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  responses of monocytes to R848 and LPS stimulation were significantly different among sites, with the largest differences due to the variation between South Africa and Canada, Ecuador, and Belgium. As noted by other investigators (21), we saw IFN- $\gamma$  production by monocytes in response to strong stimuli like LPS and R848. The production of each cytokine (IL-6, IL-12, IFN- $\gamma$ , and TNF- $\alpha$ ) in response to PAM was significantly different in monocytes from children from the four sites; the major contributor to this variation was the difference between South African subjects and those from Ecuador. The responses to PAM of South African versus Belgian and Canadian subjects also were significantly different, but only for IL-6. PGN-stimulated monocytes produced IL-6, IFN- $\gamma$ , and TNF- $\alpha$ , with a significant difference between South African and Canadian children. MDP stimulation of monocytes induced IL-6 and TNF- $\alpha$  production, which was only statistically different between Belgian and South African children (Supplemental Table I).

*Conventional dendritic cells.* A strong IL-6, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  response was induced in cDCs in response to the TLR and NOD ligands R848, LPS, PAM, PGN, and MDP. As for monocytes, cDCs did not produce any of the cytokines that we measured by flow cytometry in response to stimulation with Poly I:C (TLR3) (data not shown). Each cytokine produced by cDCs differed significantly among the four sites, with cDCs from South African children containing the lowest number of cytokine-expressing cells (Fig. 3). The cytokine response of cDCs following stimulation with PGN also was significantly different among sites, as a result of the large variation between South African children and Canadian, Ecuadorian, or Belgian children for IL-6 and TNF- $\alpha$ ; for IL-12 and IFN- $\gamma$ , the variation was most pronounced between South African and Canadian or Ecuadorian children (Supplemental Table I).

*Plasmacytoid dendritic cells.* pDCs only responded to the TLR7/8 ligand R848, producing IFN- $\alpha$ , IFN- $\gamma$ , IL-6, and TNF- $\alpha$  but not IL-12 (Fig. 3). The major differences originated from subjects from Canada versus Ecuador for IL-6, Canada versus South Africa for IFN- $\alpha$ , and Belgium versus South Africa for TNF- $\alpha$  (Supplemental Table I).

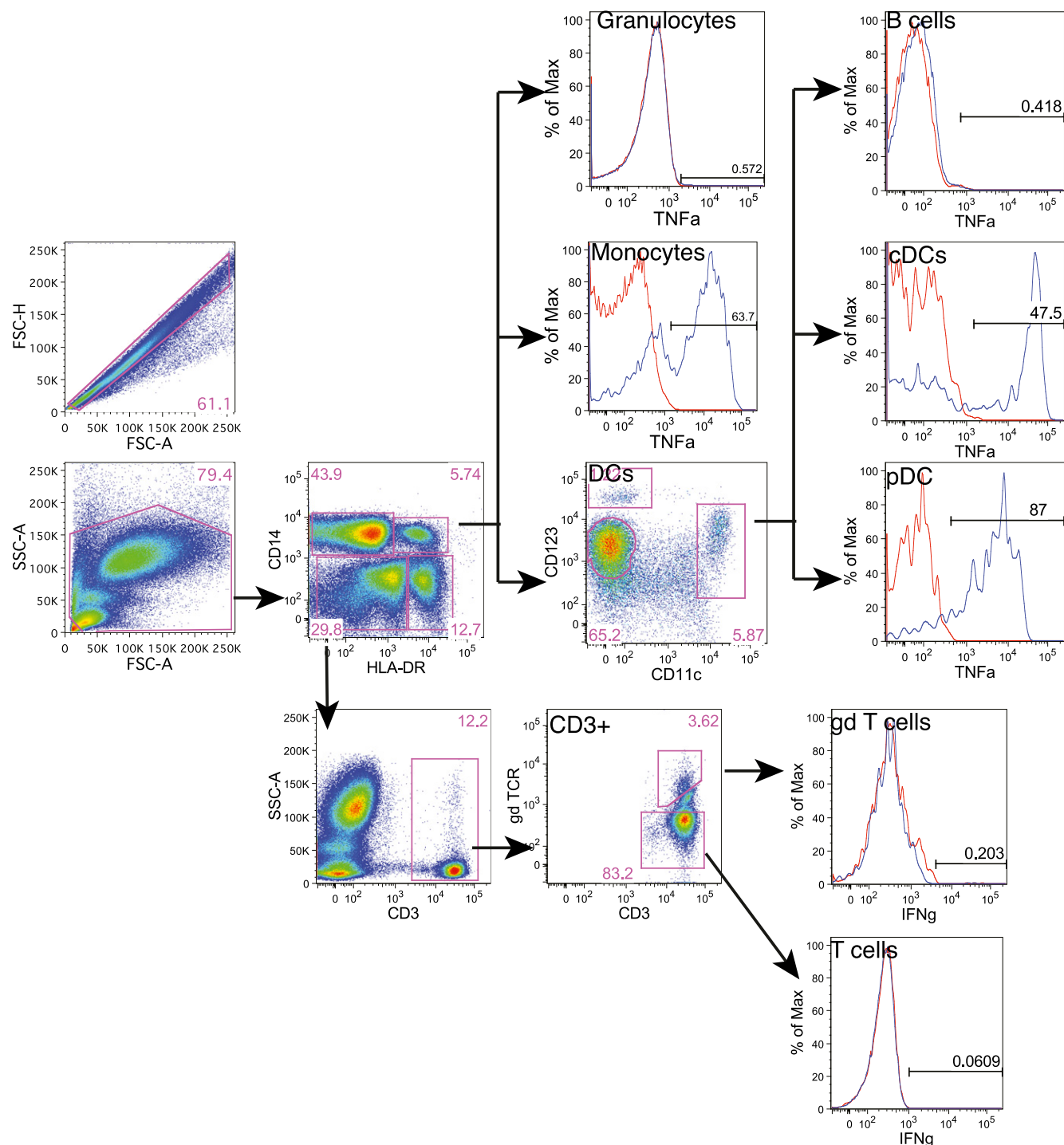
#### Multi-cytokine analysis

We set out to assess the capacity of cells in subjects from each of the four sites to produce multiple cytokines at the same time.

Table I. Demographics of the subjects at each of the four sites

Infant Characteristics	Belgium (n = 14)	Canada (n = 24)	Ecuador (n = 43)	South Africa (n = 20)
Age (mo): mean (SD)	24.7 (4.3)	19.3 (0.9)	26.7 (1.3)	24.7 (0.6)
Birth weight (g): mean (SD)	2,996.2 (796.3)	3,337.9 (435.1)	3,475.1 (988.3)	3,018.4 (383.6)
Birth mode (vaginal/cesarean section)	13/1	11/13	34/9	20/0
Gestational age (wk): mean (SD)	38.4 (3.4)	39.1 (1.7)	38.9 (1.1)	37.8 (2.4)
Premature <37 wk: n (% of total)	2 (14)	1 (4)	0 (0)	3 (15%)
Weight (g): mean (SD)	13,364.30 (1,786.1)	11,425.00 (1,553.5)	11,501.16 (1,010.7)	11,205.00 (1,300.7)
Height (cm): mean (SD)	92.2 (4.6)	82.7 (3.4)	84.3 (2.5)	84.4 (0.91)
Mean WAZ (SD)	0.69 (1.2)	0.04 (1.0)	-0.32 (0.93)	-0.58 (0.95)
Mean LAZ (SD)	1.56 (0.8)	0.29 (0.96)	-0.78 (1.49)	-1.07 (1.20)
Mean WLZ (SD)	-0.18 (1.4)	0.35 (1.04)	0.16 (0.79)	-0.03 (0.87)





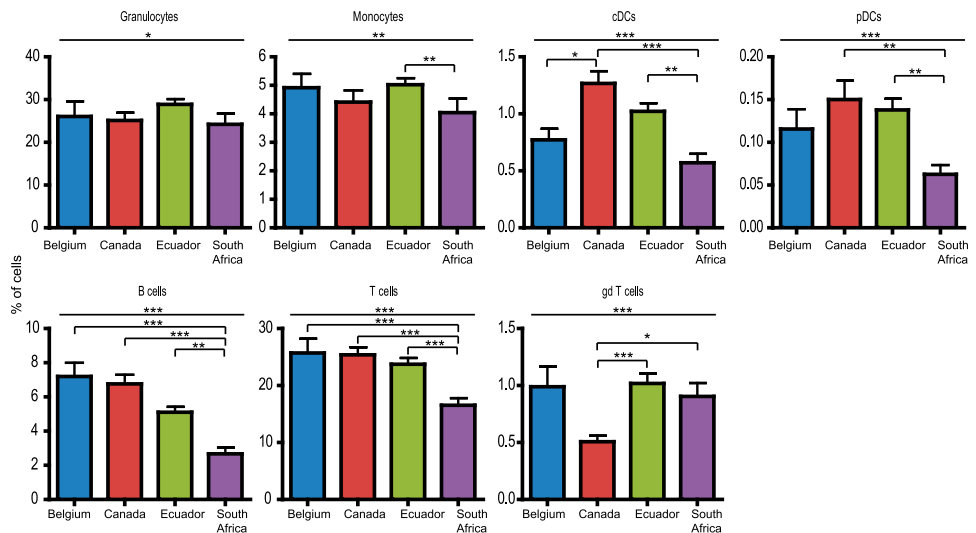
**FIGURE 1.** Gating strategy for the 11-color panel for flow cytometry.

**Monocytes. R848, LPS, and PAM.** The IL-6, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  response to all three stimulations were different among sites, with the largest differences due to the variation between South Africa and Canada, Ecuador, or Belgium (Fig. 4). Canadian and Ecuadorian children produced the highest polyfunctional response, including double cytokine-producing (TNF $\alpha$ <sup>+</sup>IL6<sup>+</sup>), triple cytokine-producing (TNF $\alpha$ <sup>+</sup>IFN $\gamma$ <sup>+</sup>IL6<sup>+</sup>, TNF $\alpha$ <sup>+</sup>IL12<sup>+</sup>IL6<sup>+</sup>), and quadruple cytokine-producing (TNF $\alpha$ <sup>+</sup>IFN $\gamma$ <sup>+</sup>IL12<sup>+</sup>IL6<sup>+</sup>) populations. South African children had an overall lower response (lowest height of the stacked bar) and responded primarily with single cytokine-producing cells (TNF $\alpha$ <sup>+</sup> or IL12<sup>+</sup>), with very few polyfunctional cytokine-producing monocytes detected.

**PGN and MDP.** Single cytokine-producing cells dominated the response to NOD2 stimulation across all four sites. PGN-stimulated monocytes responded mainly with TNF $\alpha$ <sup>+</sup>, followed by TNF $\alpha$ <sup>+</sup>IL6<sup>+</sup> production. Belgian and South African children displayed an overall lower response compared with Canadian and Ecuadorian children (Fig. 4). MDP evoked only a minimal response, but IL-6 and TNF- $\alpha$  production was still different among sites and was most pronounced between Belgian and South African children (Supplemental Fig. 2B).

The difference in monocyte cytokine production between children from different continents becomes more readily apparent when plotting the polyfunctional trends in a line graph (Fig. 4,

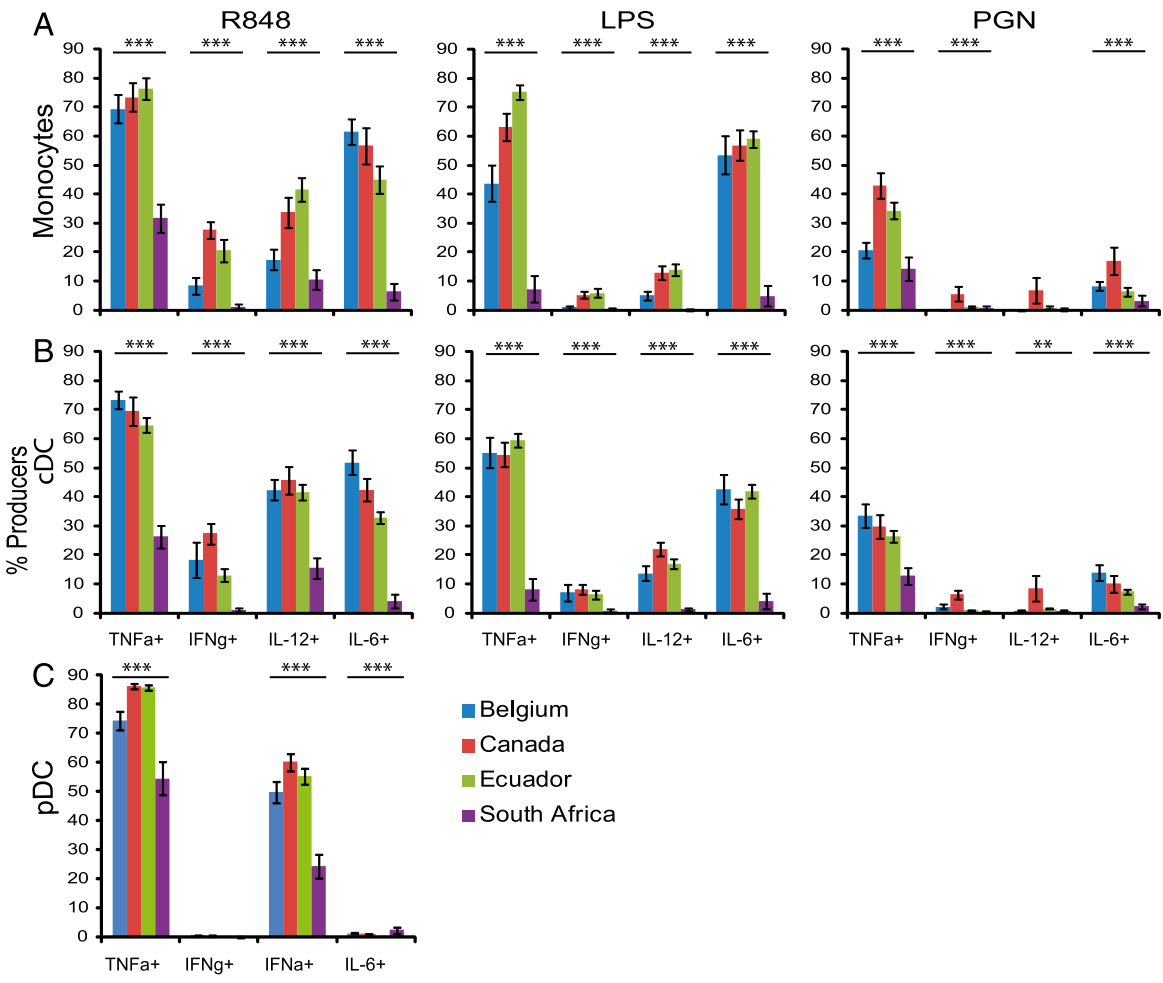
**FIGURE 2.** Cellular composition of whole blood cells at each of the four sites. \*\*\* $p < 0.005$ , \*\* $p < 0.01$ , \* $p < 0.05$ .



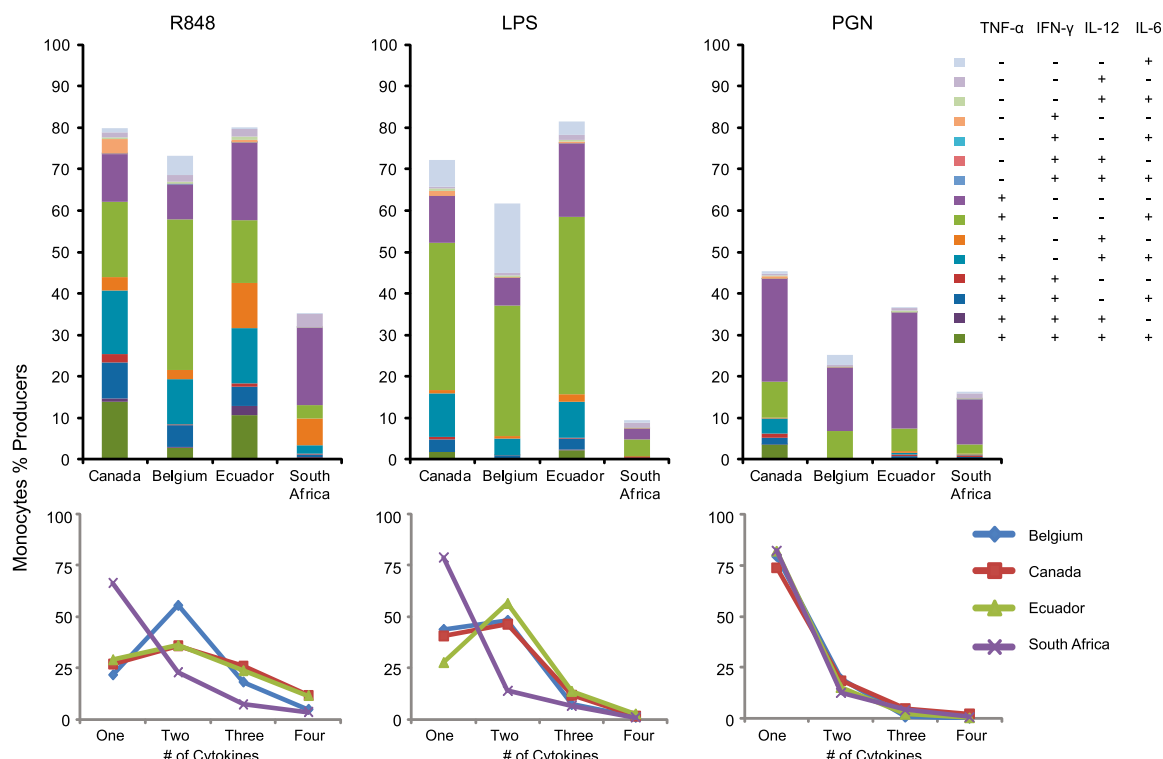
Supplemental Fig. 2B). The cytokine response of monocytes to R848 or LPS stimulation was dominated by single cytokine producers for South African children, whereas monocytes from the children at other sites contained single, double, and triple cytokine producers following each stimulation. PAM stimulation resulted in only a slight difference between South Africa and the other three sites,

whereas the polyfunctionality of monocytes stimulated with PGN and MDP did not vary significantly among sites.

**Conventional dendritic cells. R848, LPS, and PAM.** The relative fraction of multicytokine-producing cDCs in response to R848, LPS, and PAM stimulation was similar across all sites, with the exception of South Africa. Although cDCs from Canadian,



**FIGURE 3.** South African children have a weaker single cell-specific cytokine response to TLR or NLR ligand stimulation. Whole blood obtained from children from four sites was stimulated with R848, LPS, or PGN ligands and measured by flow cytometry for IL-6, IL-12, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$  production by monocytes (A), cDCs (B), and pDCs (C). The Kruskal-Wallis test was performed to examine the statistical differences of each cytokine/cell type/stimulation, followed by the Dunn post hoc test. \*\*\* $p < 0.005$ , \*\* $p < 0.01$ .



**FIGURE 4.** Multicytokine response of monocyte single cell type showed that South African children exhibited weaker and less diverse responses to TLR and NLR ligands. Whole blood obtained from children from four sites were stimulated with R848, LPS, or PGN ligands and measured by flow cytometry for IL-6, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  levels. The stacked bar graphs represent the combination of cytokine contributions/cell type (*upper panels*). The line graphs indicate the polyfunctional state of the cell type, summarizing the percentages of cells producing one, two, three, or four cytokines in response to stimulation (*lower panels*).

Belgian, and Ecuadorian children responded with a large number of single, double, or triple cytokine-producing cells, South African cDCs responded only with single cytokine-producing cells (IL12<sup>+</sup> and TNF $\alpha$ <sup>+</sup>) (Fig. 5, Supplemental Fig. 2B).

**PGN and MDP.** cDCs responding to PGN stimulation were dominated by the TNF $\alpha$ <sup>+</sup> response, followed by TNF $\alpha$ <sup>+</sup>IL6 double-producing cells. The largest diversity in the cytokine response was seen in Canadian children (Fig. 5). MDP stimulation primarily resulted in a TNF $\alpha$ <sup>+</sup> single cytokine-producing cDC population at each site (Supplemental Fig. 2B).

The polyfunctional line graph trend lines confirmed that responses of cDCs to R848, LPS, and PAM stimulation were dominated by a single cytokine response in the cDCs from South African children, whereas those from the other sites contained both single cytokine- and multicytokine-producing cells. However, cDCs stimulated with PGN and MDP did not show a difference between sites (Fig. 5, Supplemental Fig. 2B).

**Plasmacytoid dendritic cells.** Following R848 stimulation, the largest fraction of pDC responses consisted of double cytokine-producing cells (TNF $\alpha$ <sup>+</sup>IFN $\alpha$ <sup>+</sup>); the single-positive cells comprised the second largest fraction. The polyfunctional line graph revealed that pDCs from South African children predominantly produced single cytokines in response to R848 stimulation response, whereas pDCs from children at the other three sites responded primarily with the production of double cytokines (Fig. 6).

#### Polyfunctional index

The above-summarized single-cell approach to determine the production of multiple cytokines in response to PRR stimulation allowed us to statistically assess the ability of each cell to produce more than one cytokine at the same time (i.e., their PI) (Fig. 7) (17).

**Monocytes.** The PI for monocytes from South African children was lowest compared with all other sites, whereas it was similar among Belgian, Canadian, and Ecuadorian children. This difference for South African children was most pronounced in response to R848, LPS, PAM, and PGN and was less pronounced in response to MDP (Fig. 7, Supplemental Fig. 3).

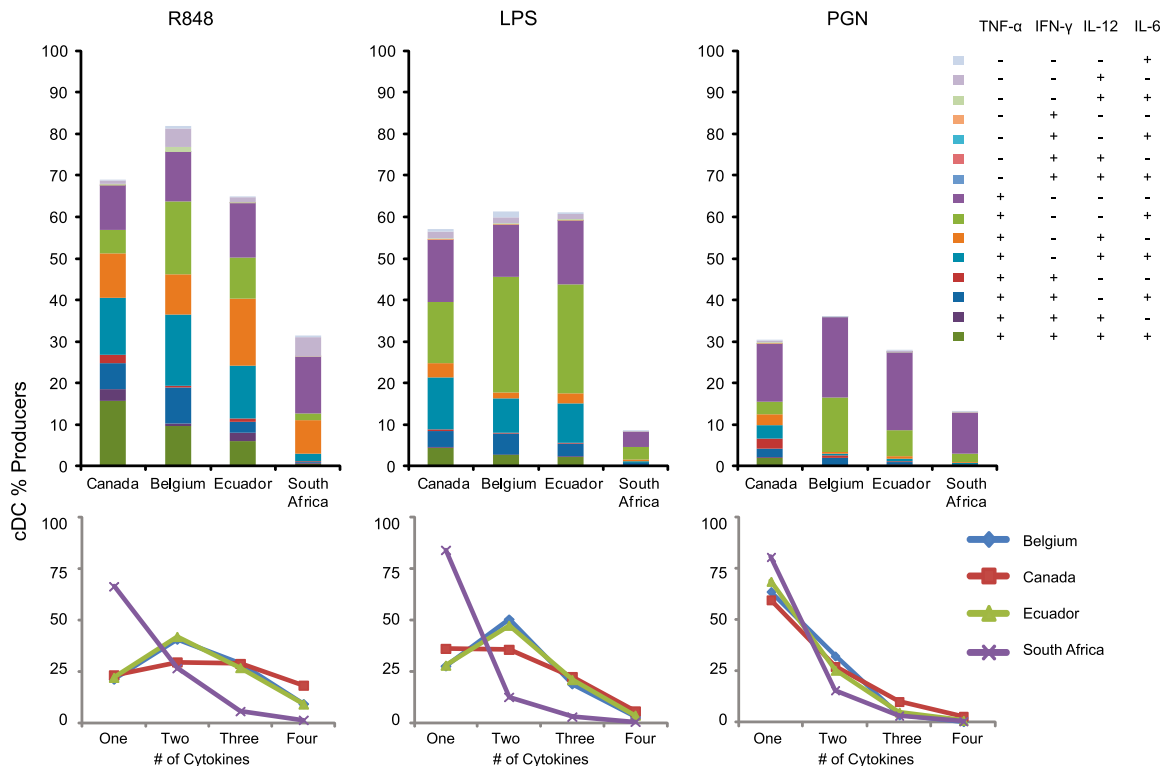
**Conventional dendritic cells.** In response to R848, LPS, PAM, PGN, and MDP stimulation, the South African children mounted cDC polyfunctional responses that were significantly lower than those in children from the other sites. Statistical analysis revealed that this difference was due to variation in responses between South African and Belgian, Canadian, and Ecuadorian children (Fig. 7, Supplemental Fig. 3).

**Plasmacytoid dendritic cells.** Statistical analysis of the PI for pDCs following R848 stimulation identified a significant difference between South African and Canadian, Ecuadorian, and Belgian children (Fig. 7). South African subjects showed the lowest response.

## Discussion

We recently identified that innate immune responses early in life differ among children from different continents, with a cohort of South African children secreting significantly less cytokine following PRR stimulation compared with children in cohorts from Ecuador, Belgium, or Canada (5). Using a stringently controlled, high-throughput intracellular cytokine flow cytometry-based analysis, we determined that this difference was the result of an overall lower fraction of innate cells in the peripheral circulation, as well as the lower fraction of innate cells producing cytokines.

In our previous study, we analyzed cytokine secretion into culture medium following in vitro stimulation; however, this approach



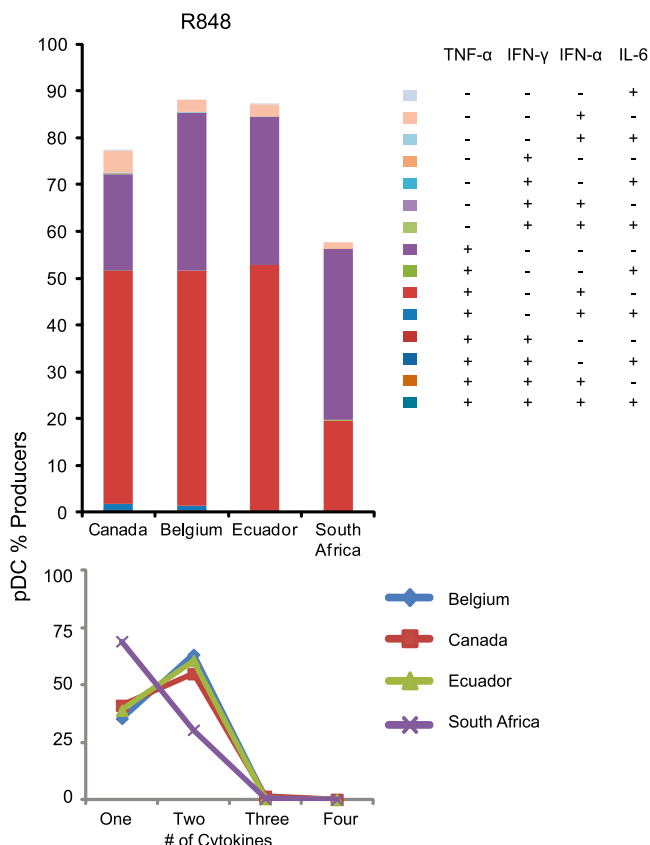
**FIGURE 5.** Multicytokine responses of cDC single cell type showed that South African children exhibited weaker and less diverse responses to TLR and NLR ligands. Whole blood obtained from children from four sites was stimulated with R848, LPS, or PGN ligands and measured by flow cytometry for IL-6, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  levels. The stacked bar graph represents the combination of cytokine contributions/cell type (upper panel). The line graph indicates the polyfunctional state of the cell type, summarizing the percentages of cells producing one, two, three, or four cytokines in response to stimulation (lower panel).

does not provide the detail necessary to guide further delineation of underlying mechanism(s). We now set out to identify the response at the single-cell level to determine whether differences in global cytokine secretion were due to differences in blood cell composition, innate cell subset-specific differences in response to PRR stimulation, or both. We found that the composition of the peripheral WBC compartment varied among children across all four continents, with the peripheral blood from South African children harboring the lowest fraction of the main PRR responder cell types: monocytes, cDCs, and pDCs. Because granulocytes,  $\gamma\delta$  T cells,  $\alpha\beta$  T cells, B cells, and the few remaining unidentified cells did not produce levels of cytokines above background, the notable quantitative differences in these cell populations between children from different sites were unlikely to contribute directly to the observed difference in secreted cytokines. Although our data support the notion that differences in cellular composition could have contributed to differences in cytokine secretion after PRR stimulation, whole blood from South African children contained as many monocytes, cDCs, and pDCs as did the whole blood of children from other sites. This suggests that differences in cell composition alone were unlikely to be fully accountable for the lower secreted cytokine response detected in blood from South African children. Population-based differences in cellular composition were described previously (22, 23). A comparison of European and Ugandan children noted lower lymphocyte counts in Ugandan children compared with black European children, whereas neutrophil counts were similar (23). Overall, these findings appear to be consistent with our data.

The single-cell-based approach of our current study allowed us to identify cell population-specific differences at the single-cell level of functional PRR stimulation responses and con-

trast them between geographic sites. Previously, we identified an age-dependent change in innate responsiveness to PRR stimulation of monocytes, cDCs, and pDCs (24). More importantly, our previous longitudinal cohort studies following Canadian (5, 10) and South African children (11) from birth over the first few years of life suggested that the developmental trajectories in response to PRR stimulation might differ between children from these two countries. However, these previous studies were not conducted using the same reagents or protocols, precluding a direct comparison. The cross-sectional study presented in this article was set up to allow precisely this kind of direct comparison. Our data clearly indicate that the innate immune response with respect to PRR stimulation of monocytes, cDCs, and pDCs differed significantly between our cohorts of South African and Canadian children. By conducting this stringently controlled side-by-side comparison for all of our cohorts across four continents, we can now extend this conclusion to state that, at the single-cell level, the South African cohort's innate immune response differed from the Canadian cohort, as well as the Belgian and Ecuadorian cohorts. Although there were differences in monocyte, cDC, and pDC responses to PRR stimulation among children from Canada, Belgium, and Ecuador, these differences were relatively minor compared with the strikingly and consistently lower response of South African children. This lower functional response to PRR stimulation in our cohort of South African children extended across all of the PRR stimulation-responsive cell types, applied to all PRR stimuli tested, and included all cytokines measured, including the degree of polyfunctionality. Together, these data begin to outline a state of relative innate immune suppression in our cohort of South African children compared with children from other parts of the world.





**FIGURE 6.** Multicytokine response of pDC single-cell type showed that South African children exhibited weaker and less diverse responses to TLR7/8 ligand. Whole blood was stimulated with R848 ligand and measured by flow cytometry for IL-6, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$  levels. The stacked bar graph represents the combination of cytokine contributions/cell type (upper panel). The line graph indicates the polyfunctional state of the cell type, summarizing the percentages of cells producing one, two, three, or four cytokines in response to stimulation (lower panel).

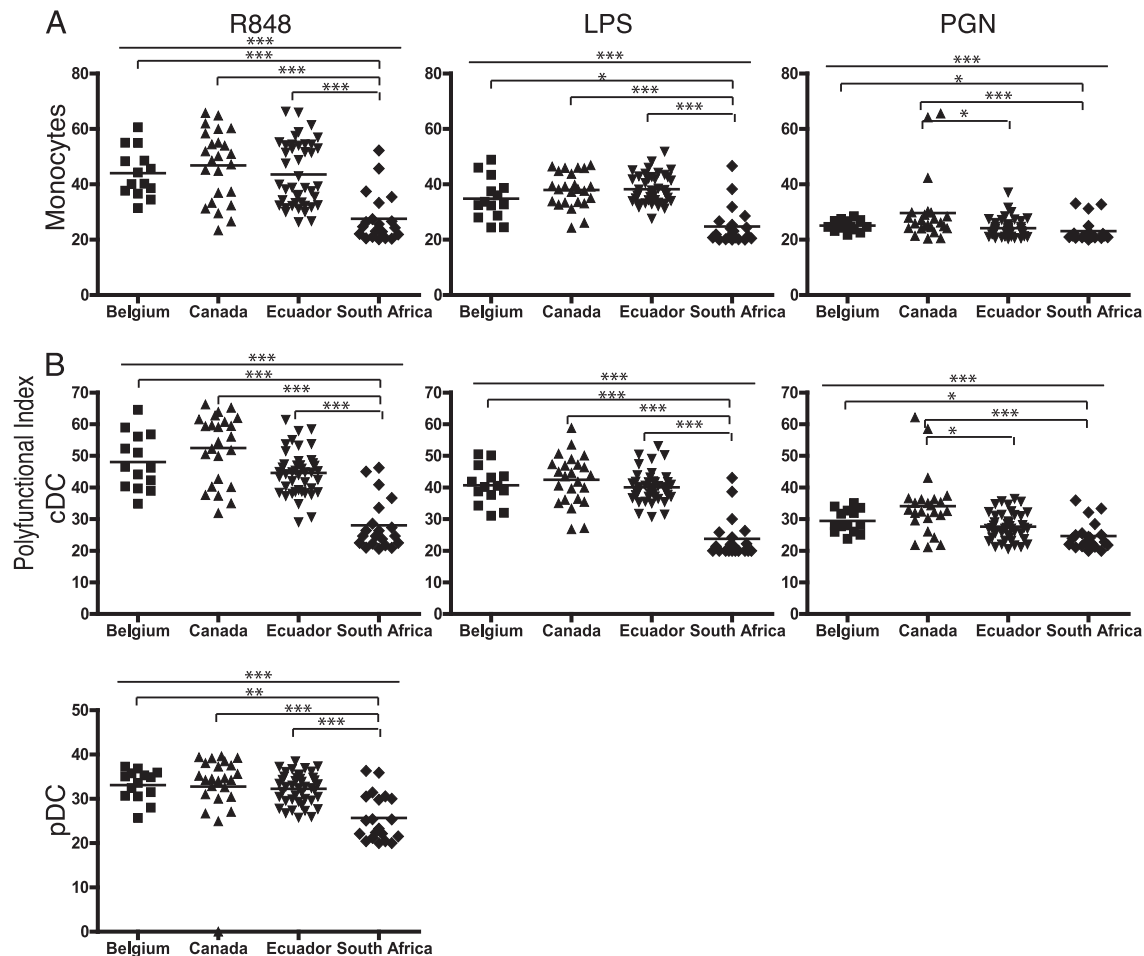
A reduction in polyfunctionality has been described for T cells following chronic infections, such as HIV, hepatitis C virus, and EBV (25, 26), or administration of immune suppressive medications (27). Functionally, this lower degree of T cell polyfunctionality has been linked to an increased risk for infection in transplant patients (27) and to decreased control of HIV replication in HIV-infected subjects (28). To our knowledge, our data are the first to identify differences in the degree of polyfunctionality of innate immune cells. Han et al. (29) showed that T cell stimulation initiates cytokine responses in an asynchronous manner, with a dynamic trajectory of responses occurring in a sequential manner. We have yet to conduct a time course evaluation of the intracellular cytokine response in our subjects to determine whether differences in kinetics contribute to differences in polyfunctionality.

The lower response of our South African cohort versus the other cohort children to PRR stimulation could be due to variations in host genetics and/or environmental differences. Differences in host genetic composition are known to influence innate immunity (30). We recently showed that variation in innate immune responses can be influenced by single nucleotide polymorphisms within the PRR pathways and that the prevalence of these single nucleotide polymorphisms varies among different racial backgrounds (31). Thus, it is entirely possible that genetic differences among our populations contributed to the differences in functional responses that we measured among sites. However, given the wide variation in racial background of the parents in our

South African and Belgian cohorts (including African, white, Asian and mixed), we do not believe that differences in genetics alone would explain the consistently lower innate cytokine response of the South African children compared with the children from other sites.

Our data support the conclusions of Lisciandro et al. (32, 33), who suggested that APC immune responses are lower in traditional environments versus modern environments. However, our data do not support simple division into resource-rich versus resource-poor individuals or a distinction based on latitude, because children from Ecuador (considered resource poor) displayed equivalent intracellular cytokine responses as did children from Belgium or Canada (considered resource rich) but a much higher response compared with children from South Africa (considered resource poor). Thus, environmental factors leading to the lower response of South African children would have to be more specific to South Africa, possibly even to the area of the Western Cape within South Africa from which our cohort was recruited (34). Although the exact nature of the environmental factor(s) is unknown, we can exclude a range of possible candidates. Environmental factors, such as vaccination, feeding mode, birth mode, birth weight, and age, may impact innate immune ontogeny (3, 8, 35–38). However, among our four global cohorts, vaccine formulations and schedules were very similar and adhered to the Expanded Program on Immunization (39). The most notable difference among our cohorts was the use of neonatal BCG vaccination. Although BCG was not given to newborns in Belgium and Canada, it was administered to the children from South Africa and Ecuador. Given that Ecuadorian children displayed an innate immune response more akin to Belgian and Canadian children than to South African children, neonatal BCG seems an unlikely culprit to explain the difference in child innate immune status around 2 y of age. Djuradi et al. also found no clear effect of BCG vaccination on the innate immune ontogeny. However, although the overall vaccination schedule was similar for all children at each site, differences in vaccine composition (e.g., acellular versus whole-cell pertussis) or exact age of vaccination differed among sites. As a result of this, we cannot exclude that variation in standard childhood vaccination might contribute to the observed differences in innate immune development.

Given that all of our children were enrolled according to the same well-defined inclusion and exclusion criteria, differences in medical illness over the first 2 y of life are unlikely to have contributed to the differences among sites that we detected. We also interpret our data to indicate that differences in feeding mode (e.g., duration of breast-feeding, breastfeeding versus bottle feeding) were unlikely to be major contributors to the differences among the sites that we observed. Although we cannot exclude that feeding mode could lead to subtle differences, our data suggest that it has minimal impact because it differed vastly both among and within sites, whereas the innate immune response variation within sites was negligible. Furthermore, variation in innate immune response to PRR stimulation did not correlate with feeding mode when comparing individuals within one site (data not shown). Although studies showed that birth mode can impact the children's immune system up to the age of 5 y (3, 36), we were unable to detect an association between birth mode and innate immune response (data not shown). Specifically, approximately half of the Canadian children were born via cesarean section, whereas nearly all of the South African, Ecuadorian, and Belgian children were born vaginally. Although birth weight and gestational age were shown to correlate with a higher risk for infant mortality (38), their impact on postnatal innate immune trajectory has not been delineated. However, the average birth weight was similar for all



**FIGURE 7.** PI values of children at each site/cell type in response to TLR and NLR ligand stimulation. **(A)** PI numerically evaluated the degree and variation of polyfunctionality within the four-site cohort. It allows for the difference among cytokines produced by the different cell type to defined stimulations (R848, LPS, PGN) to be observed. **(B)** The Kruskal–Wallis test was used to compare the four sites per cell type and stimulation. The Dunn post hoc test was applied to each site pairing. \*\*\* $p < 0.005$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

of our cohorts and within the “normal” range for each site. Importantly, all of the subjects in our cohort fell within the average WHO Child Growth Indices (for all WAZ, LAZ, and WLZ). Additionally, the total number of children enrolled in our cohorts who were born prematurely (<37 gestational weeks of age) was very low ( $n = 3$  in South Africa and  $n = 2$  in Belgium, ~15% per cohort). This suggests that neither birth weight nor gestational age could explain the differences that we detected among our cohorts. Lastly, although several studies identified differences in immune status based on sex, analysis of our data stratified by sex of the subjects did not reveal any significant differences (data not shown). This may be due to the small sample size, but it precludes sex as the major determinant for the differences that we detected among our global cohorts with regard to innate immune response to PRR stimulation.

The major limitation of this study is the relatively small number of subjects per site. Nevertheless, our data identifying lower innate cytokine responses in our cohort of South African children were consistent across multiple stimuli and for multiple cell types, suggesting that our findings are likely biologically relevant and possibly clinically meaningful. Clearly, our findings will need to be replicated on a larger scale. It should also be borne in mind that our cohort recruitment was not representative of the entire population at each site. For instance, the Ecuadorian children were selected from a population-based rural cohort and do not represent all Ecuadorian children from a wide variety of backgrounds and

environments. Despite these limitations, our data strongly support the existence of profoundly reduced innate immune responsiveness to PRR stimulation in South African children. Whether such quantitative and qualitative innate immune deficiency compared with other regions of the world has clinical implications is not entirely clear, but it warrants further exploration.

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## Disclosures

The authors have no financial conflicts of interest.

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