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Single cell analysis of innate cytokine responses to pattern recognition receptor stimulation in children across four continents

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Abstract

Innate immunity instructs adaptive immunity, and suppression of innate immunity is associated with increased risk for infection. We had previously shown that whole blood cellular components from a cohort of South African children secreted significantly lower levels of most cytokines

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following stimulation of pattern recognition receptors (PRR) as compared to whole blood from cohorts of Ecuadorian, Belgian, or Canadian children. To begin dissecting the responsible molecular mechanisms, we now 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, significant reduction of the intracellular cytokine production on the single cell level was only detected in South African childrens' monocytes, cDC, and pDC. We also uncovered a marked reduction in polyfunctionality for each of these cellular compartments in South African children as compared to children from 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.

Introduction

Susceptibility 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). As 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. To date, no worldwide comparison of cell-specific innate immune response has been conducted.

The innate immune system relays on germline-encoded Pattern Recognition Receptor (PRR), which recognize conserved molecular motifs in microbes known as pathogen-associated molecular patterns (PAMPs). Sensing of PAMPs 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 had previously found that children from a South African cohort secreted markedly lower amounts of nearly every cytokine measured following PRR stimulation as compared to 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 or the response of particular cell subsets, or both. As the first step towards identifying the responsible cellular and molecular mechanisms, we here employed single-cell intracellular cytokine cytometry in order to identify the cellular compartment(s) from which the observed differences arose. Given our eventual goal to determine the impact of innate immune variation on vaccine responses, we focused on the main antigen-presenting cells, namely conventional and plasmacytoid dendritic cells (cDC and pDC) as well as monocytes; however, we also included granulocytes, TCR-αβ and -γδ T cells as well as 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

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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 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. This study was approved by the University of British Columbia Ethics Board (protocol: H11-01423). Additionally each site involved had obtained ethics approval in their respective research centre. Informed written consent from the next of kin, care givers or guardians on the behalf of the minors involved in this study was obtained for all study participants.

Participants Recruitment and Enrollment

This study compared children 2 years of age from four different sites: Vancouver, 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). Children from Ecuador were recruited within a rural-based population cohort study (6), while 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 months, 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 units of sodium-heparin (Becton Dickinson (BD) Biosciences, catalog no. 8019839). Blood samples were kept at room temperature and processed within < 4 hrs 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 pattern recognition receptor (PRR) stimulation is vulnerable to technical artifacts (8). In order to minimize technical artifacts we employed 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 1μg/ml; Polyinosinic-polycytidylic acid (Poly I:C; TLR3; Amersham Biosciences) at 100μg/ml; Lipopolysaccharide (LPS; TLR4, InvivoGen) at 10ng/ml; R848 (R848; TLR7/8, InvivoGen) at 10μM; Peptidoglycan (PGN; NOD1/2, InvivoGen) at 10μg/ml; Muramyl dipeptide (MDP; Nucleotide-binding oligomerization domain (NOD) 2, InvivoGen) at 0.1μg/ml; and media alone. All of the wells contained Brefeldin A (BFA; Sigma-Aldrich) at 10ug/ml. The same person (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 pre-warmed RPMI 1640, and 200μl 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% 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 1.4ml of 1× BD FACS Lysing Solution, placed into fresh tubes, and stored at −80°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 μl of 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 100 μl of PBSAN for 45 min at room temperature. After two additional washes with PBSAN, cells were resuspended in PBSAN and analyzed on an LSRII Flow Cytometer (BD Biosciences). Cytometer Setup and Tracking beads (CST; BD Biosciences) were used to calibrate the machine prior to each use to ensure machine performance remained the same. Compensation beads (CompBeads; BD Biosciences) were used to standardize voltage settings and 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 if the median fluorescence intensity of cytokine populations remained the same between experiments, i.e. ensuring the CST-based set-up performed according to the manufacturer's specifications. A total of 500,000 uncompensated events were acquired per sample. Compensation was set in FlowJo (Tree Star) 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 (FSC/SSC) appearance as described previously (8, 9); no difference in viability was noted between samples from the four sites.

Standardization

Given its role to rapidly sense environmental change (15, 16), technical artifacts can quickly impact innate immune assessment (8). We thus implemented an experimental approach with stringent rigor and focus on quality assurance, in order 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 absence of innate immune activating substances (8, 9). Samples post-processing were shipped on dry ice with temperature monitors; this revealed that temperatures remained stable at −80°C during all shipments. Upon arrival at the central analysis site (Vancouver, Canada), samples were stored frozen in liquid nitrogen. All samples were run within 12 months 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 (PI)

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. Larsen (Paris, France).

Statistical Analysis

Kruskal-Wallis analysis was performed to compare the four sites for significant variance among the median cytokine response. Dunn’s post-test was used to determine which of the sites contributed to the significant differences. Statistical analysis was conducted in Prism Version 6 (GraphPad Software, La Jolla, CA, USA).

Z-score Analysis

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

Results

Cohort Characteristics

Four different 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 approximately 24 months. 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 normal range with no more than +/−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 antigen presenting cell (APC) target populations in whole blood. These include monocytes (HLA-DR+, CD14+), cDCs (HLA-DR+, CD14−, CD11c+, CD123−), and pDCs (HLA-DR+, CD14−, CD11c−, CD123+). We also identified αβ-T cells (CD3+, γδTCR−), γδ-T cells (CD3+, γδTCR+), B cells (HLA-DR+, CD14−, CD11c−, CD123−), and granulocytes (HLA-DR−, CD14+) in the same sample. An example of the gating strategy employed to identify cell populations and their cytokine response following PRR stimulation is shown in Figure 1. The use of these comprehensive anchor markers allowed direct comparison of cell composition between sites (Figure 2). This comparison identified several differences between cellular subpopulations. For example, while samples from South African children had similar percentages of granulocyes as the other three sites, they contained fewer monocytes, cDC, pDC, αβ-T cells, γδ-T cells, as well as B cells, while children from the Canadian cohort displayed lowest percentage of γδ-T cells.

Single-Cytokine Analysis

The expression of major innate cytokines, specifically IL6, IL12, IFNα, IFNγ, and TNFα was next identified at the single cell level for each of our subpopulations of cells. We focused on these cytokines as they permit assessment of a broad range of immune functions (5, 10) An example of cytokine gating is shown in Figure 1 and Supplementary Figure 1.

γδ-T cells, αβ-T cells, B-cells, and Granulocytes—Cytokine secretion following PRR stimulation was not observed above the level of unstimulated samples for granulocytes, γδ-T cells, αβ-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 (TLR 7/8), LPS (TLR 4), PAM (TLR 1/2), PGN (TLR 2 and NOD 1/2), and MDP (NOD 2), by producing IL6, IL12, IFNγ, and TNFα but not IFNα. No response was detected to stimulation with PolyI:C (TLR 3) (data not shown); PolyI:C was not included in further analysis. Monocyte production of cytokines in response to R848, LPS, PAM, PGN, and MDP were found to differ significantly between groups, with subjects from South Africa harboring lower numbers of cytokine-expressing monocytes compared to the other three sites (Figure 3, Supplementary Figure 2a). The IL6, IL12, IFNγ, and TNFα responses of monocytes to R848 and LPS stimulation were found to be significantly different between sites, with the largest differences due to the variation between South Africa vs. Canada, Ecuador, and Belgium. As seen by others (21), we also saw IFNγ production by monocytes in response to strong stimuli like LPS and R848. Response to PAM showed that production of each cytokine (IL6, IL12, IFNγ, and TNFα) was significantly different in monocytes from children of the four sites; the major contributor to this variation was the difference between South African subjects vs. those from Ecuador. The responses to PAM of South African vs. Belgian and Canadian subjects were also significantly different but only for IL6. PGN stimulated monocytes produced IL6, IFNγ, and TNFα, with significant difference between South African and Canadian children. MDP stimulation of monocytes induced IL6 and TNFα production, which was statistically different between Belgian vs. South African children only (Supplementary Table I).

cDC—A strong IL6, IL12, IFNγ, and TNFα response was induced in cDC in response to the TLR and NOD ligands R848, LPS, PAM, PGN, and MDP. As for monocytes, cDC did not produce any of the cytokines we measured by flow cytometry in response to stimulation with PolyI:C (TLR 3) (data not shown). Each cytokine produced by cDC differed significantly between the four sites, with cDC from South African children containing the lowest number of cytokine expressing cells (Figure 3). The cytokine response of cDC following stimulation with PGN was also significantly different between sites, as a result of the large variation between South African children compared to Canadian, Ecuadorian, or Belgian children for IL6 and TNFα for IL12 and IFNγ the variation was most pronounced between South African vs. Canadian or Ecuadorian children (Supplementary Table I).

pDC—pDCs only responded to the TLR7/8 ligand R848, producing IFNα, IFNγ, IL6 and TNFα, but not IL12 (Figure 3). The major differences originated from subjects from Canada vs. Ecuador for IL6, Canada vs. South Africa for IFNα, and Belgium vs. South Africa for TNFα (Supplementary 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.

Monocytes

R848, LPS, PAM: The IL6, IL12, IFNγ, and TNFα response to all three stimulations were found to be different between sites, with the largest differences due to the variation between South Africa vs. Canada, Ecuador, or Belgium (Figure 4). Canadian and Ecuadorian children produced the highest polyfunctional response including double-(TNFα+IL6+), triple-(TNFα+IFNγ+IL6+, TNFα+IL12+IL6+), and quadruple-(TNFα+IFNγ+IL12+IL6+) cytokine producing populations. South African children had an overall lower response (lowest height of the stacked bar) and responded primarily with single-cytokine producing cells (TNFα+ or IL12+), with very few polyfunctional-cytokine producing monocytes detected.

PGN, MDP: Single-cytokine producing cells dominated the response to NOD2 stimulation across all four sites. PGN stimulated monocytes responded mainly with TNFα+, followed by TNFα+IL6+ production. Belgian and South African children displayed an overall lower response compared to Canadian and Ecuadorian children (Figure 4). MDP evoked only a minimal response, but IL6 and TNFα production was still different between sites, and most pronounced between Belgian vs. South African children (Supplemental Figure 2b).

The difference in monocyte cytokine production between children from different continents becomes visually more readily apparent when plotting the polyfunctional trends in a line graph (Figure 4, Supplemental Figure 2b). The cytokine response of monocytes to R848 or LPS stimulation was dominated by single cytokine producers for South African children, while 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 African and the other three sites, while the polyfunctionality of monocytes stimulated with PGN and MDP did not vary significantly between sites.

cDC

R848, LPS, PAM: The relative fraction of multi-cytokine producing cDC in response to R848, LPS, and PAM stimulations were similar across all sites except for South African responses. While cDCs from Canadian, Belgian, and Ecuadorian children responded with a large number of single-, double-, or triple-cytokine producing cells, South African cDC responded with only single-cytokine producing cells (IL12+ and TNFα+) Figure 5, Supplementary Figure 2b.

PGN, MDP: cDCs responding to PGN stimulation were dominated by TNFα+ response, followed by TNFα+IL6 double-producing cells. The largest diversity of cytokine response was seen in Canadian children (TNFα Figure 5, Supplemental Figure 2b).

The polyfunctional line graph trend lines confirmed that responses of cDC to R848, LPS, and PAM stimulation were dominated by a single cytokine response in the South African children cDC, while cDC from the other sites contained both single as well as a multi-cytokine producing cells. cDC stimulated with PGN and MDP however did not show a difference between sites (Figure 5, Supplemental Figure 2b).

pDC—Following R848 stimulation, the largest fraction of pDC responses consisted of double-cytokine producing cells (TNFα+IFNα+), the second largest fraction were the single-positive cells. The polyfunctional line graph revealed that the cytokine response of pDC to R848 stimulation was predominately a single-cytokine response for the South African children, while pDC from children of the other three sites primarily responded with double-cytokine production (Figure 6).

Polyfunctional Index (PI)

The above summarized single-cell approach to determine 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 polyfunctional index (PI) Figure 7 (17).

Monocytes—The PI for monocytes from South African children was lowest compared to all other sites, while it was similar between Belgian, Canadian, and Ecuadorian children. This difference of South African children was most pronounced in response to R848, LPS, PAM, so in response to MDP and PGN less (Figure 7, Supplemental Figure 3).

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

pDC—Statistical analysis of the PI for pDC following R848 stimulation identified difference between South African vs. Canadian, Ecuadorian, as well as Belgian children as significant (Figure 7). South African subjects showed the lowest response.

Discussion

We have 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 to children in cohorts from Ecuador, Belgium or Canada [5]. Employing a stringently controlled, high-throughput intracellular cytokine flow cytometry-based analysis we have now determined that this difference was the result of an overall lower fraction of innate cells in the peripheral circulation as well as 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 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 in order to determine if 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 white blood cell compartment varied between children across all four continents, with the peripheral blood from South African children harboring the lowest fraction of the main PRR responder cell types, namely monocytes, cDC and pDC. Since neither granulocytes, γδ-T cells, αβ-T cells, B cells nor the few remaining unidentified cells produced levels of cytokines above background, the notable quantitative differences in these cell populations between children from different sites were unlikely to directly contribute to the observed difference in secreted cytokines. While 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, cDC and pDC as 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 have been previously described (22, 23). A comparison of European vs. Ugandan children noted lower lymphocyte counts in Ugandan children compared to black European children, while neutrophil counts were similar (23). These findings overall appear 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 contrast them between geographic sites. We previously identified an age-dependent change in innate responsiveness to PRR stimulation of monocytes, cDC, and pDC (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 here 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, cDC, and pDC 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 4 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 not only the Canadian but also the Belgian and Ecuadorian cohorts. While there were differences in monocyte, cDC, and pDC responses to PRR stimulation between children from Canada, Belgium and Ecuador, these differences were relatively minor compared to 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 as compared to children from other parts of the world.

A reduction in polyfunctionality has been described for T cells following chronic infections such as HIV, HCV and EBV (25, 26) or administration of immune suppressive medications (27). Functionally, this lower degree of T cell polyfunctionality has been linked to increased risk for infection in transplant patients (27), and 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 Q et al. have shown that T cell stimulation initiates cytokine responses in an asynchronous manner with a dynamic trajectory of responses occurring in a sequential manner (29). We have yet to conduct a time course evaluation of the intracellular cytokine response in our subjects, to determine if differences in kinetics contribute to differences in polyfunctionality.

The lower response of our South African vs. other cohort children to PRR stimulation could be due to variation in host genetics and/or environmental differences. Differences in host genetic composition are known to influence innate immunity (30). We have recently shown that variation in innate immune responses can be influenced by single nucleotide polymorphisms (SNPs) within the PRR pathways, and that the prevalence of these SNPs varies between different racial backgrounds (31). It is thus entirely possible that genetic differences between our populations contributed to the differences in functional responses we measured between sites. However, given the wide variation in racial background of the parents in our South African and Belgian cohorts (including African, Caucasian, 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 as compared to children from the other sites.

Our data support the conclusions of Lisciandro et al., who suggested that APC immune responses are lower in traditional vs. modern environments (32, 33). However, our data do not support simple division into resource-rich vs. resource poor individuals, nor a distinction based on latitude, as children from Ecuador (considered resource-poor) displayed equivalent intracellular cytokine responses to children from Belgium or Canada (considered resource-rich), but a much higher response than children from South Africa (considered resource-

poor). Environmental factors leading to the lower response of South African children would thus have to be more specific to South Africa, and possibly even to the area of the Western Cape within South Africa from which our cohort was recruited (34). While the exact nature of the environmental factor(s) is presently unknown, we can already exclude a range of possible candidates. Environmental factors, such as vaccination, feeding mode, birth mode, birth weight and age, all may impact innate immune ontogeny (3, 8, 35-38). However, amongst our four global cohorts, vaccine formulations and schedules were very similar, and adhered to the Expanded Programme on Immunization (EPI). The most notable difference between our cohorts was the use of neonatal BCG vaccination. While BCG was not given to newborns in Belgium and Canada, it was administered to the children from both South Africa and Ecuador. Given that Ecuadorian children displayed an innate immune response more akin to Belgian and Canadian than South African children, neonatal BCG seems an unlikely culprit to explain the difference in child innate immune status around 2 years of age. Djuardi et al. also found no clear effect of BCG vaccination on the innate immune ontogeny (39). However, although the overall vaccination schedule was similar for all children at each site, differences in vaccine composition (e.g. acellular vs. whole-cell pertussis) or exact age of vaccination differed somewhat between sites. As a result of this we cannot exclude that variation in standard childhood vaccination might contribute to the observed differences of innate immune development.

Given that our children were all enrolled according to the same well-defined inclusion and exclusion criteria, differences in medical illness over the first two years of life are unlikely to have contributed to the differences between sites we detected. We also interpret our data to indicate that differences in feeding-mode (duration of breast-feeding; breast-vs. bottle-feeding etc.) were unlikely major contributors to the differences between the sites we observed. While we cannot exclude that feeding-mode could lead to subtle differences, our data suggest it has minimal impact since, feeding mode differed vastly both between and within sites while innate immune response variation within sites was negligible. Furthermore, variation in innate immune response to PRR stimulation did not correlate with feeding mode-comparing individuals within one site (data not shown). Although studies have previously shown that birth mode can impact the children' immune system up to the age of five years (3, 36), we were unable to detect an association of birth-mode with innate immune response (data not shown). Specifically, approximately half of our Canadian children were born via Caesarian-section, while nearly all of the South African, Ecuadorian and Belgian children were born vaginally. While birth weight and gestational age have been shown to correlate with a higher risk of infant mortality (38), their impact on postnatal innate immune trajectory has not been delineated. However, the average birth weight was similar for all 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 that were born prematurely (< 37 gestational weeks of age) was very low (3 in South Africa and 2 in Belgium, about 15% per cohort). This suggests that neither birth-weight nor gestational age could explain the differences we detected between our cohorts. Lastly, although several studies have 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).

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This may be due to low sample size, but precludes sex as the major determinant for the differences we detected between our global cohorts in 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 as compared to other regions of the world has clinical implications is at the moment not entirely clear, but warrants further exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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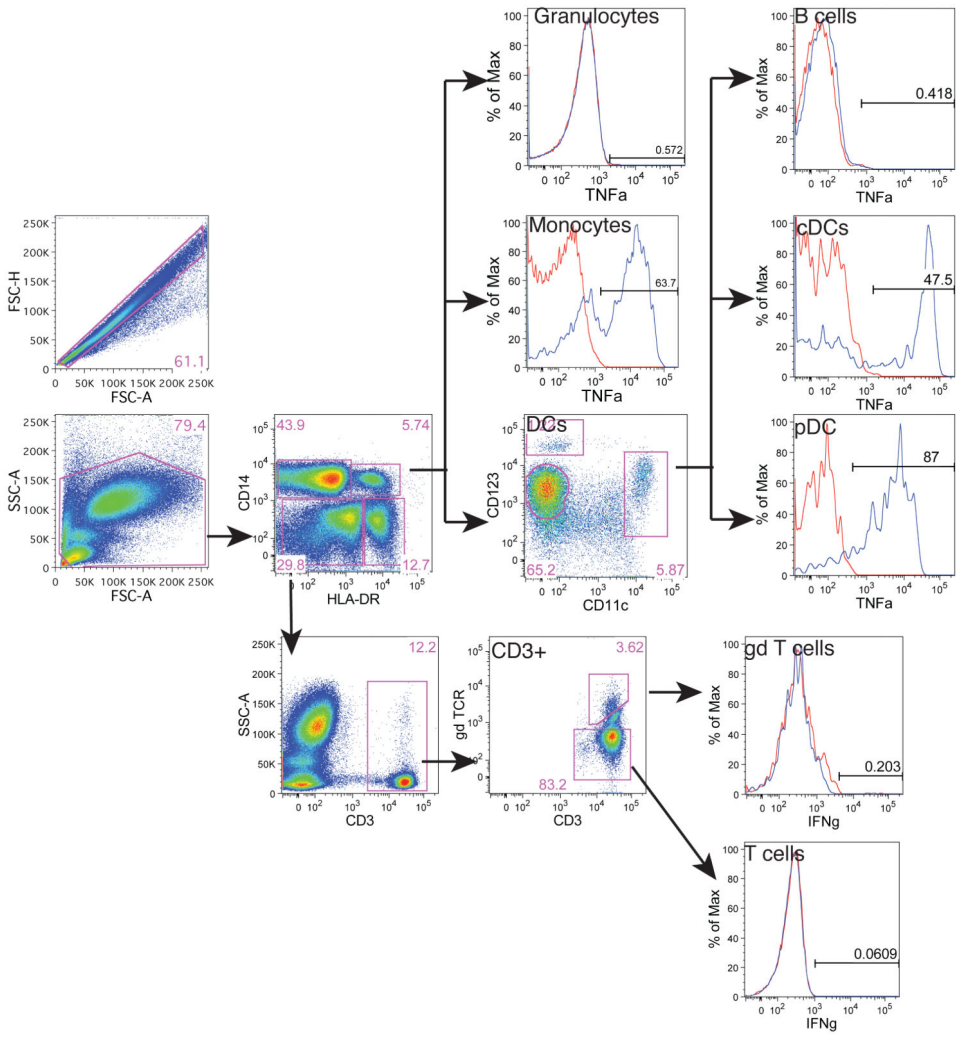

Figure 1.
Gating strategy for the 11-colour panel for flow cytometry.

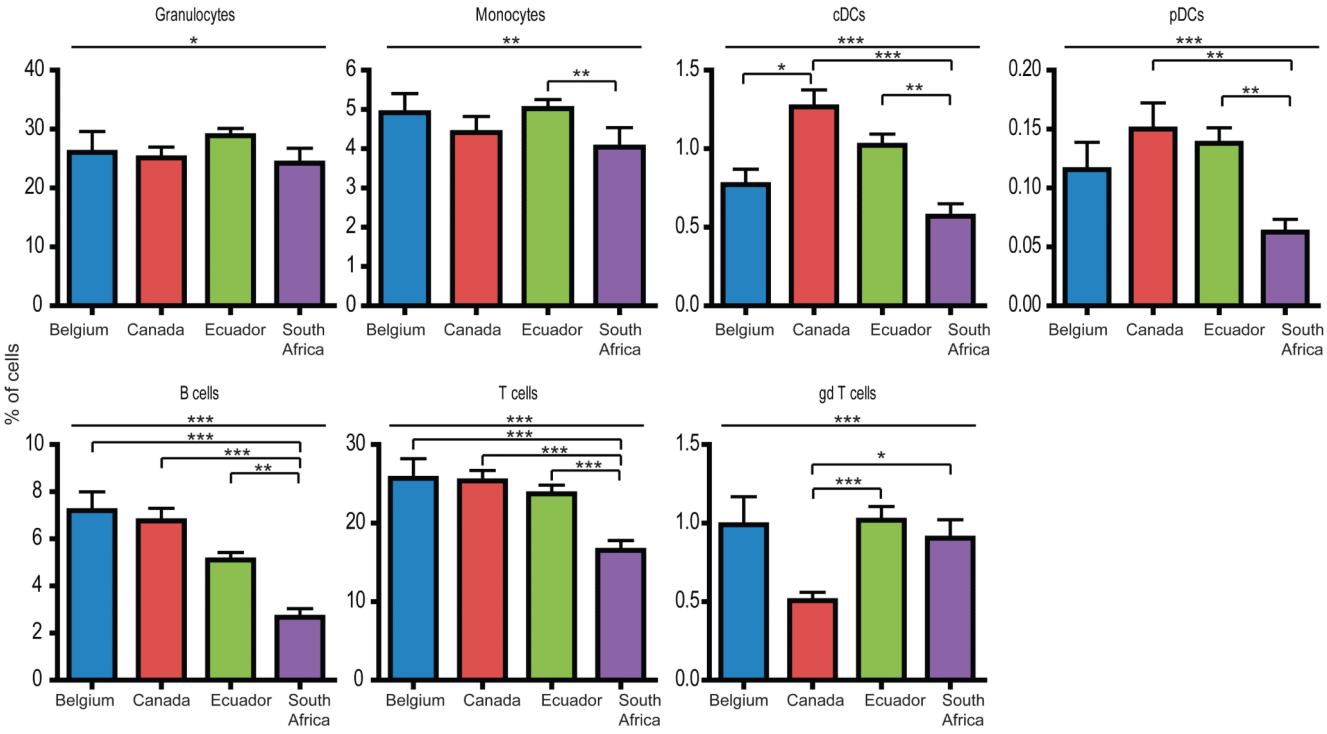

Figure 2.

Cellular composition of whole blood cells at each of the 4 sites (statistical significance p value was *** < 0.005, ** <0.01, * <0.05).

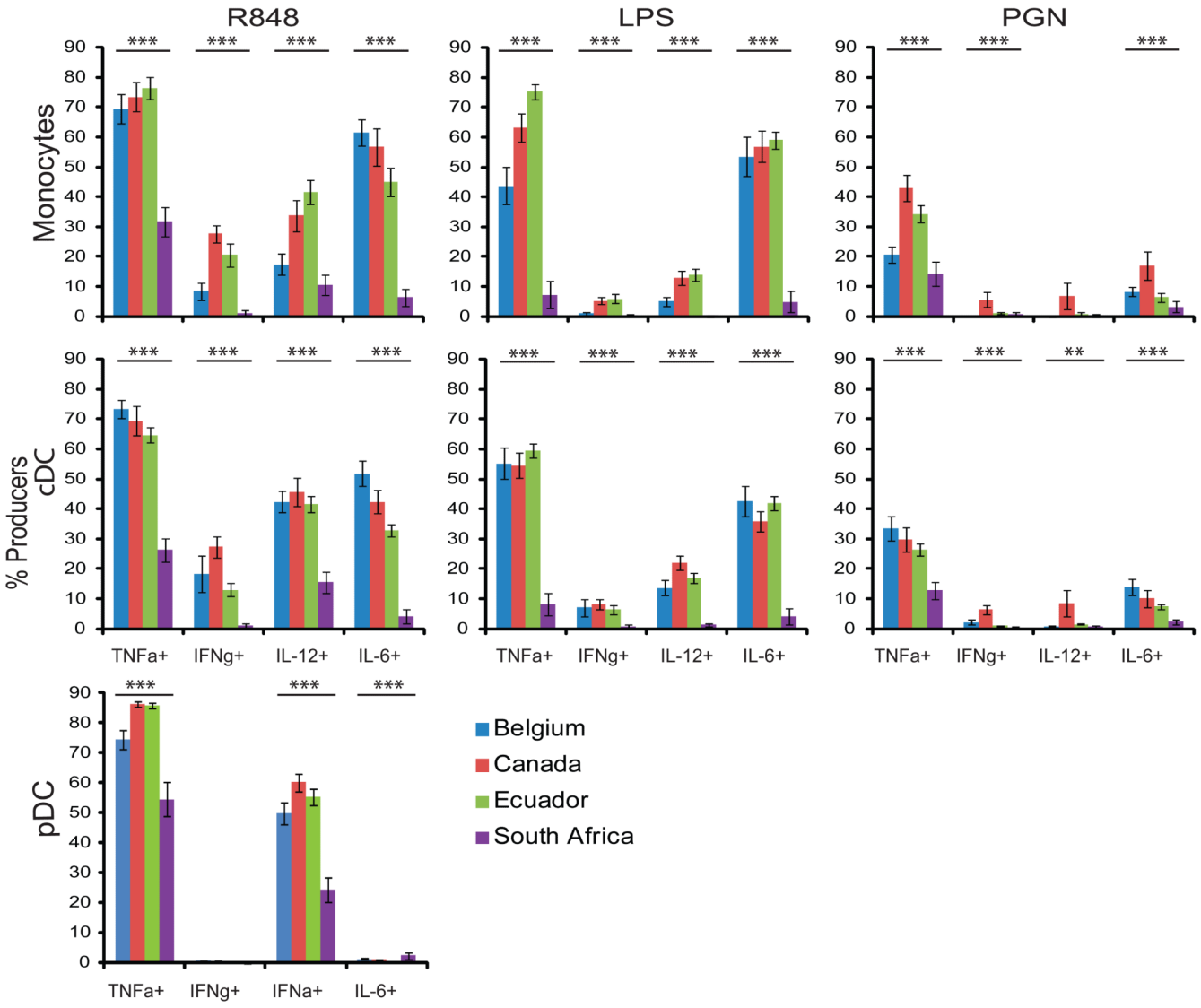

Figure 3.

Single-cell cytokine in response to TLR or NLR ligand stimulations show that South African children have a weaker single cell-specific cytokine response. Whole blood obtained from children from 4 different sites were stimulated with R848, LPS, and PGN ligands and measured by flow cytometry for IL6, IL12, IFNα, IFNγ, and TNFα production. **A.** Monocytes, **B.** cDCs, C. pDCs. Kruskal-Wallis test was done to look at statistical differences of each cytokine per cell type per stimulation, followed by Dunn's post-hoc test was applied to each site pairing (statistical significance p value was *** < 0.005, ** <0.01, * <0.05).

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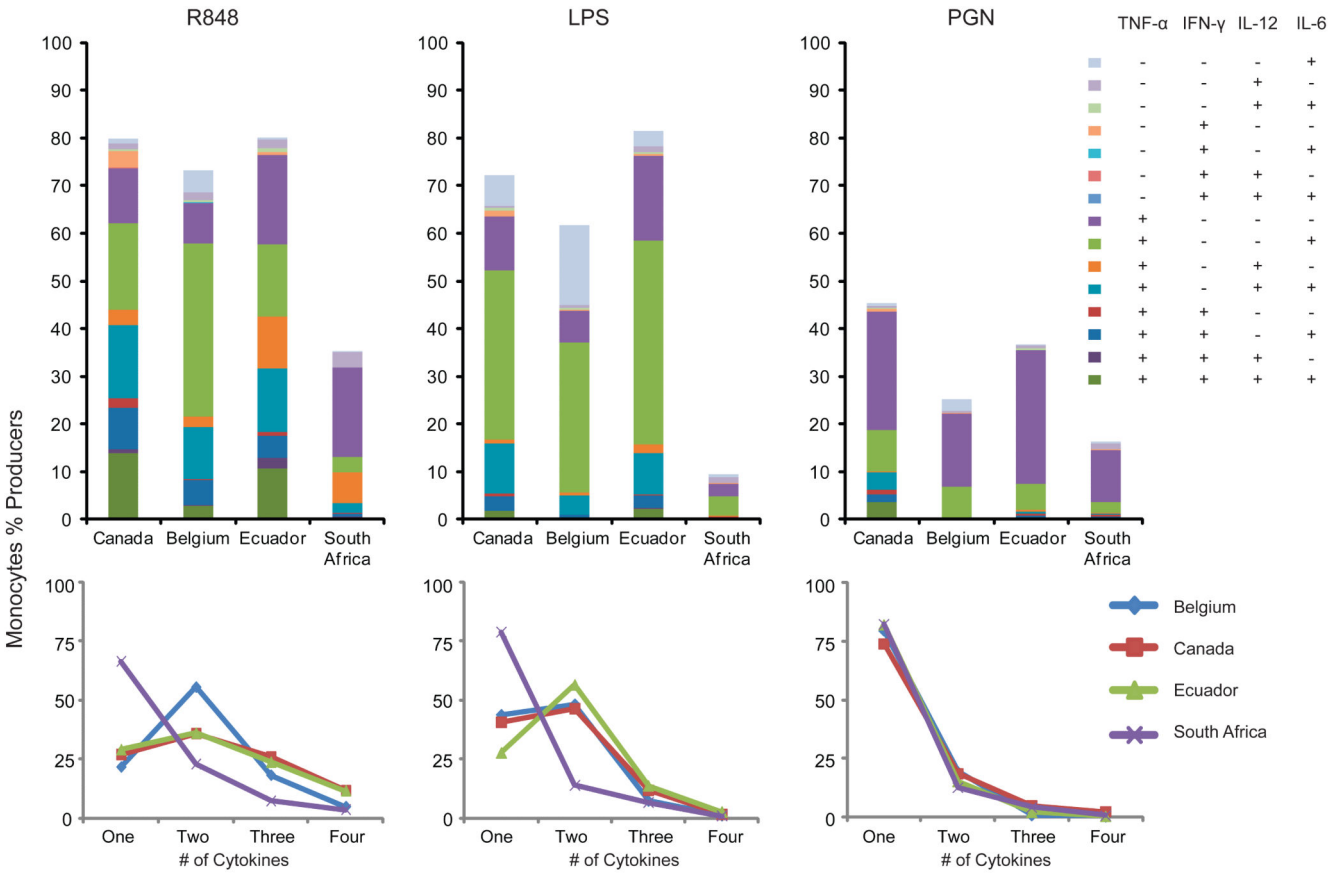

Figure 4.

Multi-cytokine response of Monocyte-single cell type showed that South Africa is weaker and less diverse in response to TLR and NLR ligands. Whole blood obtained from children from 4 different sites were stimulated with R848, LPS, or PGN ligands and measured by flow cytometry for IL6, IL12, IFNγ, and TNFα levels. The stacked bar graph represents the combination of cytokine contributions per cell type. The line graph indicates the polyfunctional state of the cell type, summarizing the percentage of cell producing a single- (one), double- (two), triple- (three), or quadruple- (four) cytokines in response to stimulation.

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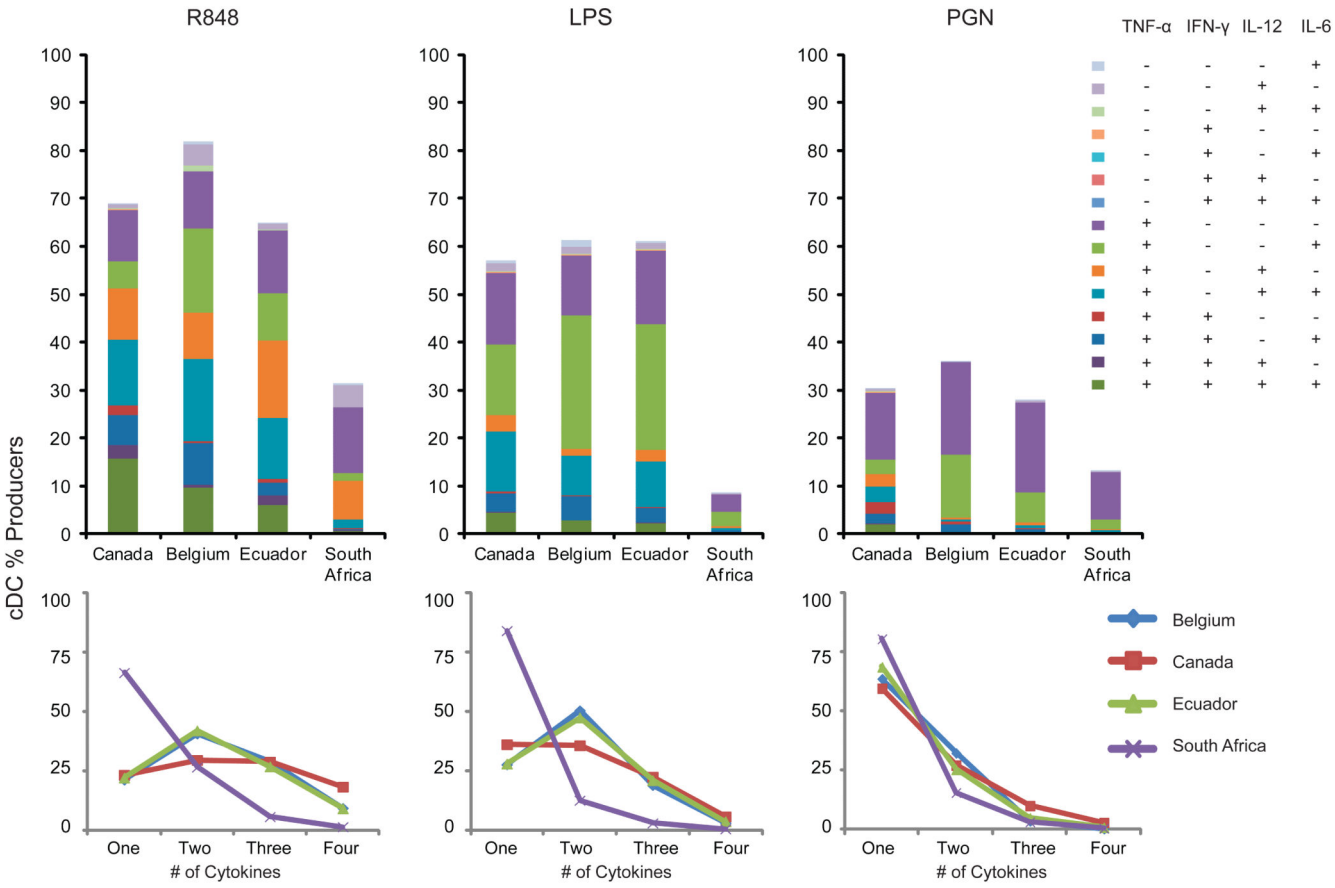

Figure 5.

Multi-cytokine response of cDC-single cell type showed that South Africa is weaker and less diverse in response to TLR and NLR ligands. Whole blood obtained from children from 4 different sites were stimulated with R848, LPS, or PGN ligands and measured by flow cytometry for IL6, IL12, IFNγ, and TNFα levels. The stacked bar graph represents the combination of cytokine contributions per cell type. The line graph indicates the polyfunctional state of the cell type, summarizing the percentage of cell producing a single- (one), double- (two), triple- (three), or quadruple- (four) cytokines in response to stimulation.

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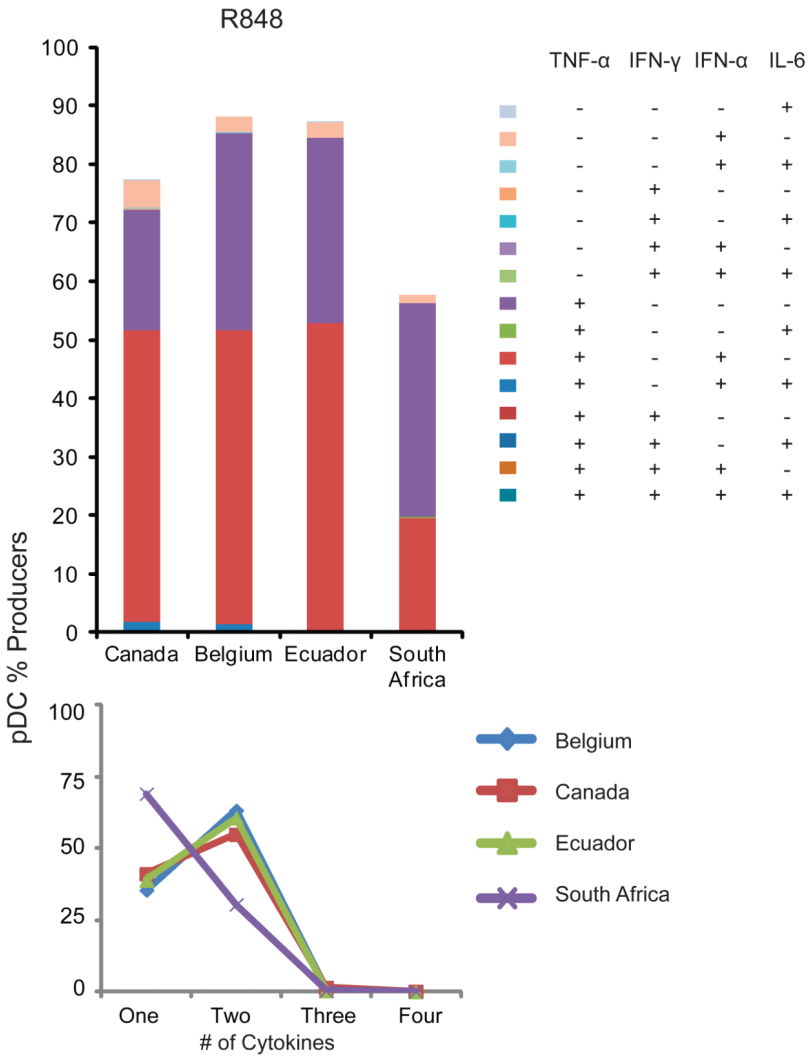

Figure 6.

Multi-cytokine response of pDC-single cell type showed that South Africa is weaker and less diverse in response to TLR 7/8 ligand. Whole blood stimulated with R848 ligand and measured by flow cytometry for IL6, IFNα, IFNγ, and TNFα levels. The stacked bar graph represents the combination of cytokine contributions per cell type. The line graph indicates the polyfunctional state of the cell type, summarizing the percentage of cell producing a single- (one), double- (two), triple- (three), or quadruple- (four) cytokines in response to stimulation.

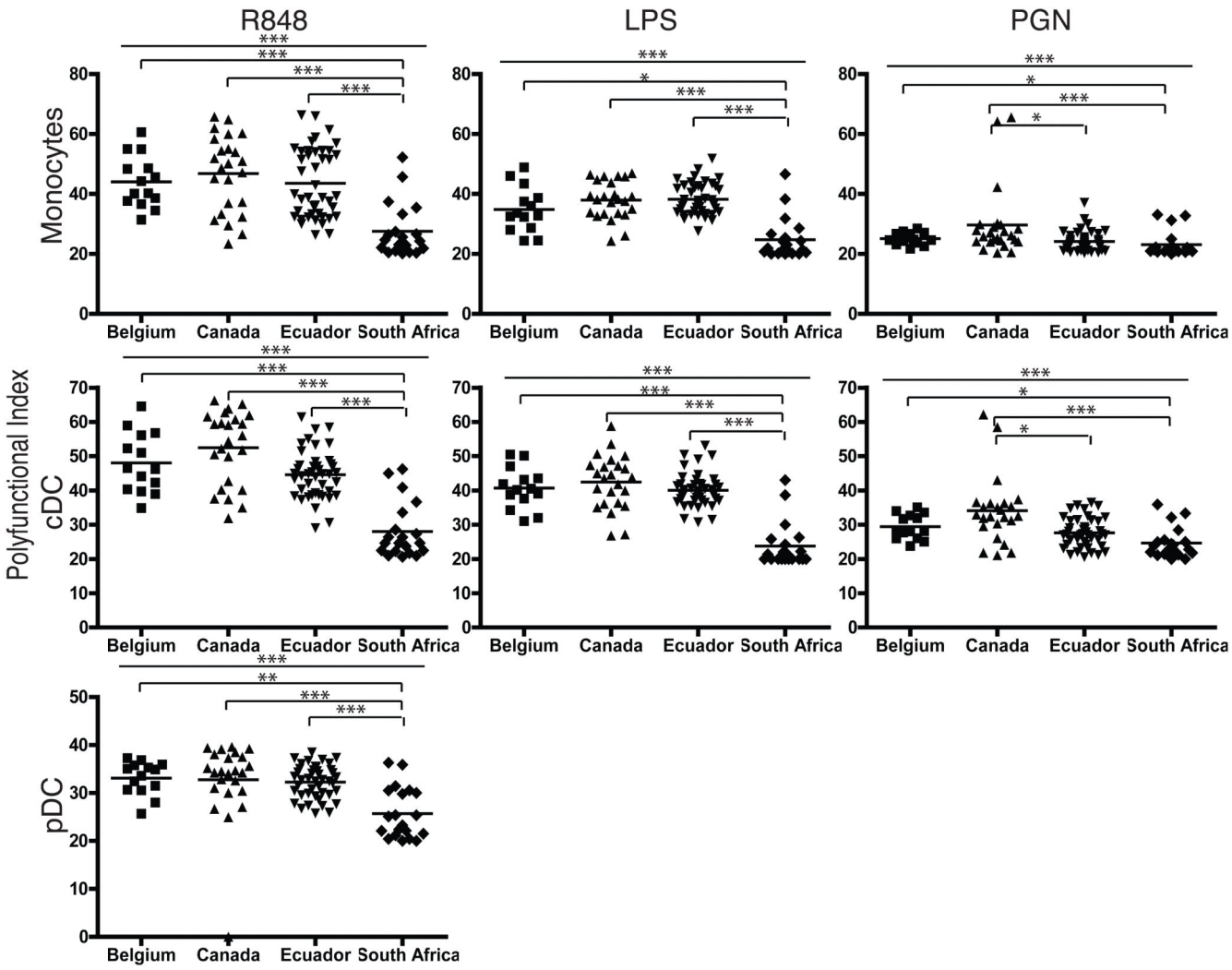

Figure 7.

Polyfunctional index (PI) values of children at each site per cell type in response to TLR and NLR ligand stimulation. **A.** PI numerically evaluated the degree and variation of polyfunctionality within the 4-site cohort. It allows for difference between cytokines produced by the different cell type to defined stimulations (R848, LPS, PGN) to be observed. **B.** Kruskal-Wallis test was used to compare the 4 sites per cell type and stimulation. Dunn's post-hoc test was applied to each site paring (statistical significance p value was *** <0.005, ** <0.01, * <0.05).

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TABLE I

Demographics of the subjects at each of the 4 sites.

	Belgium	Canada	Ecuador	South Africa
n=	14	24	43	20
Infant Characteristics				
Mean 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)	2996.2 (796.3)	3337.9 (435.1)	3475.1 (988.3)	3018.4 (383.6)
Birth mode (vaginal/c-section)	13/1	11/13	34/9	20/0
Gestational Age - mean (SD)	38.4 (3.4)	39.1 (1.7)	38.9 (1.1)	37.8 (2.4)
Premature < 37wks (% of total)	2 (14%)	1 (4%)	0 (0%)	3 (15%)
Weight (g) - mean (SD)	13364.30 (1786.1)	11425.00 (1553.5)	11501.16 (1010.7)	11205.00 (1300.7)
Height (cm) - mean (SD)	92.2 (4.6)	82.7 (3.4)	84.3 (2.5)	84.4 (0.91)
WAZ (SD)	0.69 (1.2)	0.04 (1.0)	−0.32 (0.93)	−0.58 (0.95)
LAZ (SD)	1.56 (0.8)	0.29 (0.96)	−0.78 (1.49)	−1.07 (1.20)
WLZ (SD)	-0.18 (1.4)	0.35 (1.04)	0.16 (0.79)	−0.03 (0.87)

Abbreviations: WAZ, for weight-for-age Z-score, LAZ, length-for-age Z-score, WLZ, weight-for-length Z-score