INNATE IMMUNE RESPONSE TO PATTERN RECOGNITION RECEPTORS IN INFANTS ACROSS FOUR CONTINENTS

by

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Abstract

Susceptibility to infection as well as response to vaccination varies among populations. To date, the underlying mechanisms responsible for these clinical observations have not been fully delineated. Since innate immunity instructs adaptive immunity, I hypothesized that differences between populations in innate immune responses may represent a mechanistic link to variation in susceptibility to infection or response to vaccination. To determine if differences in innate immune responses exist among infants from different continents of the world, I investigated the innate cytokine production of whole blood as well as the single cell level response following pattern recognition receptor (PRR) stimulation of blood samples obtained from two-year old infants across four continents (Africa, North America, South America and Europe). I found that despite the many possible genetic and environmental exposure differences in infants between the four cohorts, whole blood cellular components of only South African children secreted significantly lower levels of most cytokines following stimulation of pattern recognition receptors (PRR) as compared to whole blood from cohorts of Ecuadorian, Belgian, or Canadian children, specifically following stimulation of extracellular- and endosomal-, but not cytosolic-PRRs. To begin dissection of the responsible molecular mechanisms, I identified the relevant cellular source of these differences. While a significant variation in the cellular composition of whole blood was found, reduction of the intracellular cytokine production on the single cell level was only detected in South African infants' monocytes, cDC, and pDC. I also uncovered a marked reduction in polyfunctionality for each of these cell types in South African children as compared to children from other continents. Together this data indicates the existence of

differences in cell composition as well as profoundly lower functional responses of innate cells in a cohort of South African children. With a difference in number and function of innate immunity in South African children identified, we can now proceed to determine the link between altered innate immunity and increased risk for infection or lower response to vaccines in South African infants.

Preface

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List of Abbreviations

γδ T cell	gamma delta T cell
APC	antigen presenting cell
BCG	bacillus Calmette-Guérin
BFA	Berfeldin A
BRC	B cell receptor
cDC	conventional dendritic cells
CD	cluster of differentiation
CI	confidence interval
CMI	cell mediated immunity
DC	dendritic cell
DPBS	Dulbecco's phosphate buffered saline
EPI	Expanded Programme of Immunization
FCS	fetal calf serum
FMO	fluorescence-minus-one
ICS	intracellular cytokine staining

IFN	interferon
IL-	interleukine
IR	interquartile range
LPS	lipopolysaccharide
Mal	MyD88 adaptor-like protein
MHC	major histocompatibility complex
MyD88	myeloid differentiation gene factor 88
µl	microlitre
NLRs	NOD-Like receptor
NOD	nucleotide-binding oligomerisation domain
RT	room temperature
MMR	mumps, measles, and rubella vaccine
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cells

PHA	phytohaemagglutinin
PRR	pattern recognition receptor
SNPs	Single Nucleotide Polymorphisms
TB	Tuberculosis
TCR	T cell receptor
Th	helper T cell
TIRAP	TIR-associated protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TRIF	TIR domain-containing adaptor protein inducing interferon
WB	whole blood
WHO	World Health Organization

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Dedication

I dedicate this thesis to the

loving memory of M. Ciecinska and M. Smolen.

1 Chapter: Introduction

The first few years of life represent a period of marked susceptibility to infectious diseases [1–3]. Such vulnerability at least partially reflects a state of age-dependent, suboptimal immune mediated protection in early life [1]. However, the degree of this heightened age-dependent increase in mortality early in life varies greatly between different regions and populations of the world[3]; the underlying cause for this variation has not been determined.

Vaccines represent one of the most effective medical interventions, leading to a significant reduction in morbidity and mortality for many infectious diseases [4]. Specifically, childhood vaccines have drastically reduced early life infection, suffering and dying. The World Health Organization (WHO) and regional regulatory bodies have supported and implemented a standard set of the most effective vaccines worldwide. Why then are there still so many early life deaths from vaccine preventable disease? There likely are several reasons, from programmatic failures to insufficient insight into the basic mechanisms required for vaccines to optimally protect.

My thesis attempts to begin to understand the underlying science that leads to this phenomenon. As a first step towards this eventual goal, I wished to determine if variation in innate immunity exists between different populations of children around the globe. The rationale for the innate immunity focus stems from the fact that innate immunity is the first line of defense against infection (also the first arm of the immune system to respond to vaccination) and directs the ensuing adaptive immune system to clear any invading pathogen (and direct the long lived immune memory following vaccination).

1.1 Immunization Programs

The Expanded Program of Immunization (EPI), established in 1974, was modeled after the success of the smallpox eradication program to ensure that all children in the world would benefit from vaccines. EPI and similar regional or national programs direct the immunization of infants against some of the most important infectious threats worldwide [5,6]. These disease threats include polio, diphtheria, tetanus, Haemophilfus influenzae type b (Hib), hepatitis B

(HepB), tuberculosis (TB), pneumococcus, measles, rubella, and rotavirus [5,6]. Together these public health programs have greatly contributed to the reduction of infectious mortality and morbidity in early life [7].

EPI success has traditionally been measured as an operational improvement in vaccine coverage, with the assumption that average individual vaccine response is the same for children in all populations. However, this assumption did not to hold true [8]. Vaccines are generally assessed based on their effectiveness and efficacy:

- *Effectiveness* of a vaccine is defined as the reduction in the transmission rate for an average individual in a population with a vaccination program at a given level of coverage compared to an average individual in a comparable population with no vaccination program [9]. Vaccine effectiveness is a measure of the real world success of a vaccine. Considering it is impacted by program variables such as vaccine coverage, cold-chain breakage, or insufficient delivery of the vaccinations, the reasons for lower effectiveness could be many [8].

- *Efficacy* measures the protective effects of the vaccination by the reduction in infectious risk of a vaccinated individual relative to that of a susceptible, unvaccinated individual. It is determined via direct measurements following immunization under well-controlled ('trial') settings. This also is the method used to evaluate vaccines for licensure [10].

Since most vaccine programs worldwide differ very little in the schedule, administration, and formulation of each vaccination, the inherent assumption is that all infants living in different parts of the world respond in the same way to vaccines. As a result, only the 'representative populations' (those tested in the original vaccine evaluation study) are used for the assessment of vaccine programs. However, the failure of any vaccine in a region could be due in part to poor vaccine effectiveness (e.g. sub-optional vaccine coverage) as well as low efficacy (e.g. different response of the vaccinated population to the original study

population). It is this potential low efficacy, resulting from variation of responses in different populations, that represents one of the foci of this thesis.

Efficacy is influenced by many factors such as age, gender, ethnicity, antigen (Ag) dose, adjuvant, number of administered doses, and route of immunization [11–13](Appendix A). The EPI and most national programs administer similar vaccines via a similar route, at approximately the same age, and with the same number of doses. This argues that mode of immunization, age of recipient, and number of doses are unlikely to account for the variation seen in different populations. This in turn suggests that the variation in vaccine response in different populations is a result of host- and/or environmental-influences that differ between populations. In this chapter, I will summarize the current state of knowledge regarding the different responses to most standard childhood vaccines.

1.1.1 Bacille Calmette-Guerin

Bacille Calmette-Guerin (BCG) is an attenuated strain of *Mycobacterium bovis*, currently the only licensed vaccine for use against Tuberculosis (TB) worldwide. It is recommended as part of the EPI since the 1970's, and is among the most studied vaccines currently available.

BCG vaccine-trials have shown high efficacy against severe forms of systemic childhood TB, namely military TB and TB meningitis. However, clinical trials of BCG have determined its efficacy to range between 0-83% protection against adult pulmonary TB [14,15]. A significant portion of these variations can be attributed to the geographic region (latitude effect) of the studied patient populations [14]. Patient populations in the UK, Denmark, and Ireland showed a vaccine efficacy of greater than 75%, while patient populations in Malawi, India, and the USA (Georgia) showed a vaccine efficacy of less than 20% [14].

A direct comparison of European and African responses to BCG vaccination has shown that there can be large variations in immune responses between different populations even within similar latitudes. Lalor et al. showed that both infants from Malawi and the UK display cytokine responses following BCG vaccination, but that the overall profile of the two populations is very different [16]. The UK infants showed a strong Ag-specific T-helper 1 (e.g.

interferon gamma; IFN-γ) dominated cytokine response, while the Malawian infants showed a different response, producing higher innate proinflammatory cytokines, regulatory cytokines, interleukin (IL-)-17, Th2-cytokines (IL-4), chemokines, and growth factors [16]. This different immune profile following a BCG vaccination for each population could be indicative of the variability of protective efficacy in different parts of the world.

While genetic differences between various populations may play a role in the variable response to BCG, the differences in the immune response to the BCG vaccine are also likely to be heavily influence by environmental variability. Factors such as exposure to environmental mycobacteria [14,17] or particular prenatal and perinatal environments have an influence on long-term adaptive responses of infant immunized with BCG [18]. Specifically, Miles DJC et al., followed the development of CD4 T cell response in infants vaccinated with BCG at birth for the first two years of life. This study found that IFN-γ production was the predominant response in the first year of life, while the production of IL-2 remained low [18]. They also found that factors such as season of birth of the infant, maternal Body-Mass-Index (BMI), and ethnicity influenced the immune response to BCG vaccination [18,19].

1.1.2 Hepatitis B

Hepatitis B (HepB) vaccination is used to prevent Hepatitis B virus (HBV) infection, a global public health threat that has infected over 350 million people worldwide [20]. The vaccine is given to infants at birth or at 6-8 weeks of age, using a 3-dose regime and although the timing of vaccination varies, high levels of protective responses are seen in most populations. Yet population based differences have been described.

A study looking at the Ladino and Native Indian Guatemalan infants revealed that both infant groups develop high levels of protective Ab titers, but only the Native Indian Guatemalan infants develop high geometric mean anti-hepatitis B surface Ab [21]. The study speculated that this may be due to environmental exposure, but genetics factors were not examined and could not be excluded.

In China, researchers examined students from different regions to determine whether immunological memory against hyperendemic HBV infection persists for upward of 15 years post vaccination, and whether socio-economic factors influence immunity to hyperendemic HBV. The study found differences in persistence of immune memory between students from different regions. Further differences were found between rural and urban areas for the Han Chinese subgroup. The study concluded that for hyperendemic HBV infection, long-term immunogenicity of vaccination was modified by host genetic factors and urbanization and that the vaccine was less effective in socio-economically disadvantaged areas [22].

A study comparing the HepB vaccine responses of infants in Senegal and Cameroon showed that infants in Senegal had significantly weaker vaccine responses. In this particular study, Rey-Cuille M. et al. noted that children from Senegal, who had a low response to HepB vaccine, were also at risk for other vaccine preventable diseases such as diphtheria, tetanus, pertussis, as well as Hib [23], suggesting a broader variation in the vaccine immune response.

Unlike many other vaccinations, some of the host-related genetic factors affecting the HepB response have been described. Approximately 10% of vaccinated individuals fail to mount an immune response to hepatitis B surface antigen (HBsAg) mixed with aluminum as an adjuvant [24]. This has long been assumed to be due to variation in major histocompatibility complex (MHC) link traits [25]. However, this vaccine failure has more recently been linked to an altered response to the adjuvant, as adding monophospholipid A (MPL), a TLR4 agonist, induced robust responses in all those that had failed the standard vaccine containing Alum[26–28].

1.1.3 Measles

The measles vaccine is administered as a single or combination vaccine (measles-mumps-rubella (MMR), or as a measles-mumps-rubella-varicella (MMRV) combination vaccine.

The response to measles vaccinations has shown significant variation amongst different populations; most of the differences could be linked to variation in host genetics. Specifically, studies in the USA compared Caucasian and African-American subjects for

immunogenicity and found genetic related differences [29]. Another study analyzed the response of 12-months old Bedouin and Jewish children living in Israel to MMR vaccination [30]; the study showed that seroconversion to measles was 99% in Bedouin and 79% in Jewish children. The levels of neutralizing antibody (Ab) were twice as high in Bedouin compared to Jewish children. Yet another study by the WHO identified that Peruvian children mounted excellent humoral responses to measles vaccination (MV) with Ab titers equal to or superior to those reported for North American children [31,32]; but only 23% of these same children generated detectable lymphoproliferative responses to measles Ag compared to North American children, where approximately 65% responded[33]. Further studies indicated a heritability of >90% in the immune response to MV [34].

1.1.4 Oral Vaccines

The responses to oral vaccines such as polio, rotavirus, cholera, salmonella, and shigella, have shown to vary significantly between populations from different parts of the world. For example, after receiving 3 doses of oral polio vaccine, children in industrialized countries have greater than 90%, 100%, and 100% seroconversion rates to polio types 1, 2, and 3 respectively, while children from developing countries have respective seroconversion rates of approximately 70%, 90%, and 70% [35]. Most of these differences have been presumed to be associated with the differing intestinal microbial colonization. However, the precise causes that lead to a reduced oral vaccine efficacy in resource-poor regions have not yet been identified.

1.2 Variation in Immune Cell Numbers in Populations Across the Globe

Outside of vaccine studies, very few studies have compared immune system differences across the globe. Population-based differences in cellular composition were described in studies establishing levels to help in the diagnosis and monitoring of regular childhood diseases [36,37]. These studies found that the patterns and values of peripheral while blood cells varied in the first 3 years of life within a European cohort [38]. A comparison of European and Ugandan children noted lower CD4+, CD8+, and lymphocyte counts in Ugandan children as compared to black European children, while neutrophil counts were similar [36]. Thus, variation in cellular composition could contribute to variation in response to vaccination.

However, there has not yet been a study directly comparing specific aspects of the immune system functionally between populations of children from different parts of the world.

1.3 The Immune System

The immune system defends the host through a dynamic and highly regulated network of cells and molecules, which work synergistically. When a threat is encountered, the immune system responds with a diverse arsenal to eliminate this threat. The immune system is principally divided into two separate arms, the innate and the adaptive immune system. Both arms constitute an essential component of the overall response to vaccination [39].

1.3.1 The Adaptive Immune System

The adaptive immune system consists of two separate branches: the cellular and the humoral response. The cellular response (or cell mediated immunity; CMI) consists mainly of T cells. The main subsets are CD4+ helper and CD8+ killer T cells. The humoral response consists mainly of antibodies (Abs) produced by B cells. Both T and B cells contain unique receptors that allow for high specificity of binding to microbial antigens; T cells contain T cell receptors (TCR), B cells contain B cell receptors (BCR). The TCR and BCR are able to recognize a wide array of antigens. The recognition of such a diversity of antigens is a result of specific gene rearrangements that occur during lymphocyte development, a process that is highly controlled with positive and negative selection.

For an adaptive response to occur, an antigenic stimulation is required to activate and expand the specific cell population, as instructed by the innate immune system. This clonal expansion allows T-cell or B-cell dependent effector functions like (increased phagocytosis of microbes (supported by T helper cells), killing of infected cells (by T killer cells), and neutralization (e.g. by antibodies)) to eradicate pathogens. After the infectious threat is cleared, a small portion of the expanded cells remains as memory cells. These immune memory cells stay in the system to protect the host against re-infection by the same pathogen, with faster response time, greater specificity, and higher efficiency than the original innate immune response. This immune memory forms the basis for effective protection from infection and/or disease following immunization [39].

1.3.2 The Innate Immune System

For the adaptive immune system to clear pathogens during an infection or develop immune memory following vaccination, innate immune mechanisms need to be set into motion that lead to execution of the adaptive program. Furthermore, the innate immune system, which has been evolutionarily conserved, provides the first line of defense against invading pathogens.

The innate immune system consists of many different parts that heavily and intensely interact: physical and physiological barriers, cellular components and humoral components. The physical and physiological barriers provide the primary defense against pathogens. These barriers included intact skin, mucociliary clearance mechanism, low stomach pH, and bacteriolytic and lysozome components of various secretions.

The second layer consists of the cellular and humoral components. The cellular components include macrophage, dendritic cells, mast cells, granulocytes (neutrophils, eosinophils), natural killer (NK) cells, and NK T cells. The humoral components include complement proteins, LPS binding protein, C-reactive protein, mannose-binding lectin, and antimicrobial peptides. All of these components are involved in both sensing of microbes as well as effector mechanisms to facilitate clearing of the invading or infectious pathogens.

Centrally important for the innate immune system, and the focus of this thesis, are the germline-encoded pattern recognition receptors (PRRs). The innate immune system is activated when PRRs recognize conserved molecular motifs in microbes collectively known as pathogen-associated molecular patterns (PAMPs). The sensing of PAMPs by PRRs triggers an immediate effector response (e.g. phagocytosis, radical production, cytokine release) aimed at eliminating or reducing the spread of pathogens [40–42]. This requires the successful coordination of molecular events by multiple cell subsets, which must be tightly regulated to avoid excessive activation and maintain homeostasis. PRRs are also present on antigen presenting cells (APC) such as monocytes and dendrtic cells (DCs). The binding of a PAMP to its specific PRR on APCs triggers an innate immune response that culminates in

antigen presentation in the context of MHC, costimulatory molecule expression, and cytokine secretion [1].

1.3.3 Pattern Recognition Receptors

The discovery of pattern-recognition receptors (PRR) has expanded our insight into the mechanisms and central role of the innate immune system in immune mediated protection [43]. The main PRRs families of the innate immune system are the Toll-Like receptor (TLRs), the NOD-Like receptor (NLRs), the RIG-I-Like receptor (RLRs), cytosolic DNA sensor (CDS)[44], and the C-type lectin receptors (CLRs).

1.3.4 Toll-like Receptors

TLRs are type I transmembrane proteins with an extracellular amino-terminus and an intracellular carboxy-terminus. They are composed of several domains including, the characteristics extracellular leucine rich repeat (LRR), one or two cysteine-rich regions as well as an intracellular toll/ interleukin-1 receptor (TIR) domain.

In the mammalian TLR family there are currently 13 described members, of which 10 are characterized in humans [44,126]. TLR family can be separated into two distinct groups based on the relative expression by the cell of interest and the recognition of specific PAMPs or ligands. The two groups are: extracellular and endosomal.

- The extracellular TLRs consist of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, all of which are expressed on the cell surface [44]. These TLRs detect primarily lipids and lipoproteins derived from bacterial products, and are more highly conserved [44], with the exception of TLR 5 which detects flagellin.
- The endosomal TLRs consist of TLR3, TLR4, TLR7, TLR8, and TLR9, which are located inside of endsomes. These TLRs predominantly recognize nucleic acids from several different pathogens [44].

These receptors recognize specific PAMPs derived from various microbial pathogens, including bacteria, viruses, protozoa, and fungi [44].

1.3.5 TLR Signaling Pathway

The signaling events initiated by TLR activation are mediated by the interactions between TIR domain-containing cytosolic adaptors. These cyosolic adaptor molecules include myeloid differentiation gene factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88 adaptor-like (Mal) protein, TIR domain-containing adaptor protein inducing interferon (TRIF) and TRIF-associated adaptor molecule [45].

The selective recruitment of the adaptor molecules results in the specific response of each TLR in a distinct pathway, MyD88-dependent and TRIF-dependent pathways. The MyD88-dependent pathway, with the exception of TLR3 which uses the TRIF-dependent pathway, controls most of the TLR-mediated responses. TLR4 uses both pathways.

1.3.5.1 MyD88 Dependent Signaling Pathway

Except for TLR3, the MyD88-dependent signaling pathway is activated downstream for all TLRs. TLR2, TLR4, and TLR5 MyD88 stimulation, primarily drives inflammatory gene expression. In response to an appropriate PAMP, MyD88 associates with the cytoplasmic TIR domain and recruits IL-1 receptor kinase (IRAK) 1/2 and IRAK4 through homophilic interaction. This is shown to be essential in the NF-κB activation and in IL-R1/TLR signaling in response to these TLR stimulation [45,46]. The MyD88 adaptor binds to IRAK4 and IRAK1/2, resulting in the activation of IRAK1. The activated IRAK1 associates with TRAF6. The IRAK1–TRAF6 complex then disengages from the receptor and interacts with the TAK1/TAB1/2/3 complex at the plasma membrane. TRAF6 complex activates and phosphorylates TAK1/TAB1/2/3, which then translocates to the cytoplasm and phosphorylates the IKK-γ/NF-κB essential modulator (NEMO). Two distinct pathways are stimulated; IKK complex and MAPK pathway.

First, the IKK complex results in the phosphorylation of the inhibitory IκB, IκB undergoes proteasomal degradation to allow activation and translocation of NF-κB to the nucleus. Secondly, the MAPK pathway allowed TAK1 phosphorylates members of the MAPK kinase (MKK) family, including MKK3, -4, -6, and -7. MKK3/6 subsequently phosphorylate and activate p38, whereas MKK4/7 activate c-Jun N-terminal kinase (JNK).

The phosphorylation of IKK-β and MAP-kinases results in the modulation of NF-ϰB and MAP kinases, which translocates to the nucleus and initiates expression of proinflammatory cytokine genes [45,46].

1.3.5.2 MyD88 Dependent Signaling Pathway – TLR 7 and TLR9

TLR7 and TLR9 induce the production of type I IFN in addition to other proinflammatory cytokines. Upon stimulation, MyD88 associates IRAK4, IRAK1, TRAF6 and IFN regulatory factors (IRF)-7 to form a complex. The signaling results in the IRAK1-mediated phosphorylation of IRF-7 and production of type I IFNs [45,46].

1.3.5.3 TRIF Dependent Signaling Pathway

TLR3 and TLR4 are able to induce IFN-α and IFN-β production in a MyD88 independent manner, through the recruitment of TRIF as well as TRAM, with acts as a bridging adaptor. Upon stimulation, TRIF associates with receptor-interacting protein-1 (RIP-1), which is responsible for activation of NF-ϰB, or TRIF-family member-associated NF-ϰB activator (TANK) binding kinase-1 (TBK1) via TRAF3. TBK1 is comprised of a family of inducible IϰB kinases that directly phosphorylates IRF-3 and IRF-7. IRF-3 and IRF-7 are translocate to the nucleus and bind to the IFN-stimulated response elements, resulting in the expression IFN-inducible genes. IRF-3 and IRF-7 are essential in the production of type I IFNs in the viral mediated responses [45,46]. IRFs along with NF-ϰB and AP1 form a multiprotein complex termed the enhanceosome, which induces IFN- b gene transcription. TBK1 is not involved with the TLR-mediated NF-ϰB activation [45,46].

1.3.6 NOD-like Receptors

NOD-like Receptors (NLR) are found in the cytosol of the cell. They consist of three characteristic domains: amino-terminal, central, and carboxyl-terminal domain. First, the N-terminal effector domain is responsible for signal transduction and the activation of the inflammatory response. Four different N-terminal domains have been identified: acidic transactivation domain, caspase activation and recruitment domain (CARD), pyrin domain (PYD), and baculoviral inhibitor of apoptosis protein (IAP) repeat (BIR) domain. Second, the

central nucleotide binding and oligomerization (NACHT; NBD; NOD) domain shares similarities with the NB-ARC motif of the apoptotic mediator APAF1. Lastly, the C-terminal leucine-rich repeat (LRR) domain is responsible for ligand sensing and autoinhibition.

NOD1 (CARD4; NLRC1) and NOD2 (CARD15; NLRC2) are the main NLR family members. Whereas NOD1 is widely expressed by many cell types, the expression of NOD2 is restricted to monocytes/macrophages, dendritic cells, Paneth cells, keratinocytes, and epithelial cells of the oral cavity, intestine and lung. NOD1 and NOD2 are both implicated in sensing the presence of bacterial peptidoglycan (PGN) fragments. NOD1 senses meso-diaminopimelic acid (meso-DAP)-containing PGN fragments, which are present in most Gram-negative bacteria. NOD2 however, seems to be a general sensor which becomes activated by muramyl dipeptide (MDP), the minimal motif common to nearly all Gram-positive and Gram-negative bacteria.

1.3.7 NOD Signaling

Upon activation, NOD1 and NOD2 initiate a pro-inflammatory response through the recruitment of the receptor interacting protein 2 (RIP2; RICK; CARDIAK). Binding of ubiquitinated RIP2 leads to the direct binding to NEMO (IKKγ), which then gets ubiquitinated and degraded in a proteasome-dependent manner. Ultimately, this leads to the activation of the catalytic subunits IKKα and IKKb. The activated IKK complex phosphorylates the inhibitor IkB, leading to its proteasomal degradation and allowing NF-kB to translocate into the nucleus and exerting its function. Additionally, K63-polyubiquitinated RIP2 recruits TAK1, which is also essential for IKK complex activation. Furthermore, NOD1 and NOD2 mediated MAPK activation is also dependent on RIP2 and TAK1.

1.4 Causes for the Differences in Immune Response Across the Globe

Vaccine responses mostly measure changes in adaptive immune functions, which is rational since the specified goal of a vaccine is to induce adaptive immune memory. But it is the innate immune system that instructs the adaptive response [47], and by extension controls the induction of adaptive immune memory. It follows that my attempt to identify the underlying cause of differences in immune status should begin with a comparison of innate immunity

around the globe. Furthermore, within the innate immune response, the recognition of PAMPs by the PRRs is the first step of activation. Thus to assess the variation within the immunes response to vaccination or differences in infectious morbidity and mortality around the world, the first step would be to determine the variation in populations of the innate immune response to PRR stimulation. As explained above, differences in vaccine response have been attributed to variations in host genetic background as well as variation in environmental exposures [8,48]. Given the initiating function of the innate immune system in general for any immune response, and the PRRs in particular, my thesis thus focused on dissecting the role of PRR-induced innate immune responses in the variation of immune responses early in life between different populations.

1.4.1 Genetics

With the sequencing of the human genome it has become clear how different and yet how similar all human beings are to one another. Genetic differences between subjects have allowed us to trace ancestry [49] as well as the migration of humans and their evolution around the globe [50]. Throughout evolution our genetic makeup has been profoundly influenced by the environment, affecting our health and predisposition to disease. Previous studies have shown that variation in innate immune responses can be influenced by as small a change as single nucleotide polymorphism (SNP) within the PRR pathways. For example, our lab reported that variation in SNPs across different racial backgrounds can profoundly affect the response to TLR stimulation early in life [51]. Thus, genetic differences between populations clearly can influence innate immunity.

1.4.2 Environment

Given that the innate immune system is meant to detect changes in the environment (sentinel function), environmental variation is likely to play a very important role in shaping the innate immune system. Not only is the type of exposure important, the timing and duration of exposure are also important. Given the focus of this thesis on early life, the environment can be broken down into three distinct periods: prenatal, perinatal, and postnatal environments.

1.4.2.1 Prenatal Environment

The prenatal environment influences the developing fetal immune system in utero [52]. Lisciandro et al. suggested that infants conceived in modern (developed) environments exhibit an increased APC reactivity at birth (as measured in cord blood) compared to infants conceived and born in more traditional environments. They hypothesized that the quiescent nature of the infants born under traditional environments may perhaps be protective from the potential inflammatory response in early life [53].

However, prenatal exposure is largely dependent on environmental factors affecting the mother. For instance, maternal nutrition during pregnancy has been shown to have a profound effect on the emerging immune system. Variation in maternal nutritional status from malnourished to well-nourished during pregnancy can affect the placental development, modify nutrient transport available for embryonic and fetal development, affect vascular development, and alter the transfer of immunity to the child [54]. All of these factors have a profound impact on the emerging immune system of the child.

Immune development in utero is further influenced by the timing and nature of the 'stressors' on the child. This has more recently been espoused under the 'developmental origin of health and disease (DOHaD) theorem[55].

Maternal exposure to infectious stressors during pregnancy is thought to have an effect on the mother's immune system as well as the fetal immune system. The timing and duration of exposure has a large impact on infant immunity. As the fetus develops many changes are observed within the immune system. The complement proteins are present in the first trimester (~50-70% of adult conc.), and the innate effector cells are detectable in developing organs by the end of the first trimester [54]. With the correct stimuli, granulocyte division is able to surpass adult levels within 48-72h, with limited exhaustion capacity and surface protein expression until early infancy, while other innate characteristics, such as tissue macrophage, NK cells, NK cytolytic activity, do not reach levels of adulthood until much later in childhood [54].

Chronic exposure to infectious or non-infectious agents appears to have a profound impact on innate immune responses. For instance, maternal HIV infection negatively impacts the immune system of offspring, which may lead to a reduced magnitude in the child's immune response to vaccinations given in early life [56]. On the other hand maternal exposure to farm animals has been shown to be strongly associated with up-regulation of the innate immune receptors and lower degree of allergic sensitization. Maternal exposure to microbial compounds and consumption can result in increased T helper 1 (Th1) IFN-γ and TNF-α cytokine in cord blood.

An acute exposure appears to be more complex in its response. The complexity of the acute exposure is in part due to the rapidly changing development of the fetal immune system, making the response difficult to predict. The best-studied role of prenatal infectious exposure on infant immune development has been parasitic infections.

Exposure to parasites can vary greatly in outcome depending on the type of infection, the age of gestation at point of infection, and the duration of the infection. In the case of malaria, the time of maternal exposure has a profound effect on the innate immune response of the fetus once born. For example, cells of neonates born to mothers who acquired *P.falciparum* infection ≤1 month before delivery had significantly altered IFN-γ and TNF-α responses after stimulation with LPS and pIC, compared with cells of neonates born to uninfected mothers or to mothers who were successfully treated for malaria during pregnancy [57]. Helminth infections can affect the fetal innate immune response to vaccination. For instance, certain helminthes affect the immune response to vaccines while others do not, suggesting that the type of parasitic infection and/or the location might have an effect [56].

Not only does the chronic or acute infection matter, but also at what stage of the pregnancy it occurred because the components which comprise the front lines of the innate defense appear in early gestation and mature rapidly in the 3rd trimester to ensure adequate protection around the time of birth [54].

1.4.2.2 Perinatal Environment

Factors such as mode of delivery, seasons of birth, birth weight, as well as sex of the newborn have been shown to impact the initial immune phenotype. For example, the vaginal birth process is a controlled acute inflammatory process, which stimulates an acute phase reaction in the newborn. This reaction can be seen in the newborn cord blood as an elevated cytokine response compared to the newborns delivered by a cesarean section without labor.

Season of birth also appears to impact the early life innate response [52]. Specifically, births in the winter months are associated with lower vaccine responses and tend to have lower TLR3 mediated IL-12p70 production, but a higher IL-10 production when stimulated with TLR7 [58]. TLR9 stimulation revealed that IFN-α and IL-12-p40 responses were high in spring and summer birth months, while IL-8, IL-10, and TNF-α responses were higher in fall and winter months [52]. IFN-α responses were not only seasonally dependent, but also stimulation dependent, where TLR9 induced IFN-α response were high in the spring and summer, while TLR1/2 and TLR4 induced IFN-α response was high in the fall winter [52]. Importantly, birth weight and season of birth have been shown to predict response to vaccination not only in infancy but also well into adulthood [59].

Lastly, the sex of the infant has also shown to affect vaccine responses. Numerous abnormalities in the innate and adaptive immune response have been shown to be suppressed by measles, sex hormone differences have been noted in the first months of life and have been suggested as a potential factor in the observed difference [56].

1.4.2.3 Postnatal Environment

The postnatal environment is difficult to define, measure, and compare among populations. Socio-economic factors would be the most confounding contributor. Additionally, nutrition is important at every stage of life, and for a developing immune system it is important *in utero* as well as after birth. Acute as well as chronic effects of malnutrition (protein-calorie, mirco-nutrient under-nutrition, vitamin deficiency) have a long lasting effect on the immune system. Although fascinating, these aspects are not discussed in this thesis.

A number of studies have shown that differences in rural and urban life style can directly affect the child postnatally. It is presumed that a loss of exposure to a high biodiversity (range of different microbes) within the urban setting can have a profound effect on the immune system; this concept has been described under the 'hygiene hypothesis' theorem. In high-income, urban settings most of the biodiversity of microbes has vanished and with that, potential immune modulating factors, resulting in an increased incidence of allergies, inflammatory bowl disease, autoimmune disease, and other inflammatory states [60]. For example, children of farmers versus urbanites are at decreased risk of developing allergies [61]. When compared to non-farmers' children, farmers' children have an increased expression of CD14 and TLR2 in whole blood, suggesting that the innate immune system has responded to some microbial exposure [62]. Additionally, children living in rural environments, exposed to un-boiled farm milk in the first year of life showed a strong association with up-regulating mRNA expression of CD14, TLR4, TLR5, TLR6, and TLR7 compared to no farm milk consumption[63].

A study investigating ontogeny of the innate immune system in Papa New Guinean (PNG) versus Australian infants, presumed to reflect a traditional vs. modern environment showed a constant or increasing IL-10 production in PNG infants. This suggests that persistent IL-10 production is seen in populations experiencing exposure to a higher biodiversity associated with traditional environments [64]. However, the impact of microbial exposure surely is more complex, and can not be reduced to simple quantitative measures, but likely depends on the type of microbial exposure [14].

Parasites

Parasitic infections have a profound effect on the immune system. Parasitic infection can induce an immune response that is able to polarize the immune system, affecting the ability of the host to deal with new infections or vaccines. A number of studies have shown that helminth infections were found to influence TLR responses or expression levels. For example, *Schistosoma haematobium* (*S.haematobium*)-infected Gabonese children develop a stronger pro-inflammatory TLR2 mediated response, suggesting that parasite infection does

not suppress but rather alters the host innate immune system in the context of a single TLR ligation [65,66]. On the other hand, filarial infections can diminish expression and function of multiple TLRs [67]. Such single-pathogen evaluations likely are oversimplified, as in real life, infections often occur as co- or concurrent infections, and even one single pathogen can activate several immune pathways[48].

Vaccines

Vaccines have been shown to have profoundly different effects depending on the overall health, sex, and age of the child, to whom they were administered [59]. Although still somewhat controversial, findings by Aaby et al. suggest that vaccines have a non-specific (i.e. not involving the targeted microbe (antigen-specific) of the vaccine) effect on morbidity and mortality, and that these effects appear to be more pronounced in girls than boys [68]. For example, BCG protects from non-TB related infectious death in areas of the world with high infant mortality [69]. Although the exact mechanisms are not yet understood, it is speculated that BCG alters innate immunity, which in turn seems to have advantageous effects on the host system toward other pathological stressors.

1.4.2.4 Microbiota

The microbes that live upon and within the average individuals represent the most intimate environmental exposure challenge of the immune system. Microbiota colonizes the human gut, skin, and mucosal membranes, all of which are important for energy harvest, metabolism, and immunological education [127,128,129,130,131]. Alternations to the colonization process have been shown to predispose and increase the risk of the individual to disease later in life [132]. Currently there is a limited amount of research investigating the role of eukaryote in microbial communities, and future work will be required to understand its role in health and disease. It is known, however, that the human microbiota is acquired from local environment and its variability depend on the environmental influences, such as geography, diet, and lifestyle [133].

1.5 Aim

The main aim of this thesis was to begin to decipher the underlying mechanisms leading to variation in the immune responses of children from diverse geographic locations. Given the initiating function of the innate immune system, my thesis focused in particular on variation in innate immune development in populations from different environmental backgrounds.

My thesis work constituted the central part of a comparative international infant cohort study in collaboration with University of British Columbia (Canada), Université Libre de Bruxelles (Belgium), Stellenbosch University (South Africa), and Centro de Investigaciones FEPIS (Ecuador). This collaboration was built on a highly standardized, stringently controlled innate immune phenotypic comparison platform that I optimized as part of my thesis work.

1.5.1 Objectives

To achieve this aim, the following objectives were addressed:

1. To quantify and compare the PRR-mediated functional response of whole blood, through its production of innate cytokines in infants across four continents
2. To compare the PRR-mediated single cell functional response, through the evaluation of select key innate cytokine production in innate immune cell subtypes
3. To evaluate differences between the sites as a first step towards understanding potential variation in response to vaccination or infectious threats.

1.5.2 Hypothesis

The hypothesis of this study was that differences in the innate immune response of children, living in different parts of the world exist.

2 Chapter: Materials and methods

2.1 Cohort Characteristics

2.1.1 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)(Appendix B). Additionally each site involved had obtained ethics approval in their respective research center. 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.

2.1.2 Participate Recruitment and Enrollment

This study compared infants of 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 infants participating in other ongoing research studies at the University of British Columbia[70]. Subjects in Belgium were part of a pilot study for a larger urban-based birth cohort study established at St. Pierre Hospital (Brussels) in collaboration with the Institute for Medical Immunology (IMI). Infants from Ecuador were recruited within a rural-based population cohort study [71], while South African infants had been enrolled in an urban-based birth cohort established at Stellenbosch University [72]. A subject was included in the study if the infant was considered healthy based upon a history-driven health assessment.

Subjects were excluded from the study if they had met one or more of the following criteria: significant chronic medical condition, immune deficiency, immunosuppression by disease or medication, cancer, bone marrow or organ transplantation, or receipt of blood products within 3 months, bleeding disorder or major congenital malformation or genetic disorder. Infants born to HIV positive mothers were excluded from this analysis.

2.2 Innate Immune Analysis Platform

Due to the sentinel function of the innate immune system the analysis of innate immune responses to PRR stimulation is vulnerable to technical artifacts [73]. I therefore first developed a highly standardized, stringently controlled innate immune phenotyping platform ensuring the same experimental set up for all sites.

2.2.1 Blood Collection

Given that one of the major roles of the innate immune system is sensing environmental changes [74,75], technical artifacts can easily plague innate immune assessment [73]. We thus implemented an experimental approach with stringent focus on quality control and assurance. Every step of the experiments was standardized and controlled across all four sites. All materials and reagents from blood draw to final analysis were tested to ensure absence of innate immune activation substances, as our previous work had shown lot-dependent variation [73,76]. All blood draws were performed in a hospital by a trained phlebotomist; the majority of the samples were collected from the arm, with some from the neck. 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 [73,76].

2.2.2 TLR Stimulation

Master mixes of all reagents were made in quantities adequate for the entire study, frozen and shipped under monitored conditions to all four sites. I performed all aspects of the experiments at all sites using our well-established robust, validated and quality-controlled innate immune phenotyping protocol, to ensure study consistency[73,76–79]. In brief, deep-96-well (VWR) source plates, each well containing 1.3ml of a specific TLR ligand, were prepared using sterile procedures under a laminar airflow hood. 22µl from each well of the source plate was dispensed into each well of recipient 96-well round-bottom polystyrene plates (Corning) using the Evolution P3 Precision Pipetting Platform (PerkinElmer). Recipient plates were sealed with sterile aluminum plate sealers and frozen at −80°C until use.

The 96-well plates contained the following TLR 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; NOD 2, InvivoGen) at 0.1μg/ml; and media alone. All of the ligands were diluted in RPMI medium to obtain desired concentration.

Table 2.1 Outline of the TLR stimulation plate used.

	1	2	3	4	5	6	7	8
Stimulation	PAM	POLY I:C	LPS	R848	PGN	MDP	Unstim	Unstim
Concentration	1µg/ml	100µg/ml	10ηg/ml	10µM	10µg/ml	0.1µg/ml	RPMI	RPMI

2.2.3 Luminex Assay

2.2.3.1 Blood Processing and Whole-Blood Culture

Whole blood collected from each subject was processed within 4 h of collection. The blood collected 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 ligands. Blood was incubated for 24 hrs, after which the plates were centrifuged at 600g and 100μl of supernatant was removed and frozen at –80°C for later multiplex assay analysis. Samples were shipped on dry ice via World Courier Inc., with a temperature monitor in each shipment ensuring maintenance of the desired temperature (-80ºC). Samples were stored at -80°C again in the central analysis site (Vancouver, Canada), all were run within 12 months of collection.

2.2.3.2 Instrumentation

Luminex (Upstate/Millipore) multiplex allows for up to 100 analytes to be measured simultaneously in a single microplate well. My instrument has been optimized to measure the 13 cytokines chosen for the Milipore “Flex Kit” required.

2.2.3.3 Cytokine Measurement

Supernatants were thawed at room temperature and assayed by multiplex assay technique (Luminex: Upstate/Millipore "Flex Kit" system) using the high-biotin protocol with overnight incubation at 4°C. Cytokines measured were IFN-α2, IFN-γ, CXCL10, IL-12p70, IL-12p40, IL-6, TNFα, IL-1β, CXCL8, CCL2, CCL3, CCL4, IL-10. Samples were diluted 1-to-1 (and, if needed to fall within the standard curve, 20-, 80-, or 150-fold) with RPMI 1640. Beadlytes, biotin, and streptavidin-phycoerythrin were used at half the manufacturer's recommended concentrations. Assays were read using Luminex 200 Total System (Luminex) running either the Bio-plex (Bio-Rad) or the MasterPlex (MiraiBio) software, and the downstream analysis was performed using Excel (Microsoft) and an in-house database.

2.2.3.4 Human IL-23 ELISA

To determine the IL-23 concentration, filtered supernatants were diluted 1:4 in diluent contained in the human IL-23 (p19/p40) ELISA kit (eBioscience), and assays were performed according to the manufacturer's specifications. Plates were read at 450nm with 570nm subtraction, on a SPECTRAmax Plus. A 4-parameter sigmoid logistic curve was used to generate the standard curve.

2.2.4 Flow Cytometry Assay

2.2.4.1 Blood Processing and Whole-Blood Culture

Whole blood collected from each subject was processed within 4 h of collection. The collected blood was diluted 1:1 with sterile pre-warmed RPMI 1640. 200µl of the diluted blood was added to each well of the premade plates containing the specific ligands and Brefeldin A (BFA). For the ICS assays, the blood cells were incubated for 6 h at 37°C in 5% CO_2. At 6 h, the cultured cells were 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 1x BD FACS Lysing Solution, placed into tubes, and stored frozen at –80°C until staining.

An identical set of plates was incubated in parallel for 24 h without BFA; at 24 h, these plates were spun and 100μl of supernatant was removed and frozen at –80°C for Luminex analysis.

The left over cultured cells were 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 1x BD FACS Lysing Solution, placed into tubes, and stored frozen at –80°C until staining.

2.2.4.2 Blood Processing and *in vitro* Stimulation

Blood collected from each subject was processed within 4 h of collection. The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation. PBMCs were cultured in RPMI 1640 supplemented with 100 U penicillin/ml, 100 mg streptomycin/ml (Invitrogen), and 10% human AB serum.

2.2.4.3 Instrumentation

LSRII Flow Cytometer (BD Biosciences) with four lasers was used for this study. My instrument has been optimized to measure up to 17 fluorescent parameters (Figure 2.1).

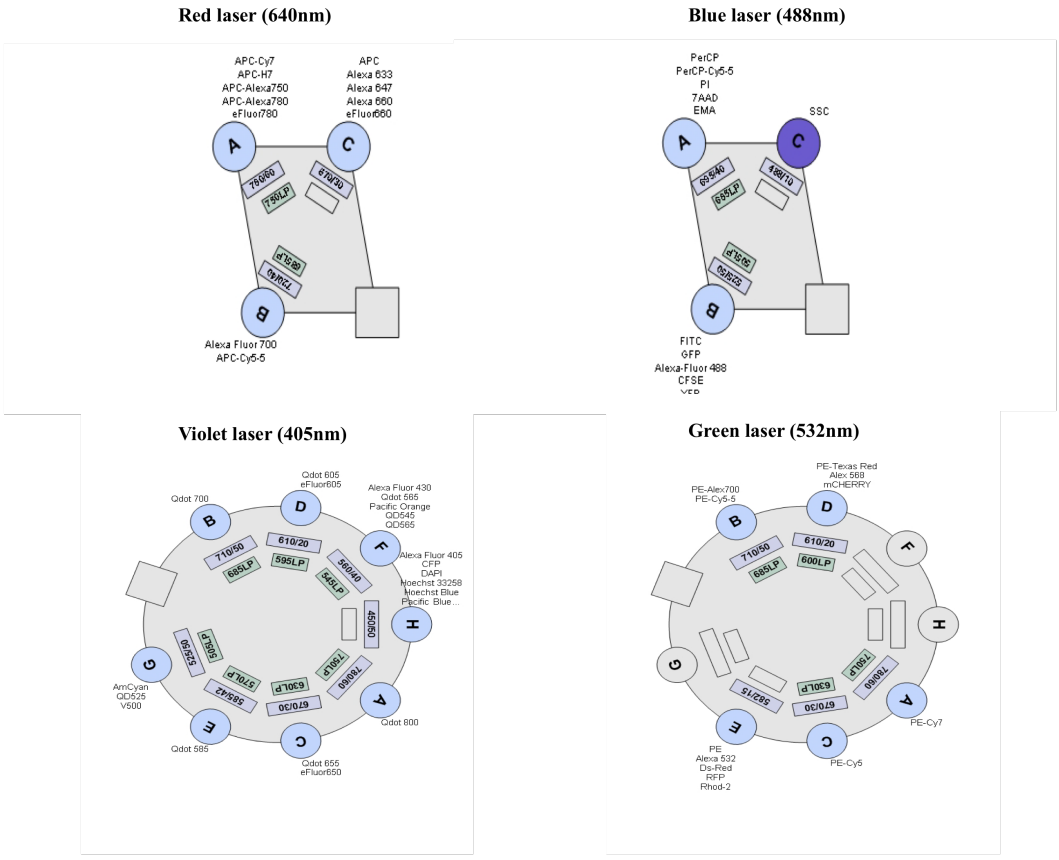


Figure 2.1 LSRII Flow Cytometer parameter layout. This is the layout of the machine used to complete the data acquisition.

2.2.4.4 Flow Cytometry Antibody Panel

To obtain all of the information desired, an 11-colour flow cytometry panel was developed for use on the LSRII Flow Cytometer (BD Biosciences). The details of the panel are listed below in Table 2.2.

Table 2.2 11-colour panel antibody specifications.

Fluorochromes	Name	Clones
APC	CD11C	
Alexa eF 700	TNFα	MAb11
APC eF 780	IFN-γ	4S.B3
Fitc	gdTCR	B1.1
PerCP eF710	IL-6	MQZ13A5
eF450	IL-12	C8.6
V500	CD14	M5EZ
eF605	HLA-DR	LN3
PE	IFN-α	7N4-1
PE-CF594	CD3	UCHTI
PE-Cy7	CD123	6H6

Extensive optimization was done to compile this 11-colour panel.

2.2.4.5 Flow Cytometric Panel Optimization

The gating strategy was extensively optimized to allow for the maximum amount of information to be obtained from the data set. Gates were set based on the fluorescence-minus-one (FMO) principle [80,81], where all but one Ab is combined into a mix. The FMO is used to identify the positively Ab stained cell populations to set the gates. I positioned the unstimulated flow cytometric samples as a negative control.

2.2.4.6 Intracellular Cytokine Staining

Preparation of the samples for flow cytometric analysis was performed as described previously [76,82,83]. The frozen FACSlyed sample tubes were thawed and spun. The pellets were resuspended in 200μl of BD FACS PermeabILizing Solution and incubated at room temperature for 10min. Washed twice in a PBSAN solution (PBSAN contains: PBS containing 0.5% BSA and 0.1% sodium azide). After washing, these cells were stained using an AB master mix, in a final volume 100μl of PBSAN for 45min at room temperature. After

two additional washes with PBSAN, cells were resuspended in PBSAN and analyzed on an LSRII Flow Cytometer (BD Biosciences) set up according to published guidelines [76,83,84].

2.2.4.7 Flow Cytometric Acquisition

All of the data were acquired with a 10-day period on the LSRII Flow Cytometer (BD Biosciences). A total of 500,000 events were acquired per sample. Compensation was set in FlowJo (Tree Star) and samples were analyzed.

2.2.4.7.1 Cell Type Identification

This 11-colour panel allowed for the identification of most of the innate immune response cells types. It also allowed for the comparison of the cell types across the four continents.

2.2.4.8 Flow Cytometric Standardization

To standardize each consecutive experiment that was run on the LSRII using the High Throughput System (HTS), certain calibrations and controls were used to ensure experimental continuity to allow for sample compensation. First, the machine was calibrated using BD CST beads (CST; BD Biosciences) prior to each experimental run. Second, compensation beads (CompBeads; BD Biosciences) were used to standardize/confirm voltage settings and used as single-stain positive and negative controls, as described previously [76,80,83]. Thirdly, FACSlyse frozen stock of a single adult whole blood sample unstimulated and stimulated with R848 were used to set up the detectors in every run to ensure MFI of cytokine positive and negative populations remained approximately the same.

2.2.4.9 Flow Cytometric Gating Strategy

The gating strategy was used to determine all the cell types and cytokines of interest. Using this panel, I was able to identify granulocytes, monocytes, DCs (pDCs and cDCs), CD3+ T cells, γδ T cells, B cells, as well as IFN-α, IFN-γ, IL-12, IL-6, and TNFα. See Figure 2.2.

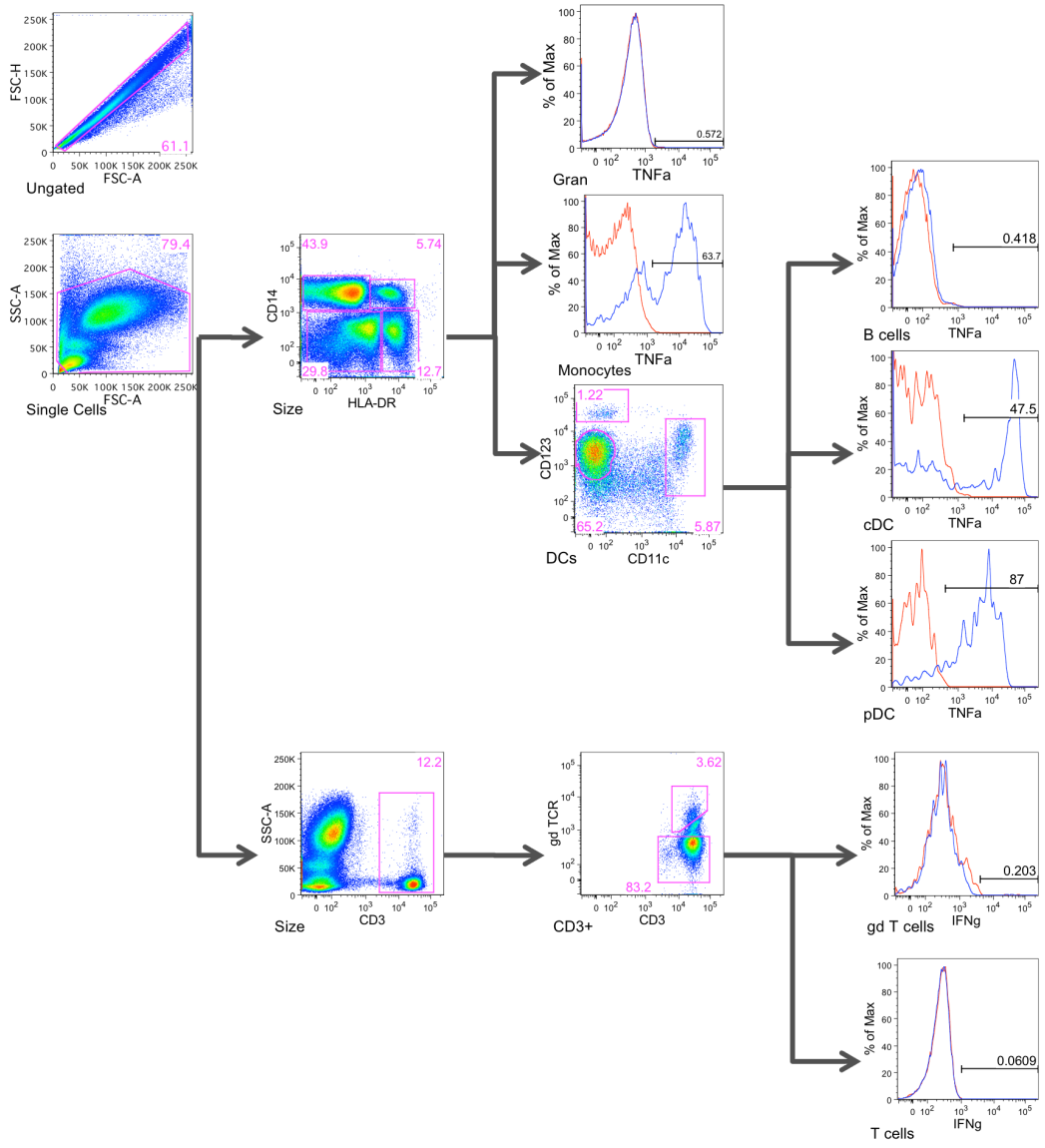


Figure 2.2 The gating strategy used to identify the cell sub-types.

2.2.4.10 Flow Cytometric Sample Analysis

The data analysis was done using FlowJo (TreeStar). The experimental compensation was done in FlowJo; the corresponding samples were analyzed once compensated (Table 2.3). Gates were based on the FMO principle. The unstimulated flow cytometric samples were used as biological negative controls, to help position the bisector gate to determine the positive cytokine responses.

Table 2.3 Compensation Matrix

	Alexa Fluor 700	APC	APC-Alexa780	eFluor 450	eFluor 605	FITC	PE	PE-Cy7	PE-Texas Red	PerCP	V500
Alexa Fluor 700		0.02431	0.2291	1.67E-03	5.94E-04	6.64E-04	0	0.04534	0	0.02668	1.23E-03
APC	0.3455		0.08101	0	8.14E-04	0	0	0.02008	0	9.51E-03	0
APC-Alexa780	0.08172	0.1189		2.48E-03	0	2.15E-03	1.16E-03	0.255	0	3.31E-03	1.74E-03
eFluor450	0	0	0		0.01216	0	0	0	0	0	0.12
eFluor605	0	0	0	1.54E-03		5.62E-04	0.0561	0	0.2002	9.78E-04	5.24E-04
FITC	0	0	0	7.10E-04	7.36E-03		0.01656	0	2.74E-03	0.01521	0.02384
PE	0	0	0	2.41E-03	0.03127	6.69E-03		8.10E-03	0.1327	0.03934	1.30E-03
PE-Cy7	1.87E-03	0	0.02714	0	9.85E-04	1.10E-03	0.05503		8.64E-03	3.68E-03	0
PE-Texas Red	2.41E-03	5.49E-03	0	0	0.1748	2.53E-03	0.3983	0.1152		0.4694	0
PerCP	0.2793	0.02246	0.06942	0	0	0	0	0.3248	0		0
V500	0	0	0	0.1767	0.4096	0.06688	0	0	0	3.24E-03	

2.2.5 Standardization

Given the role the innate immune system to rapidly sense environmental change [74], technical artifacts can easily plague innate immune assessment [73]. 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 [73,76]. Samples post-processing were shipped on dry ice with a temperature monitor in each shipment; 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 or run effects.

2.3 Analysis

2.3.1 Z-score Analysis

The WHO Anthropometric calculator was used to determine each participant's individual z-score (WHO Anthro version 3.2.2)[85].

2.3.2 Statistical Analysis

To compare the whole cytokine data, Kruskal-Wallis analysis was performed to compare the four sites for significant variance among the median cytokine concentrations; Bonferroni test was applied to correct for multiple comparisons. 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).

To compare the flow cytometric data, Kruskal-Wallis analysis was performed to compare the four sites for significant variance among the median cytokine concentrations 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).

2.3.3 Principal Component Analysis

In order to visualize the data in an intuitive fashion, we plotted the data using Principal Component Analysis (PCA). The cytokine data were log-transformed and then subjected to PCA analysis using GINKGO: Multivariate Analysis System [86,87]. The data were plotted using Tableau visualization software (Tableau Software. Inc.). Due to low sample volume, IL-23 could not be assessed for each of the enrolled subjects; the IL-23 data were thus not included into the PCA cluster analysis but were included in the box-plots and statistics.

2.3.4 Polyfunctional Analysis

Polyfunctional analysis is defined as the assessment of multiple parameters at the single cell level [88]. The Polyfunctionality Index (PI) numerically evaluates the degree and variation of polyfunctionality within a particular dataset, enabling comparative and correlative statistical analysis as described [88]. The polyfunctionality analysis was performed using the software "FunkyCells - Boolean Data Miner" developed by Dr. Larsen (Paris, France).

3 Chapter: Pattern recognition receptor-mediated cytokine response in infants across four continents

3.1 Introduction

The first few years of life represent a period of marked susceptibility to infectious diseases [1–3]. Such vulnerability reflects a state of age-dependent suboptimal immune mediated protection in early life [1,39,82]. Around the world, the Expanded Program of Immunization (EPI) and similar regional or national programs direct the immunization of infants [5,6]. These public health programs have greatly contributed to diminishing infectious mortality and morbidity in early life [7]. As the formulations and schedules of vaccination do not vary considerably amongst countries, these vaccination strategies rely on the notion that responses to the vaccination are similar amongst infants living in different regions of the world [6,11,89]. However, it has become apparent that vaccine responses differ in infants from varying geographic regions [90]. The underlying mechanisms leading to different vaccine responses in different populations remain largely unknown. This lack of understanding prevents optimization of infant vaccine responses. As innate immunity directs the adaptive response, I reasoned that the first step towards identifying the mechanistic causes leading to variation in vaccine responses in infants from diverse regions of the world would be to determine if differences in innate immunity exist among different populations from disparate regions. Several previous studies have described the ontogeny of the innate pattern recognition receptor (PRR) response in infants from different geographical regions [82]. We set out to contrast the PRR response to stimulation of infants across four continents (Africa, North America, South America, and Europe) using a highly standardized, stringently controlled innate immune phenotyping platform, ensuring the same experimental set up for all sites. We found significant differences in innate immune responses to PRR stimulation when comparing different populations of children.

3.2 Results

3.2.1 Cohort Characteristics

We selected four populations that differ in many of the elements presumed to be relevant for variation in risk for infection or vaccine responses, most importantly genetic variation amongst the hosts and differences in environmental exposure such as residence in resource-poor vs. resource -rich settings (Table 3.1). We chose to study innate immunity in infants 2 years of age to ensure all had completed locally recommended infant vaccinations (Table 3.2). The characteristics of the study population at the time of sample collection are described in Table 1. Based on the WHO 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 each subject in all four cohorts were within less than 2 standard deviations of the mean (Table 3.1). This indicated that the infants in all of our cohorts were within the average range for normal child growth standards [91,92]. Furthermore, all infants were healthy based on clinical history taken at the time of sample collection.

Table 3.1 Demographics of the infants at each site.

	Belgium	Canada	Ecuador	South Africa
n=	14	22	43	20
Infant Characteristics				
Mean Age (mo) - mean (SD)	24.7 (4.3)	19.1 (0.8)	26.7 (1.28)	24.7 (0.6)
Birth Weight (g) - mean (SD)	2996.2 (796.3)	3339.6 (448.2)	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)	39.2 (1.5)	38.9 (1.1)	37.8 (2.4)
Premature < 37wks (% of total)	2 (14%)	1 (4.5%)	0 (0%)	3 (15%)
Weight (g) - mean (SD)	13364.3 (1786.1)	11190.9 (1392.5)	11501.16 (1010.7)	11205.0 (1300.7)
Height (cm) - mean (SD)	92.2 (4.6)	82.2 (3.0)	84.3 (2.5)	84.4 (0.91)
Female/Male	1/13	10/12	29/14	12/8
WAZ (SD)	0.69(1.2)	-0.05(0.9)	-0.32 (0.93)	-0.58 (0.95)
LAZ (SD)	1.56(0.8)	-0.30(0.9)	-0.78 (1.49)	-1.07 (1.20)
WLZ (SD)	-0.18(1.4)	0.17(1.0)	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

3.2.2 Innate Cytokine Responses

We chose 13 cytokine target read-outs to broadly cover the most important functional categories: innate cytokines supporting Th1-type adaptive immunity (IFN-α, IFN-γ,

CXCL10, IL-12p70), innate cytokines supporting Th17-type adaptive immunity (IL-12p40, IL-6, IL-23), pro-inflammatory cytokines (TNFα, IL-1β), chemokines (CXCL8, CCL3, CCL4), and the regulatory cytokine IL-10. There were no significant differences in response between males vs. females (data not shown); we thus analyzed males and females as one group for each site.

Principal Component Analysis (PCA) (Fig 3.1) allowed us to compress the many dimensions (12 eigenvectors, each representing one cytokine) following response to all 7 PRR agonists for visual analysis [87,93]. In Figure 3.1, each color represents a ligand, and each dot represents one infant for a particular stimulatory condition. The percentage of PCA1 (55.45%) and PCA2 (19.45%) contributing to overall variance between subjects and stimuli is depicted on the x- and y-axis respectively. The primary component separating data points in Figure 3.1 (i.e. principle component 1 (PC1)) appears to be the overall strength of the stimulation, as the weakest stimulant (unstimulated samples) clusters furthest to the right, while the overall strongest stimulant (R848) clusters furthest to the left. PC2 on the other hand separates the clusters based on location of the PRR, i.e. endosomal PRRs (TLR3 and TLR7/8) responses cluster higher up, while cell surface and cytoplasmic PRRs (TLR2/1, TLR4, NOD) cluster lower down. The eigenvectors of the PCA are shown in Figure 3.1B. This allows further delineation of contributors for the clustering along PC2, in that the endosomal-TLR driven clusters (i.e. those located higher up in the plot) are largely composed of Th-1 supporting innate cytokines (IFN-α, IL-12p70 and IFN-γ), while the cell-surface TLR- and NOD-driven clusters (i.e. those clustering lower down but to the left) are composed of the pro-inflammatory cytokines (TNFα, IL-1β etc.). This pattern is also consistent with the known function of PRRs, in that endosomal PRRs mainly recognize intracellular pathogens and their activation leads to production of innate cytokines supporting cell mediated Th1-type immunity (i.e. IFN-α, IL-12p70) [47,82]. Visual analysis indicated that the largest variance between samples was determined by the type of PRR stimulation (Figure 3.1). Furthermore, the fact that the PRR-induced responses led to similar clustering for most infants from all four sites suggests that basic biological mechanisms functioning in all populations represented the strongest component contributing to clustering.

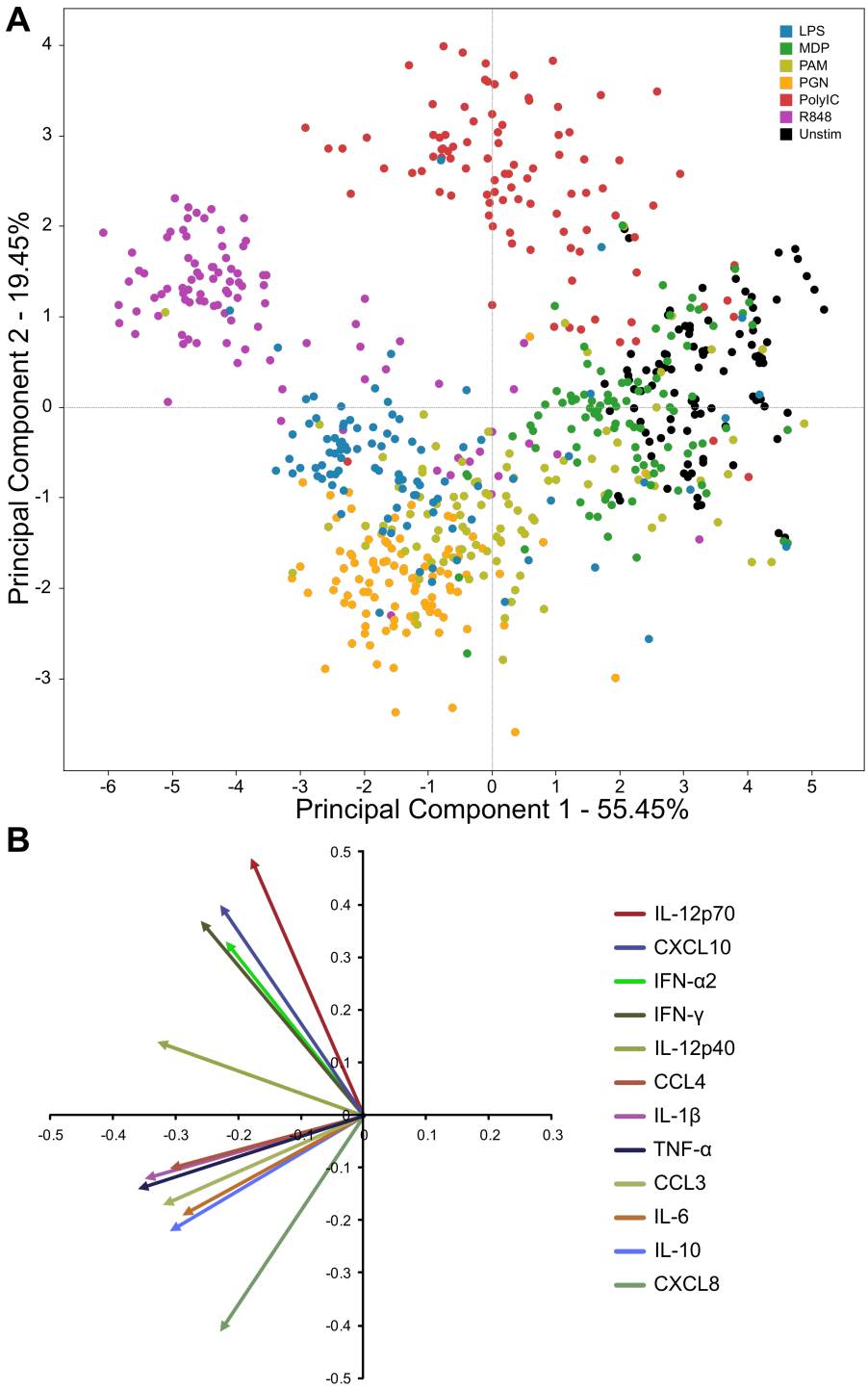


Figure 3.1 PCA ordination of the innate immune response for all subjects measured by cytokine secretion in response to PRR agonists. A. Depicts the variance in cytokine response (12 dimensions) to all ligands. Each color represents a ligand, while each dot represents one child. B. Eigenvectors show the particular correlations of individual cytokines to the ordination of the PCA in A.

3.2.3 Endosomal TLRs Responses: South African Infants Under-Responded.

3.2.3.1 TLR7/8 (R848)

In Figure 3.2A, we highlighted responses of all four cohorts to R848 (a TLR7/8 ligand) stimulation. The Belgian, Canadian, and Ecuadorian responses tightly clustered together at the upper left-hand corner of the ordination, clearly apart from the un-stimulated samples. The response of infants in the South African cohort localized as a distinct and separate cluster between the Belgian-Canadian-Ecuadorian stimulated cluster and the cluster of all the un-stimulated samples. This pattern suggests that compared to the other geographic cohorts, South African infants respond differently to stimulation with R848.

We also contrasted production of individual cytokines between populations (Figure 3.3). Kruskal-Wallis analysis (Table 3.3) revealed that, with the exception of CXCL8, all cytokines produced in response to TLR7/8 stimulation were detected at significantly different levels among sites. Dunn's post-test (Table 3.3) further revealed that South African infants' responses were solely responsible for the significant variation among sites, with the South African infants responding consistently lower than infants from the other sites. This was true for Th1-supporting innate cytokines, where production in South African vs. Ecuadorian infants differed for all, and South Africa vs. Canada for most cytokines. For Th17-supporting innate cytokines, differences in production between South African vs. Ecuadorian infants were consistently present, while IL-12p40 production displayed a significant difference between South African and Canadian infants' responses, and IL-6 between South African and Belgian subjects. For the pro-inflammatory cytokines, significant difference between sites originated between South Africa and all other sites. Production of the pro-inflammatory chemokines was also significantly different between all sites, with South African infants producing less than Belgian, Canadian or Ecuadorian subjects. Furthermore the anti-inflammatory cytokine IL-10, median concentration detected following TLR7/8 stimulation was lowest in South African infants, with Belgian infants producing the most.

3.2.3.2 TLR3 (Poly I:C)

PCA analysis of PolyI:C (a TLR 3 ligand) stimulation also led to similar responses at all sites except South Africa (Figure 3.2B). South African infants produced low Th1-supporting and pro-inflammatory cytokines. However, the magnitude of the difference in cytokine production between the response of South African subjects and those from the other sites was not as large following TLR3 stimulation as it was following TLR7/8 stimulation. For example, the median response of the Belgian cohort located marginally above the South African cohort. Furthermore, the Ecuadorian subjects stimulated with PolyI:C appeared to form two separate clusters, one grouping above and another below the main cluster composed of Belgian and Canadian subjects. The separation of the Ecuadorian subjects into two clusters is also reflected in the larger confidence interval of median cytokine concentrations depicted in Figure 3.3.

Statistical analysis (Table 3.3) revealed that cytokines were produced at significantly different levels among the four sites following TLR3 stimulation, with the exception of IL-6, CXCL8, and IL-10. Dunn's analysis of Th1-supporting and pro-inflammatory cytokine production revealed that the variation mainly originated from South African infants. Production of IL-10 following PolyI:C stimulation was not above background for subjects from any of the four sites.

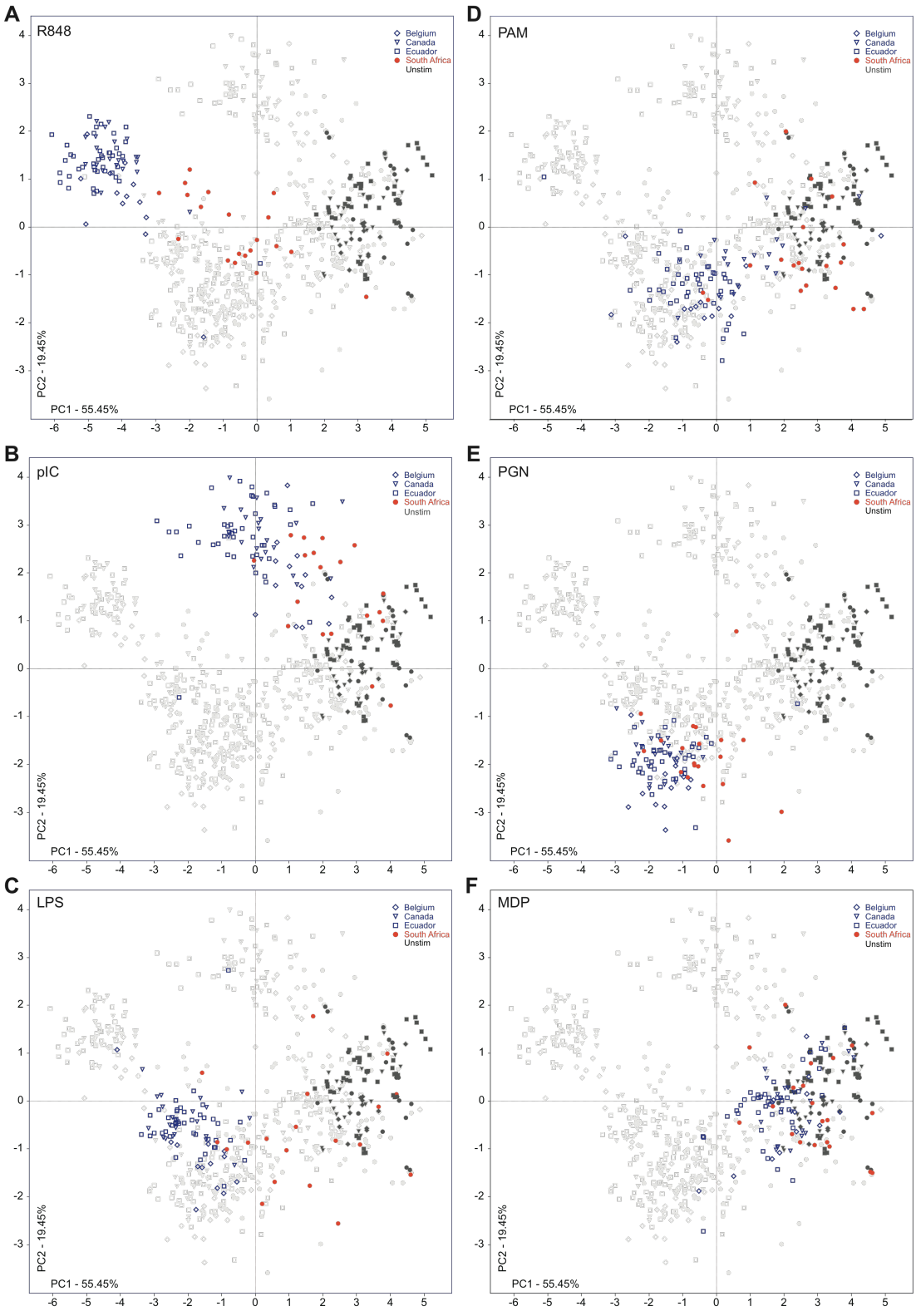


Figure 3.2 The innate immune response to PRR stimulation. PCA ordination of the R848 response is depicted in A, PolyI:C in B, LPS in C, PAM in D, PGN in E, and MDP in F. Each dot represents one subject, symbol represents a site, and color represents the stimulation (open (Belgium, Canada, Ecuador), red (South Africa) for given stimulation).

3.2.4 Responses to Cell Surface TLRs: South African Infants Under-Responded.

3.2.4.1 TLR4 (LPS)

LPS stimulation of TLR4 resulted in clustering of response for infants from all sites except from South Africa (Figure 3.2C). This IL-lustrates that compared to subjects from other sites, whole blood from South African infants also under-responded to TLR 4 stimulation. Most cytokines were detectable except IFN-α and IL-12p70, which were not produced above background by infants from any of our cohorts. The median cytokine concentration in response to TLR 4 stimulation for each individual cytokine response was consistently lowest for South African infants (Figure 3.3). Specifically, Th1 cytokine production in response to LPS revealed a low response pattern in South African subjects, while the infants from the other three sites showed higher median response. While the response overall appeared to have high variation between subjects even within a given site (note the larger spread of the CI), statistical differences were still detected (Table 3.3). Dunn's post-test identified the greatest statistical difference for IFN-γ was found between Canadian and South African or Belgian children. Production of Th17-supporting cytokines following LPS stimulation also identified South Africa infants as having the lowest response to LPS. Similarly, production of pro-inflammatory cytokines and chemokines showed the weakest response in South African and the highest response in Canadian subjects; Dunn's comparison identified the greatest statistical differences between South African vs. Canadian, and Ecuadorian children. Production of IL-10 in response to LPS was again lowest in South African children.

3.2.4.2 TLR2/1 (PAM)

Overall, PAM stimulation resulted in the least obvious PCA cluster separation between sites for surface TLRs. However, only the response of the South African cohort strongly overlapped with the unstimulated samples, indicating an overall lower response, while the response of the subjects from other sites, primarily from Ecuador and Belgium clearly clustered away from the un-stimulated samples (Figure 3.2D). With the exception of CXCL10, Th1-supporting cytokines were not produced above the background for subjects from any site in response to PAM (Figure 3.3). Dunn's test of CXCL10 production following TLR2/1 stimulation revealed that infants from South Africa and Canada did not vary from each other in their response, but infants from both sites differed significantly in their

response from Belgium and Ecuador (Table 3.3). The same relationship was found for production of Th17-supporting cytokines, with infants from Belgium and Ecuador producing higher median concentration compared to infants from South Africa and Canada; however, only South African infants varied statistically from Belgian and Ecuadorian children. The production of pro-inflammatory cytokines was lower for infants from South Africa and Canada vs. Belgium or Ecuador. The response of South African infants varied significantly from Ecuadorian and Belgian infants for all pro-inflammatory cytokines. The same statistical relationship was also detected for production of IL-10.

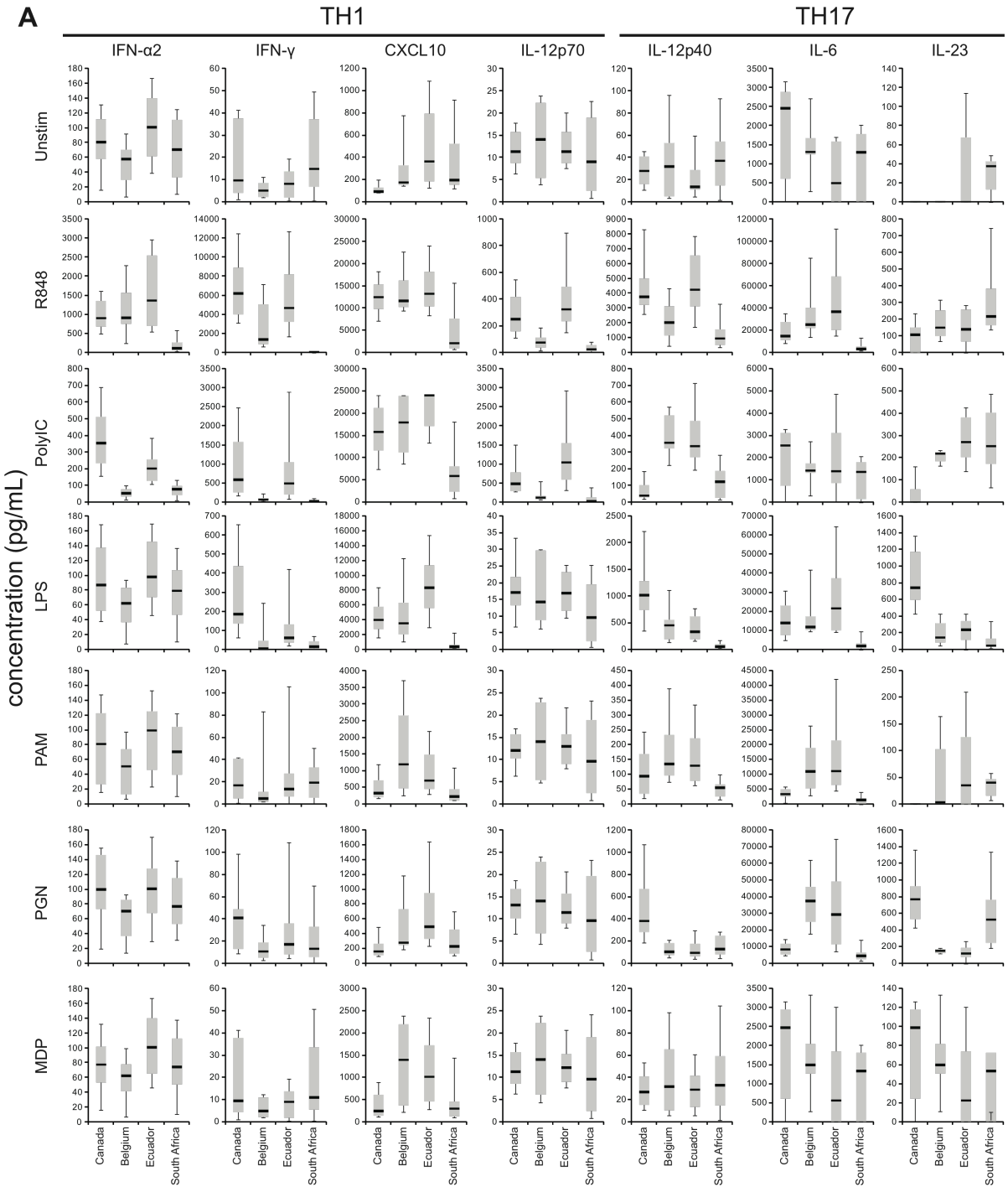


Figure 3.3 Cytokine production comparison for all four sites per cytokine and stimulation. Box-whisker plots, with the median highlighted for each site (the error bars = 90% CI). A shows the production of Th-1 and Th-17 supporting innate cytokines. B shows the production of the pro-inflammatory cytokines and chemokines, as well as the regulatory cytokine IL-10.

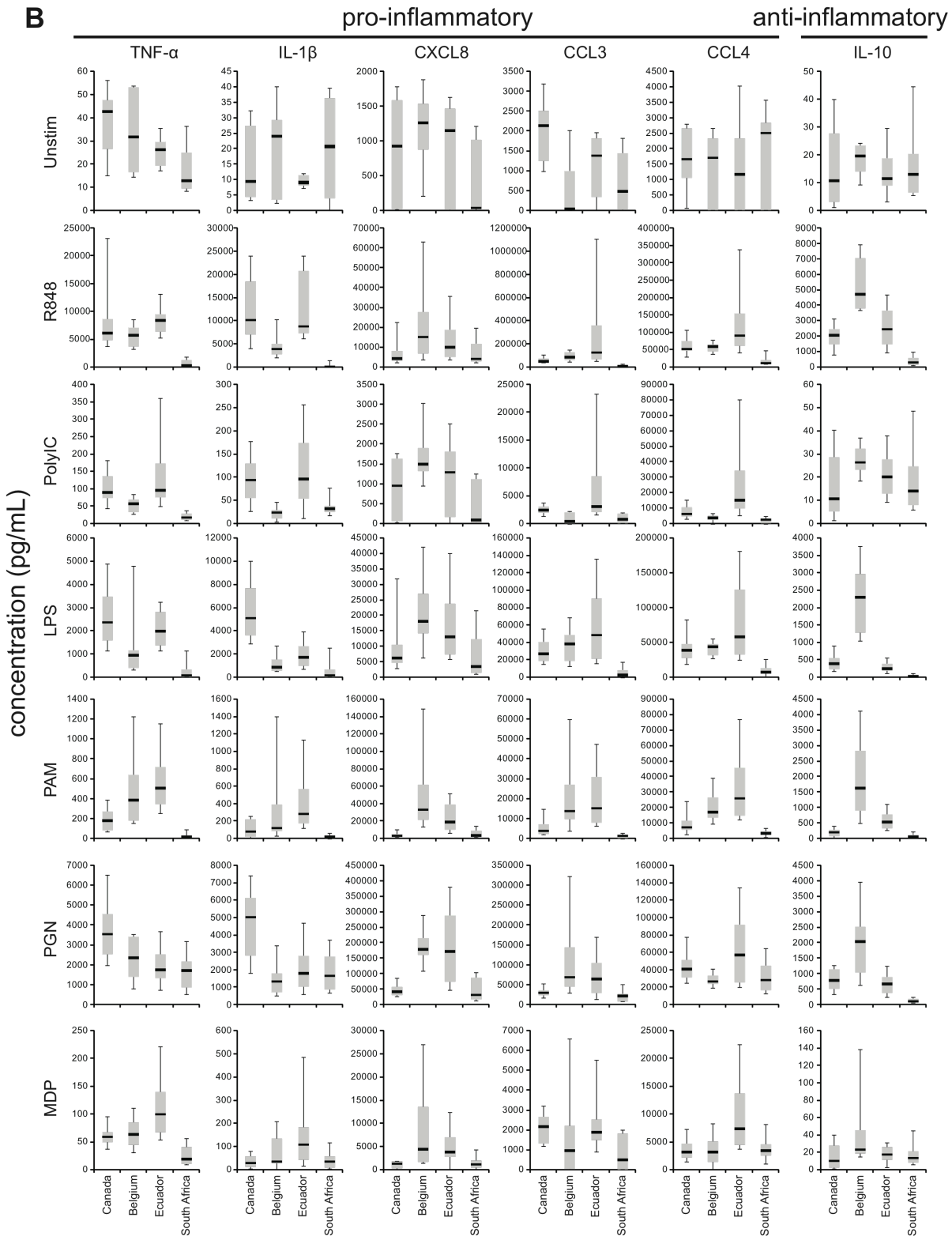

B
pro-inflammatory
anti-inflammatory
TNF-α
IL-1β
CXCL8
CCL3
CCL4
IL-10
Unstim
R848
PolyIC
LPS
PAM
PGN
MDP
concentration (pg/mL)
Canada
Belgium
Ecuador
South Africa

Table 3.2 A simplified vaccine schedule given to infants at each site.

	Infants Age (months)										
Vaccine	**Birth**	**1**	**2**	**3**	**4**	**6**	**9**	**12**	**15**	**18**	**24**
Bacillus Calmette Guerin											
Belgium											
Canada											
Ecuador	✔										
South Africa	✔										
Diphtheria, Tetanus, Pertussis											
Belgium			✔	✔	✔				✔		
Canada			✔		✔	✔				✔	
Ecuador			✔		✔					✔	
South Africa			✔	✔	✔					✔	
Inactivated Polio											
Belgium			✔	✔	✔				✔		
Canada			✔		✔	✔				✔	
Ecuador			✔(OPV)		✔(OPV)	✔(OPV)					
South Africa	✔(OPV)		✔	✔	✔					✔	
Haemophilus influenzae type b											
Belgium			✔	✔	✔				✔		
Canada			✔		✔	✔				✔	
Ecuador			✔		✔	✔					
South Africa			✔	✔	✔					✔	
Hepatitis B											
Belgium			✔	✔	✔				✔		
Canada			✔		✔	✔					
Ecuador		✔				✔					
South Africa			✔	✔	✔						
Pneumococcal											
Belgium			✔		✔			✔			
Canada			✔		✔			✔			
Ecuador			(✔)		(✔)			(✔)			
South Africa			✔		✔		✔				
Meningococcal											
Belgium									✔		
Canada			✔					✔			
Ecuador											
South Africa											
Mumps, Measles, Rubella											
Belgium								✔			
Canada								✔			
Ecuador								✔			
South Africa							✔ *			✔ *	
Rotavirus											
Belgium			✔	✔	(✔)						
Canada			✔		✔						
Ecuador			✔		✔						
South Africa			✔		✔						
Varicella											
Belgium											
Canada								✔			
Ecuador								(✔)			
South Africa											
Influenza											
Belgium											
Canada						✔					
Ecuador						(✔)					
South Africa										✔	

Notes:

In Ecuador: Varicella, Yellow fever, Influenza, and pneumococcal vaccines are only provided during vaccination campaigns and are not routinely given vaccines (✔)

In South Africa: MMR was (*) Measles only

3.2.5 Cytosolic PRR Responses: Similar Responses in All Cohorts

3.2.5.1 NOD2 & TLR2/1(PGN):

Following PGN stimulation the response of infants from all of the sites, including South Africa, clustered tightly together (Figure 3.2E). On closer inspection, we found that none of the Th1 cytokines were produced above background. Canadian and South African infants all produced significant levels Th17-supporting innate cytokines following PGN stimulation, while Belgian and Ecuadorian infants responded weakly. Production of the pro-inflammatory cytokines and chemokines was also readily detected in infants from all sites, with differences between the four sites. Dunn's comparison of IL-10 revealed strong significant variation between infants from South Africa (low) vs. those from Canada, Belgium, or Ecuador (Table 3.3).

3.2.5.2 NOD2 (MDP):

Following stimulation with MDP, the response clusters visually overlapped with the un-stimulated clusters for all sites suggesting an overall very low response (Figure 3.2F). Th1- and Th17-supporting, as well as anti-inflammatory cytokines were not produced above background; only production of CXCL10, TNFα, CXCL8 and CCL4 was detectable above background. As with the other NOD ligand (PGN), the response of South African infants was more similar to infants from the other sites as compared to the TLR stimuli. Specifically, production of CXCL10, TNFα, CXCL8, and CCL4 in South African infants was similar to Canadian children, while responses in both of these groups were significantly lower than the responses of Belgian and Ecuadorian children.

Table 3.3 Statistical analysis of each cytokine per stimulation at all sites. Kruskal-Wallis test for all four sites (Global) was corrected for multiple comparisons using Bonferroni test (significant p value is p<0.000595). Dunn's post-hoc test was applied to each site pairing (statistical significance p value was *** < 0.001, ** <0.01, * <0.05). Belgium (BLG), Canada (CND), Ecuador (ECD), and South Africa (SAF).

Unstim	**IFNa2**	**IFNg**	**IP-10**	**IL-12p70**	**IL-12p40**	**IL-6**	**IL-23**	**TNFa**	**IL-1b**	**IL-8**	**MIP-1a**	**MIP-1b**	**IL-10**
Global	0.003	0.0956	< 0.0001	0.6486	0.2065	0.0024	< 0.0001	0.0002	0.3932	0.0153	< 0.0001	0.3424	0.838
SAF vs BLG			ns				*	*			ns		
SAF vs CND			**				***	***			***		
SAF vs ECD			ns				ns	ns			ns		
BLG vs CND			*				ns	ns			**		
BLG vs ECD			ns				ns	ns			ns		
CND vs ECD			***				*	*			**		
R848	IFNa2	IFNg	IP-10	IL-12p70	IL-12p40	IL-6	IL-23	TNFa	IL-1b	IL-8	MIP-1a	MIP-1b	IL-10
Global	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0036	< 0.0001	< 0.0001	0.0053	< 0.0001	< 0.0001	< 0.0001
SAF vs BLG	**	*	**	ns	ns	***		***	*		***	**	***
SAF vs CND	***	***	**	***	***	*		***	***		**	***	**
SAF vs ECD	***	***	***	***	***	***		***	***		***	***	***
BLG vs CND	ns	ns	ns	*	ns	ns		ns	ns		ns	ns	***
BLG vs ECD	ns	ns	ns	***	*	ns		ns	*		ns	ns	**
CND vs ECD	ns	ns	ns	ns	ns	**		ns	ns		**	ns	ns
pIC	IFNa2	IFNg	IP-10	IL-12p70	IL-12p40	IL-6	IL-23	TNFa	IL-1b	IL-8	MIP-1a	MIP-1b	IL-10
Global	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1267	< 0.0001	< 0.0001	< 0.0001	0.0041	< 0.0001	< 0.0001	0.0031
SAF vs BLG	ns	ns	**	ns	***		ns	ns	ns		ns	ns	
SAF vs CND	***	***	*	**	ns		***	***	*		***	**	
SAF vs ECD	***	***	***	***	***		ns	***	**		***	***	
BLG vs CND	***	**	ns	ns	***		ns	ns	***		**	ns	
BLG vs ECD	***	**	ns	***	ns		ns	**	***		***	***	
CND vs ECD	ns	ns	ns	ns	***		***	ns	ns		ns	*	
LPS	IFNa2	IFNg	IP-10	IL-12p70	IL-12p40	IL-6	IL-23	TNFa	IL-1b	IL-8	MIP-1a	MIP-1b	IL-10
Global	0.0063	< 0.0001	< 0.0001	0.1411	< 0.0001	< 0.0001	0.0036	< 0.0001	< 0.0001	0.0002	< 0.0001	< 0.0001	< 0.0001
SAF vs BLG		ns	**		***	***		*	ns	**	***	**	***
SAF vs CND		***	*		***	***		***	***	ns	***	**	***
SAF vs ECD		**	***		***	***		***	**	**	***	***	***
BLG vs CND		***	ns		ns	ns		ns	***	ns	ns	ns	***
BLG vs ECD		ns	ns		ns	ns		ns	ns	ns	ns	ns	***
CND vs ECD		*	*		**	ns		ns	***	ns	ns	ns	ns
PAM	IFNa2	IFNg	IP-10	IL-12p70	IL-12p40	IL-6	IL-23	TNFa	IL-1b	IL-8	MIP-1a	MIP-1b	IL-10
Global	0.0145	0.6916	< 0.0001	0.6243	< 0.0001	< 0.0001	0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SAF vs BLG			**		***	***	ns	***	**	***	***	***	***
SAF vs CND			ns		ns	ns	ns	ns	ns	ns	ns	ns	ns
SAF vs ECD			***		***	***	ns	***	***	***	***	***	***
BLG vs CND			*		ns	*	***	ns	ns	***	ns	ns	***
BLG vs ECD			ns		ns	ns	ns	ns	ns	ns	ns	ns	ns
CND vs ECD			*		ns	***	ns	***	**	***	***	***	***
PGN	IFNa2	IFNg	IP-10	IL-12p70	IL-12p40	IL-6	IL-23	TNFa	IL-1b	IL-8	MIP-1a	MIP-1b	IL-10
Global	0.0174	0.042	< 0.0001	0.593	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0044	< 0.0001
SAF vs BLG			ns		ns	***	ns	ns	ns	***	**		***
SAF vs CND			ns		***	ns	ns	***	**	ns	ns		***
SAF vs ECD			*		ns	***	***	ns	ns	***	***		***
BLG vs CND			*		***	***	*	ns	***	***	ns		ns
BLG vs ECD			ns		ns	ns	ns	ns	ns	ns	ns		***
CND vs ECD			***		***	***	***	***	***	***	ns		ns
MDP	IFNa2	IFNg	IP 10	IL 12p70	IL 12p40	IL 6	IL 23	TNFa	IL 1b	IL 8	MIP 1a	MIP 1b	IL 10
Global	0.005	0.2408	< 0.0001	0.7072	0.9201	0.0254	< 0.0001	< 0.0001	0.0006	< 0.0001	0.0007	< 0.0001	0.0477
SAF vs BLG			**				ns	*		*		ns	
SAF vs CND			ns				***	*		ns		ns	
SAF vs ECD			**				ns	***		**		**	
BLG vs CND			**				ns	ns		**		ns	
BLG vs ECD			ns				ns	ns		ns		**	
CND vs ECD			***				*	**		***		***	

3.3 Discussion

Our study represents the first test of the hypothesis that innate cytokine production in infancy following PRR stimulation varies between continents. Employing a stringently controlled, robust, high-throughput innate immune phenotyping platform, we identified similarities as well as differences in innate immune response to PRR stimulation of samples collected from infants from 4 different continents. When contrasting the infant innate cytokine response based on country, it emerged that the responses of South African infants for most stimuli were distinct from responses of infants at the three other sites. This was notable both in the degree of separation of the clusters as well as in the consistency displayed across multiple stimuli. However, while the innate cytokine response to PRR stimulation in South African infants was found to be lower for nearly all parameters tested, it was similar to infants from the other three sites for the NOD2 ligands PGN and MDP. This suggests that it was not an overall inability of South African infants to respond with cytokine production to PRR stimulation, but that variation in the pattern of innate cytokine production following PRR stimulation in infancy varied by geographic region in response to the particular type of PRR stimulation. More specifically, the response to endosomal as well as cell surface PRRs varied by region, while the response to cytoplasmic stimuli was more similar among infants from different continents. This suggests differences in particular downstream signaling cascades in infants from South Africa vs. the other three sites.

Differences in innate immune status have been ascribed to variation in environmental exposures ranging from birth mode, feeding mode, infections, vaccination and resource-rich vs. –poor region of residence (reviewed in [8,82]). However, based on these previous studies the results of our current study - indicating striking differences between the innate immune response of South African infants vs. those from Ecuador, Belgium, and Canada - were not predictable. Our own work with Canadian infants revealed an overall steady increase from birth onward in the production of Th1 supporting innate cytokines following TLR stimulation, while TLR-induced anti-inflammatory and Th17 supporting innate cytokines progressively declined over the first two years of life [77,78,94]. This was consistent with findings from other resource-rich regions of the world, e.g. Belgium and the Netherlands [95,96]. In contrast, we found that a similar experimental approach revealed a decline from a high at 2

weeks of age of most TLR-induced innate cytokine responses in South African infants over the first year of life [79]. Such decline over the first year of life was consistent with previous studies from The Gambia as well as Ecuador [97]. Furthermore, cord blood mononuclear cells (CBMC) from Papua New Guinean newborns produce lower IL-6 and type-I IFN responses to TLR2 stimulation, and lower TNFα responses to TLR4 stimulation as compared to Australian newborns [98]. Over the first 2 years of their lives, Papua New Guinean infants develop increasing IL-6 and IFN-γ responses to TLR2 and TLR3 agonists in parallel with sustained high IL-10 responses [64]. Based on these data it was hypothesized that infants born in resource-rich countries (i.e. Australia) exhibit increased innate immune reactivity at birth compared with infants born in resource-poor countries (i.e. Papua New Guinea) [64,99].

The data presented here strongly argue that differences between regionally disparate groups in innate cytokine production following PRR stimulation were unlikely due to resource-rich vs. resource-poor influence [64,99] nor impacted by latitude [14], as infants from Ecuador (considered resource-poor) produced as much or more of any innate cytokine than infants from Belgium or Canada (both considered resource-rich), and certainly more than infants from South Africa (considered resource poor). Furthermore, given that the subjects in our study from South Africa and Ecuador received BCG around birth while subjects from Belgium and Canada did not (Table 3.2), newborn BCG immunization also appears unlikely to be the main driver for observed differences at 2 years of age. Of note, Djuardi et al. also found no clear effect of BCG vaccination on the innate immune ontogeny [100]. While the overall vaccination schedule was similar for all infants across the four sites, differences in vaccine composition (e.g. acellular vs. whole-cell pertussis) or exact age of receipt of vaccines differed; we thus can not exclude that such variation in standard childhood vaccination might be responsible for our observed differences. We also interpret our data to indicate that differences in feeding-mode (length of breast-feeding; breast- vs. bottle-feeding etc.) were unlikely major contributors to the differences we observed, as feeding mode differed vastly between and within sites. We can however not exclude that feeding-mode would not lead differences in innate immune development when comparing different feeding-modes within a given population [101]. Parasitic infections, which are common in South Africa and Ecuador early in life, but rare in Belgium and Canada, also were unlikely to offer

a general explanation for the variation between populations we observed. However, we did not measure this directly in either the mothers nor in our study subject, thus cannot firmly exclude this possibility [101]. Our data comparing secreted cytokine levels in supernatant of whole blood cultures does not permit identification of the source or origin of observed differences in innate cytokine production; differences could thus be due to cell-intrinsic (e.g. signaling mechanisms) or cell extrinsic (e.g. cell composition) factors, or a combination of both.

Differences in host genetic composition are known to influence innate immunity [102]. 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 within different racial backgrounds [51]. It is thus entirely possible that genetic differences (including variation in HLA) between our populations contributed to the differences in functional responses we measured between sites. The relatively small standard deviation for the cytokine responses of each cohort suggests that genetic heterogeneity within each site was smaller than between sites. We also noted that the composition of enrolled subjects within each of our cohorts included a wide range of ancestral origin. We thus do not believe that differences in genetic background alone can explain the striking difference between responses to PRR stimulation of infants born and raised in South Africa vs. our other three sites. We hypothesize that the particular constellation of microbiota in South African infants may contribute to our observed difference innate immune phenotype. This hypothesis is based on the timing of the decline of innate responsiveness in South African infants (between 6-12 months of age) and the persistence of the lower innate responsiveness into adulthood [79]. A stable human intestinal microbiota is established over the first year of life and then persists into adulthood [103,104].

Infant mortality varies greatly between different regions of the world [3]. In our four cohorts between 2005-2010 for every 1000 live births Belgium registered 5 deaths per annum before the age of 5 years, Canada 6, Ecuador 26, but South Africa 79 [105]. Low birth weight and gestational age often correlate with increased mortality [106]; however, the subjects in our cohort were of normal average birth weight, and the number of lower gestational age at

delivery in each cohort was similar between sites (Table 1). These variables are therefore less likely to have caused the low innate immune response in only South African infants. Patterns of innate immune ontogeny in North American or European infants have been shown to correlate with particular age-dependent windows of vulnerability to specific infections [94]. It is thus entirely possible, that the overall lower response of South African infants at 2 years of age to PRR stimulation reflects enhanced susceptibility to infection, and thus may be of clinical relevance.

The major limitation of our study is the small sample size; we thus cannot fully exclude the potential for a type 1 statistical error even after correcting for multiple comparisons. Our findings will need to be replicated in larger studies at the same and at additional sites. Our recruitment strategy also was not representative of each population in its entirety. Notwithstanding these limitations, our data allow formulation of a molecular mechanistic hypothesis ('population-based differences exist in signaling downstream of surface and endosomal PRRs, but not of cytoplasmic PRRs'), and has identified possible relevant clinical ramifications ('differences in vaccine responses and infant mortality may relate to differences in innate PRR response'). Each of these hypotheses can now be tested in focused studies.

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

4.1 Introduction

In the previous chapter, I have shown that children from South Africa secreted markedly lower amounts of nearly every cytokine measured following PRR stimulation when compared to children from Belgium, Canada, or Ecuador [107]. 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. I 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, I here employed single-cell intracellular cytokine cytometry in order to identify the cells from which the observed differences arose. Given our eventual goal to determine the impact of innate immune variation on vaccine responses, I focused on the main antigen-presenting cells, namely conventional and plasmacytoid dendritic cells (cDC and pDC) as well as monocytes; however, I 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. My findings reveal that altered cellular composition as well as a reduced response on the single-cell level following PRR stimulation in South African children contribute 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 remains to be assessed.

4.2 Results

4.2.1 Cohort Characteristics

Four different populations were included in this study. The characteristics of the study population are described in Table 4.1. To allow for the completed administration of locally recommended early-childhood vaccines the average age at blood draw is 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 (Flanagan, Burl, Lohman-Payne, & Plebanski, 2010). 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[92]. 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.

Table 4.1 Demographics of the children 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)	19.1 (0.8)	26.7 (1.28)	24.7 (0.6)
Birth Weight (g) - mean (SD)	2996.2 (796.3)	3339.6 (448.2)	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)	39.2 (1.5)	38.9 (1.1)	37.8 (2.4)
Premature < 37wks (% of total)	2 (14%)	1 (4.5%)	0 (0%)	3 (15%)
Weight (g) - mean (SD)	13364.3 (1786.1)	11190.9 (1392.5)	11501.16 (1010.7)	11205.0 (1300.7)
Height (cm) - mean (SD)	92.2 (4.6)	82.2 (3.0)	84.3 (2.5)	84.4 (0.91)
WAZ (SD)	0.69(1.2)	-0.05(0.9)	-0.32 (0.93)	-0.58 (0.95)
LAZ (SD)	1.56(0.8)	-0.30(0.9)	-0.78 (1.49)	-1.07 (1.20)
WLZ (SD)	-0.18(1.4)	0.17(1.0)	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

4.2.2 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+), 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 2.2 (shown in the Chapter 2: Material and Methods section). The use of these comprehensive anchor markers allowed direct comparison of cell

composition between sites (Figure 4.1). 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.

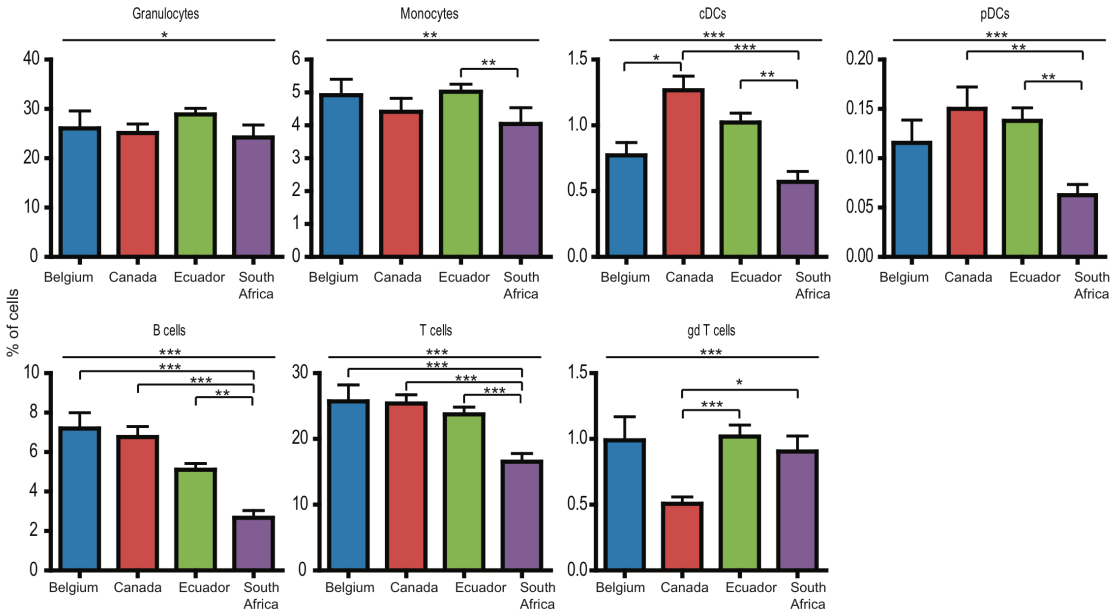


Figure 4.1 Cellular composition of whole blood cells at each of the 4 sites (statistical significance p value was * < 0.005, ** <0.01, * <0.05). The lines indicate statistically different results of the Kruskal-Wallis analysis performed to compare the all four sites for significant variance, while the brackets indicated the statically significant Dunn's post-test between paired sites.**

4.2.3 Single-Cytokine Analysis

The expression of major innate cytokines, specifically IL-6, IL-12, IFN-α, IFN-γ, 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 [77,78]. An example of cytokine-producing-cell gating is shown in Figure 2.2 and cytokines response gating Figure 4.2.

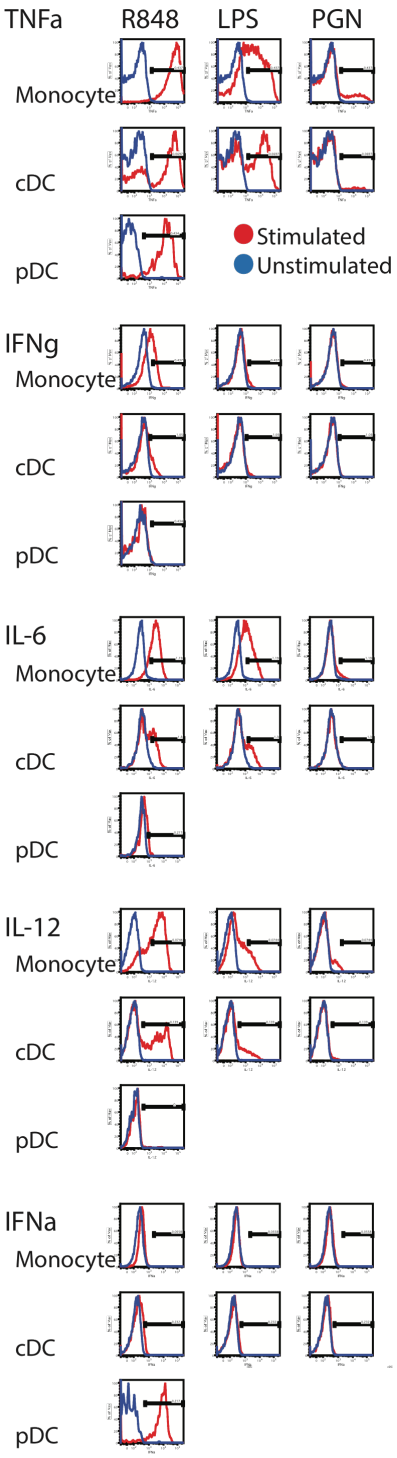


Figure 4.2 An individual's specific TNFα, IFN-γ, IL-6, IL-12, and IFN-α cytokine responses to R8484, LPS, and PGN stimulation (red) compared to unstimulated (blue) within monocytes, cDCs, and pDCs.

4.2.3.1 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.

4.2.3.2 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 IL-6, IL-12, IFN-γ, and TNF-α but not IFN-α. 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 4.3). The IL-6, IL-12, IFN-γ, and TNF-α 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 [108], we also saw IFN-γ production by monocytes in response to strong stimuli like LPS and R848. Response to PAM showed that production of each cytokine (IL-6, IL-12, IFN-γ, and TNF-α) 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 IL-6. PGN stimulated monocytes produced IL-6, IFN-γ, and TNF-α, with significant difference between South African and Canadian children. MDP stimulation of monocytes induced IL-6 and TNF-α production, which was statistically different between Belgian vs. South African children only (Table 4.2).

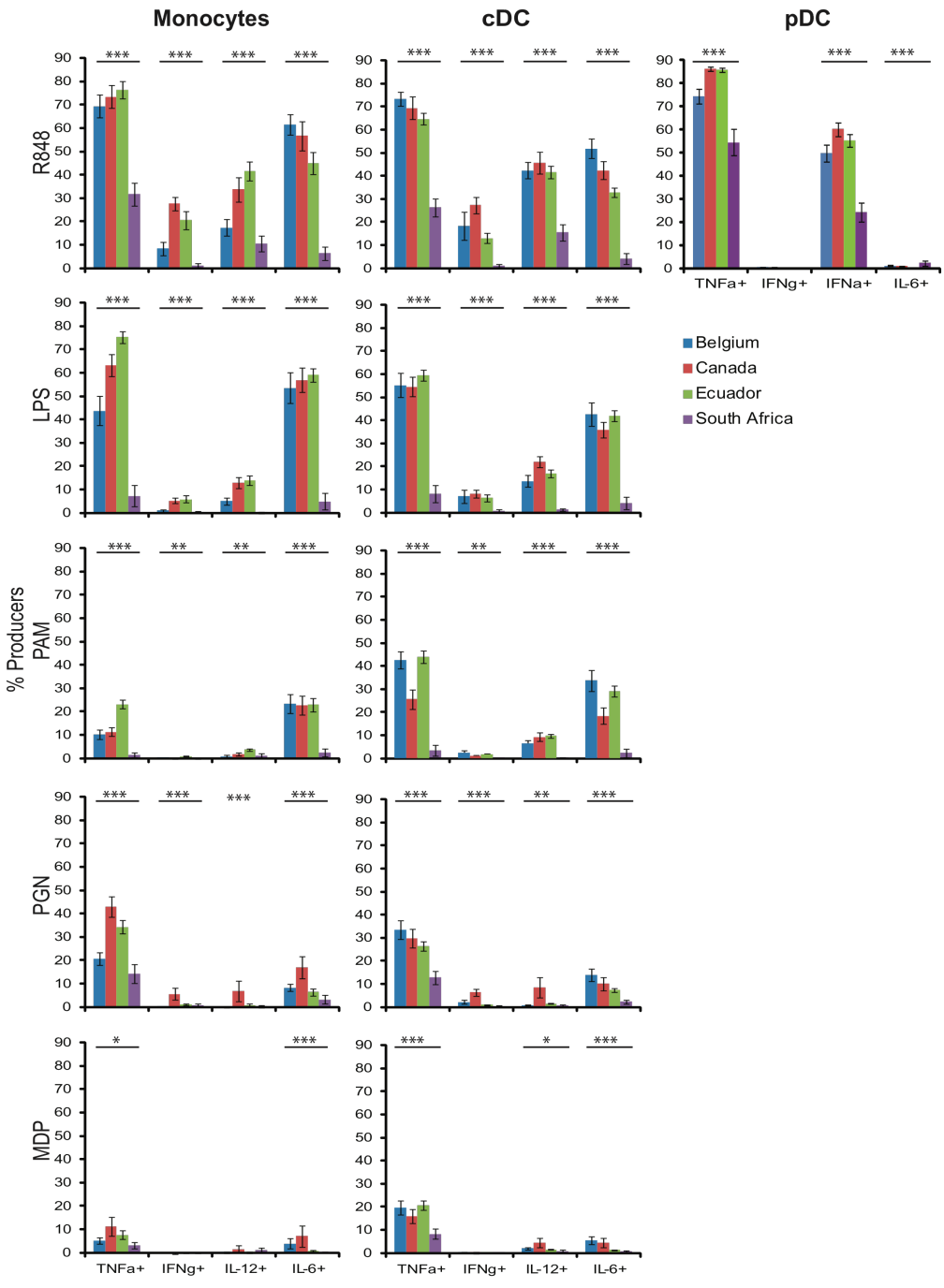


Figure 4.3 Single-cell cytokine in response to TLR or NLR ligands stimulations show that South African children have a weaker single cell-specific cytokine response. Whole blood obtained from children from 4 different sites were stimulated with R848, LPS, PAM, PGN, and MDP ligands and measured by flow cytometry for IL-6, IL-12, IFN-α, IFN-γ, and TNF-α 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 paring (statistical significance p value was *** < 0.005, ** <0.01, * <0.05).

Table 4.2 Statistical analysis of each cytokine per cell type (A. **Monocytes,** B. **cDCs,** C. **pDCs) per stimulation.** Kruskal-Wallis test was used across all four sites. Dunn's post-hoc test was applied to each site paring (statistical significance p value was *** < 0.007, ** <0.01, * <0.05).

	Monocytes									
Cytokine	cell type	stim	Kruskal-Wallis	Summary	Belgian vs Canada	Belgian vs Ecuador	Belgian vs South Africa	Canada vs Ecuador	Canada vs South Africa	Ecuador vs South Africa
IFNg+	Monocytes	R848	< 0.0001	***	ns	ns	ns	ns	***	***
IL-12+	Monocytes	R848	< 0.0001	***	ns	*	ns	ns	**	***
IL-6+	Monocytes	R848	< 0.0001	***	ns	ns	***	ns	***	***
TNFa+	Monocytes	R848	< 0.0001	***	ns	ns	*	ns	***	***
IFNg+	Monocytes	LPS	< 0.0001	***	ns	ns	ns	ns	***	***
IL-12+	Monocytes	LPS	< 0.0001	***	ns	*	ns	ns	***	***
IL-6+	Monocytes	LPS	< 0.0001	***	ns	ns	***	ns	***	***
TNFa+	Monocytes	LPS	< 0.0001	***	ns	**	ns	ns	***	***
IFNg+	Monocytes	PAM	0.0098	**	ns	ns	ns	ns	ns	**
IL-12+	Monocytes	PAM	0.0037	**	ns	*	ns	ns	ns	**
IL-6+	Monocytes	PAM	< 0.0001	***	ns	ns	***	ns	***	***
TNFa+	Monocytes	PAM	< 0.0001	***	ns	*	*	**	**	***
IFNg+	Monocytes	PGN	0.0002	***	**	ns	ns	ns	***	ns
IL-12+	Monocytes	PGN	0.3069	ns	ns	ns	ns	ns	ns	ns
IL-6+	Monocytes	PGN	< 0.0001	***	ns	ns	**	ns	***	*
TNFa+	Monocytes	PGN	< 0.0001	***	**	ns	ns	ns	***	**
IFNg+	Monocytes	MDP	0.2217	ns	ns	ns	ns	ns	ns	ns
IL-12+	Monocytes	MDP	0.9343	ns	ns	ns	ns	ns	ns	ns
IL-6+	Monocytes	MDP	0.0005	***	ns	ns	***	ns	*	ns
TNFa+	Monocytes	MDP	0.0122	*	ns	ns	ns	ns	*	*
cDC										
Cytokine	cell type	stim	Kruskal-Wallis	Summary	Belgian vs Canada	Belgian vs Ecuador	Belgian vs South Africa	Canada vs Ecuador	Canada vs South Africa	Ecuador vs South Africa
IFNg+	cDC	R848	< 0.0001	***	ns	ns	**	ns	***	***
IL-12+	cDC	R848	< 0.0001	***	ns	ns	**	ns	***	***
IL-6+	cDC	R848	< 0.0001	***	ns	*	***	ns	***	***
TNFa+	cDC	R848	< 0.0001	***	ns	ns	***	ns	***	***
IFNg+	cDC	LPS	< 0.0001	***	ns	ns	ns	ns	***	***
IL-12+	cDC	LPS	< 0.0001	***	ns	ns	**	ns	***	***
IL-6+	cDC	LPS	< 0.0001	***	ns	ns	***	ns	***	***
TNFa+	cDC	LPS	< 0.0001	***	ns	ns	***	ns	***	***
IFNg+	cDC	PAM	0.0015	**	ns	ns	ns	ns	ns	**
IL-12+	cDC	PAM	< 0.0001	***	ns	ns	**	ns	***	***
IL-6+	cDC	PAM	< 0.0001	***	ns	ns	***	ns	**	***
TNFa+	cDC	PAM	< 0.0001	***	ns	ns	***	*	**	***
IFNg+	cDC	PGN	0.0003	***	ns	ns	ns	*	***	ns
IL-12+	cDC	PGN	0.0046	**	ns	ns	ns	ns	*	**
IL-6+	cDC	PGN	< 0.0001	***	ns	ns	***	ns	**	**
TNFa+	cDC	PGN	0.0007	***	ns	ns	**	ns	*	**
IFNg+	cDC	MDP	0.9742	ns	ns	ns	ns	ns	ns	ns
IL-12+	cDC	MDP	0.0441	*	ns	ns	ns	ns	ns	ns
IL-6+	cDC	MDP	0.0004	***	ns	ns	***	ns	*	ns
TNFa+	cDC	MDP	0.0003	***	ns	ns	*	ns	ns	***
pDC										
Cytokine	cell type	stim	Kruskal-Wallis	Summary	Belgian vs Canada	Belgian vs Ecuador	Belgian vs South Africa	Canada vs Ecuador	Canada vs South Africa	Ecuador vs South Africa
IFNa+	pDC	R848	0.0003	***	ns	ns	*	ns	***	**
IFNg+	pDC	R848	0.2259	ns	ns	ns	ns	ns	ns	ns
IL-6+	pDC	R848	< 0.0001	***	ns	ns	ns	***	ns	*
TNFa+	pDC	R848	0.0002	***	ns	ns	***	ns	ns	**

4.2.3.3 cDC

A strong IL-6, IL-12, IFN-γ, and TNF-α 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 4.3). 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 IL-6 and TNF-α; for IL-12 and IFN-γ the variation was most pronounced between South African vs. Canadian or Ecuadorian children (Table 4.2).

4.2.3.4 pDC

pDCs only responded to the TLR7/8 ligand R848, producing IFN-α, IFN-γ, IL-6 and TNF-α, but not IL-12 (Figure 4.3). The major differences originated from subjects from Canada vs. Ecuador for IL-6, Canada vs. South Africa for IFN-α, and Belgium vs. South Africa for TNF-α (Table 4.2).

4.2.4 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.

4.2.4.1 Monocytes

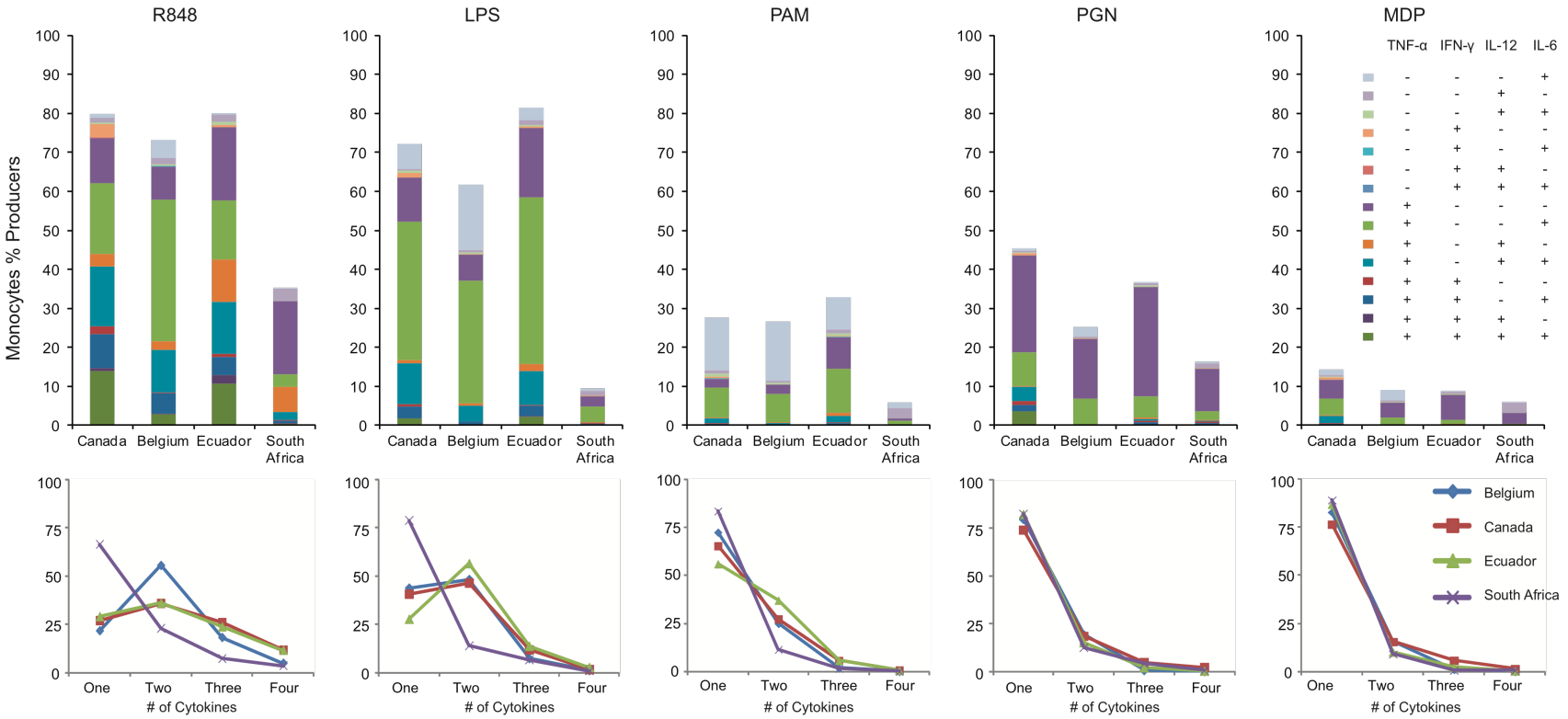
R848, LPS, PAM: The IL-6, IL-12, IFN-γ, and TNF-α response to all three stimulations were found to be different between sites, with the largest differences due to the variation between South Africa vs. Canada, Ecuador, or Belgium (Figure 4.4a). Canadian and Ecuadorian children produced the highest polyfunctional response including double-(TNF-α+IL-6+), triple-(TNF-α+IFN-γ+IL-6+, TNF-α+IL-12+IL-6+), and quadruple-(TNF-α+IFN-γ+IL-12+IL-6+) 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 (TNF-α+ or IL-12+), with very few polyfunctional-cytokine producing monocytes detected.

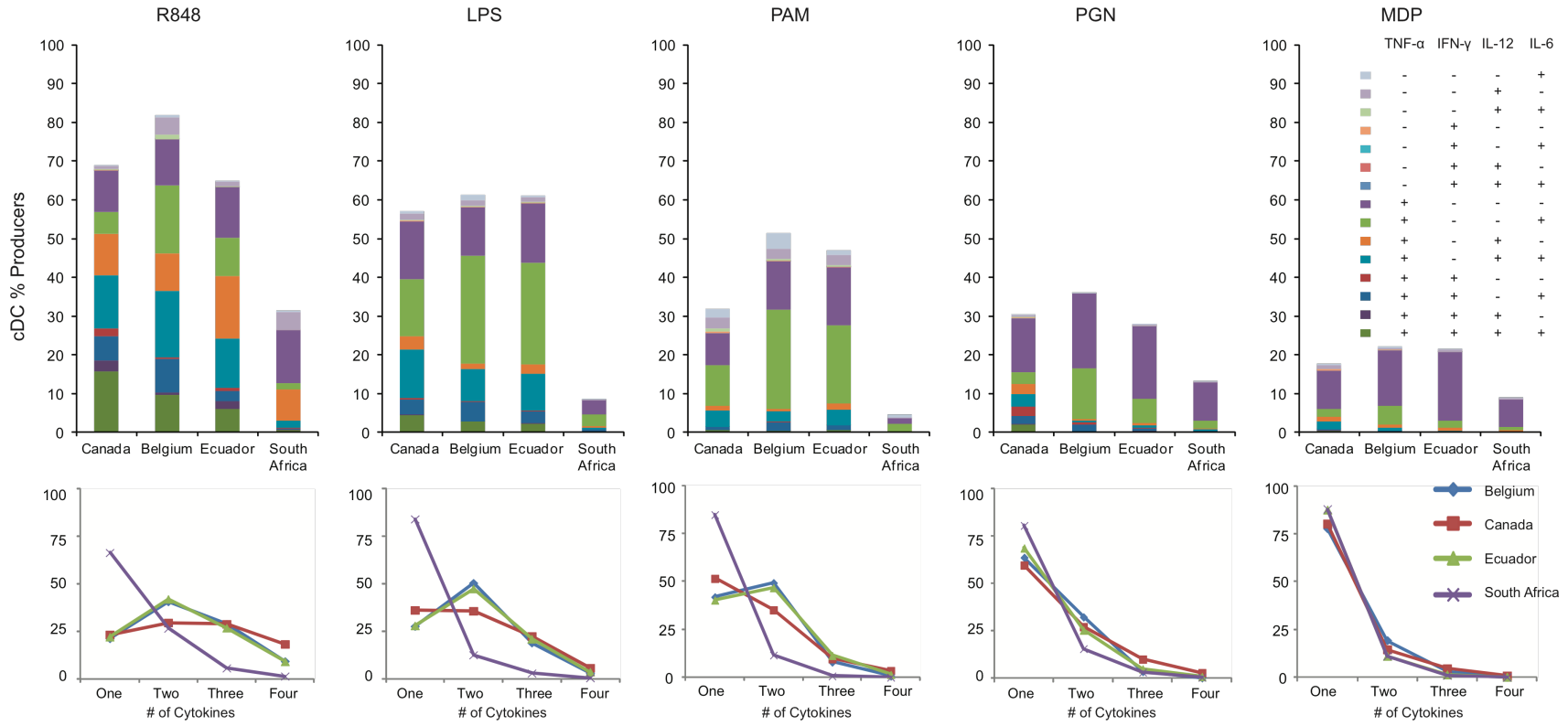
PGN, MDP: Single-cytokine producing cells dominated the response to NOD2 stimulation across all four sites. PGN stimulated monocytes responded mainly with TNF-α+, followed by TNF-α+IL-6+ production. Belgian and South African children displayed an overall lower response compared to Canadian and Ecuadorian children (Figure 4.4a). MDP evoked only a minimal response, but IL-6 and TNF-α production was still different between sites, and most pronounced between Belgian vs. South African children (Figure 4.4a).

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 4.4a). 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.

A


R848
LPS
PAM
PGN
MDP
Monocytes % Producers
Canada
Belgium
Ecuador
South Africa
TNF-α
IFN-γ
IL-12
IL-6
One
Two
Three
Four
# of Cytokines
Belgium
Canada
Ecuador
South Africa

B


R848
LPS
PAM
PGN
MDP
cDC % Producers
Canada
Belgium
Ecuador
South Africa
TNF-α
IFN-γ
IL-12
IL-6
# of Cytokines
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Two
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Belgium
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Ecuador
South Africa

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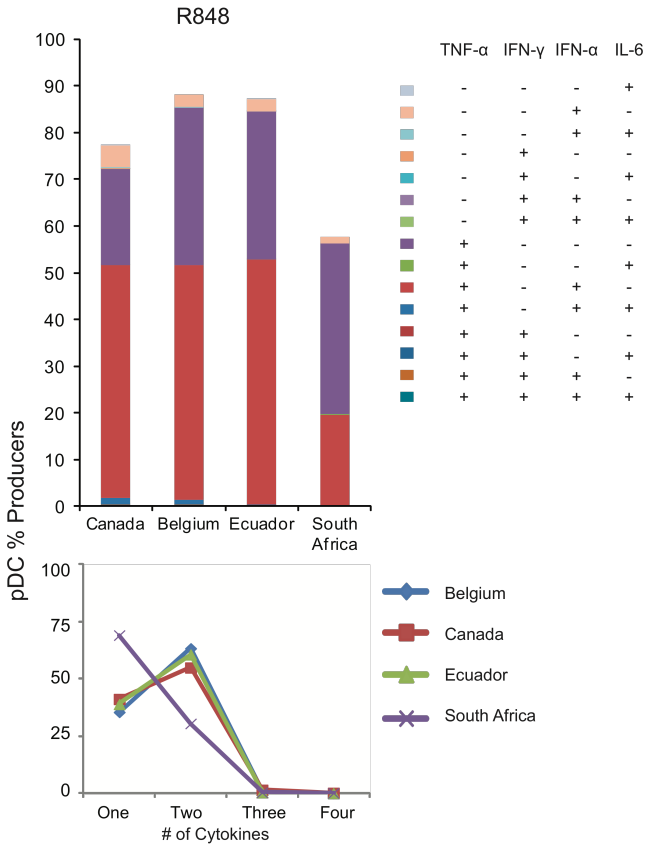

Figure 4.4 Multi-cytokine response of single cell type showed that South African children are weaker and less diverse in response to TLR and NLR ligands. Whole blood obtained from children from 4 different sites were stimulated with indicated R848, LPS, and PGN ligands and measured by flow cytometry for IL-6, IL-12, IFN-α, IFN-γ, and TNF-α levels. The stacked bar graph represents the combination of cytokine contributions per cell type. While the line graph indicated 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. Panel A. represents Monocytes, panel B. cDCs, and panel C. pDCs.

4.2.4.2 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 (IL-12+ and TNF-α+) (Figure 4.4b).

PGN, MDP: cDCs responding to PGN stimulation were dominated by TNF-α+ response, followed by TNF-α+IL-6 double-producing cells. The largest diversity of cytokine response was seen in Canadian children (Figure 4.4b). MDP stimulation primarily resulted in a TNF-α+ single-cytokine producing cDC at each site.

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 4.4b).

4.2.4.3 pDC

Following R848 stimulation, the largest fraction of pDC responses consisted of double-cytokine producing cells (TNF-α+IFN-α+), followed by 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 4.4c).

4.2.5 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 4.5 [88].

4.2.5.1 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, and PGN less so in response to MDP (Figure 4.5).

4.2.5.2 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 4.5).

4.2.5.3 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 4.5). South African subjects showed the lowest response.

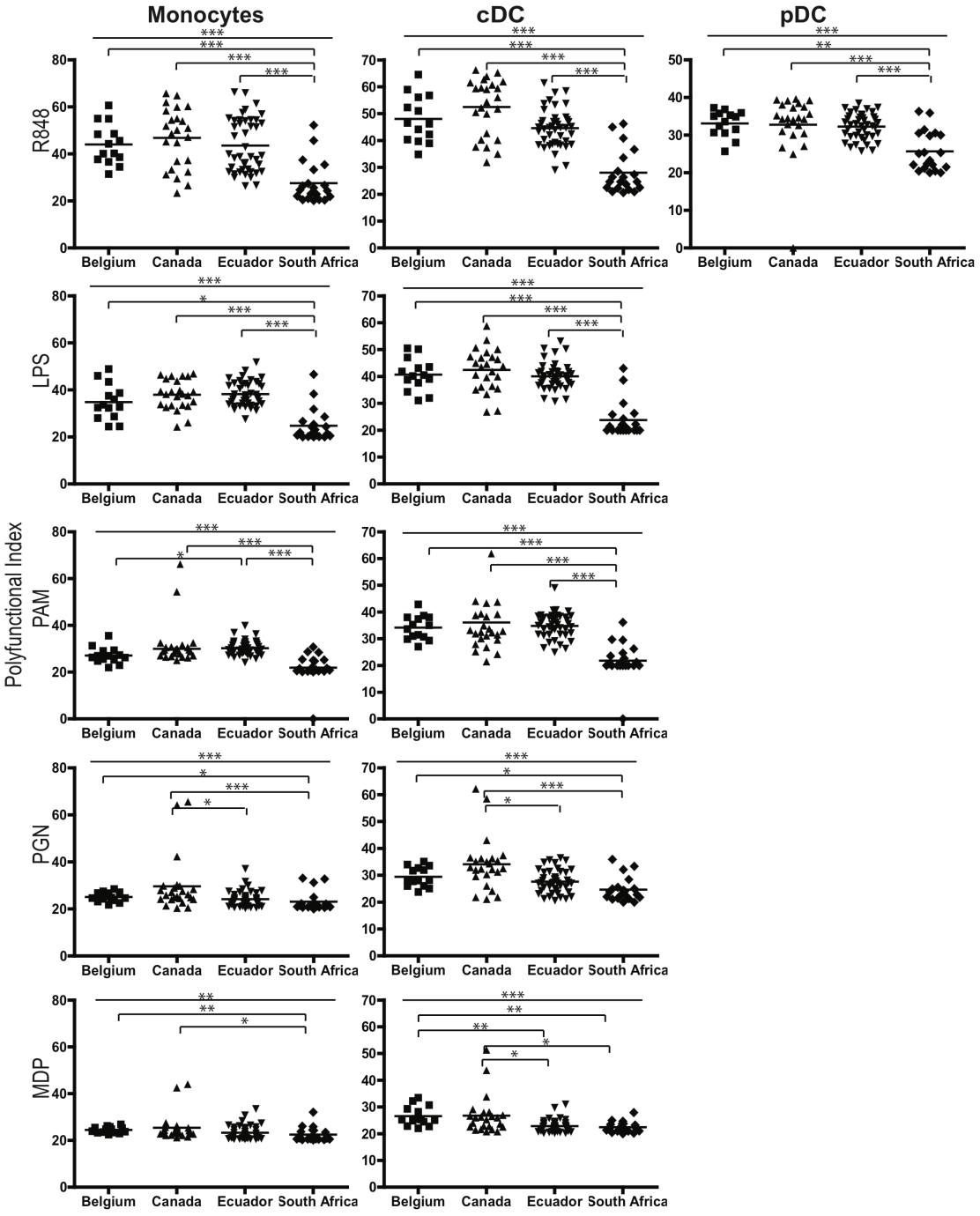

Figure 4.5 Polyfunctional index (PI) values of children at each site per cell type in response to TLR and NLR ligand stimulation. A. PI numerically evaluated the degree and variation of polyfunctionality within the 4 site cohort to allow for difference between cytokines produced by the different cell type to defined stimulations (R848, LPS, PAM, PGN, MDP) within the cohort to be seen. 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).

4.3 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[107]. 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 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 fully account for the lower secreted cytokine response detected in blood from South African children. Population-based differences in cellular composition have been previously described [36,38]. A comparison of European vs. Ugandan children noted lower lymphocyte counts in Ugandan children compared to black European children, while neutrophil counts were similar [36]. 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 cells 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 [82]. More importantly, our previous longitudinal cohort studies following Canadian [77,78] and South African children [79] 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 from 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 [109,110] or administration of immune suppressive medications [82]. Functionally, this lower degree of T cell polyfunctionality has been linked to increased risk for infection in transplant patients [111], and decreased control of HIV-replication in HIV-infected subjects[112]. 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 [113]. 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 [102]. 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 [51]. 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 APCs immune responses are lower in traditional vs. modern environments [53,99]. 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 [114]. 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[8,58,106,115–117]. However, amongst our four global cohorts, vaccine formulations and schedules were very similar, and adhered to the

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. This observation is also supported by Djuardi et al. who 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 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 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 [53,99], 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. And while birth weight and gestational age have been shown to correlate with a higher risk of infant mortality [106], 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). 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.

5 Chapter: Conclusion

The field of innate immunity is a relatively young field; the field of infant innate immunity is even younger. The field of innate immunity began to take shape in the 1990s with the discovery and characterization of TLRs. Substantial progress has rapidly been made to uncover the difference in the innate immune responses between individuals. However, there has been no comprehensive comparison regarding difference between populations. My thesis aims to begin filling this knowledge gap. The main conceptual driver for my focus on this area stems from the insight that variation in innate immune responses between populations are likely key to differences in vaccine-induced protection from infection as well as overall risk of infectious morbidity and mortality.

Previous work from our laboratory had examined the change in innate immune response to PRR stimulation as a function of age, i.e. innate immune ontogeny. Specifically, Canadian children showed an overall steady increase of Th1 supporting innate cytokines from birth onward following TLR-induced responses. These finding were consistent with findings from other regions of the world, such as Belgium and the Netherlands [95,96]. In contrast, in a similar experimental approach applied by our group to South African children we detected a striking decline over the first year of life from a high at 2 weeks of age [79]. This declining trend in cytokine response was consistent with previous studies from the Gambia [118] as well as Ecuador [97]. This suggested that differences in innate immune ontogeny might be present when contrasting children from resource-rich and resource poor regions. This suggestion was further strengthened by a study comparing children from Papua New Guinea and Australia where infants born under modern environments exhibit an increased APC reactivity at birth as compared to infants born under traditional environments [64].

However, given the sentinel function of the innate immune system, and its ability to detect changes in the environment, such cross-study comparisons are at risk of being contaminated by technical artifacts, rather than biologically relevant mechanisms. As reviewed in Dockrell HM, et al., in order to determine whether geographic variations in immune responses are real, it is essential to consider the methods employed to measure the response in each setting [119].

To assess variation in innate immune status across different populations, I reasoned, I had to develop a rigorously controlled study protocol and apply this across all sites in order to obtain reliably comparable outcomes.

My first task was to develop and implement a stringent, quality controlled approach to a study spanning 4 continents. To attain this goal, I needed to optimize an analysis platform that could travel around the world. To ensure a stringent experimental set-up at each site, a number of experimental conditions were standardized. Previous data from our lab showed how susceptible innate immune studies are to slight variations in experimental conditions [73,83]. In order to reduce artifact risk associated with a multi-site study, the experimental conditions were optimized and standardized. These included processing time, unified reagents, storage and shipment conditions, as well as analysis.

- Controlled processing time: longer delays can influence the cytokine decline. All samples were processed within 4h of procurement.
- Unified reagents and equipment: any equipment that came into contact with the blood, including tips, falcon tubes, 96-well plates, etc., were certified DNA-, RNA-, endotoxin-free, and were sourced from the same companies. Heparin in vaccutainers and loaded syringes was tested and compared prior to studies to avoid potential artifacts. Specific reagents, such as RPMI, EDTA, and FACSLyse, used directly with the blood samples were standardized, ensuring that the same lot number was used throughout the study.
- Master Mixes; once the reagents were optimized and tested under various conditions, master mixes were made to ensure no variation between stimulation plates. The stimulation master mixes were created for all subjects enrolled in the entire study. The mix was tested and stored frozen, site batches were stamped out and tested to ensure continuity prior to shipment.
- Storage conditions were carefully controlled, for all components of the study. Continuity of cold-chain was ensured for the frozen samples as potential temperature fluctuations could have compromised the samples. All of the shipped samples were shipped in temperature-controlled packages with monitoring systems in place. Longer-term storage was also controlled once samples had been returned to

Vancouver. I identified that contrary to peripheral blood mononuclear cells (PBMC) whole blood (WB) stored in FACSlysis buffer has a limited life span in the -80C freezer, as samples begin to degrade after 3 months of storage in this manner. To ensure that samples quality was maintained, I determined in extensive pilots that WB stored in LN_2 was stable without loss of quality over years.

Once the shipment arrived in Vancouver, I applied the same principles to the analysis as to the samples collected from each site. In the case of the cytokine analysis, Luminex kits were order in bulk to maintain lot number. The assay was run by the same operator to ensure continuity over a limited amount of time (2 months). In the case of flow cytometry, various papers have shown the sensitivity of the assay and the potential variation that could result. To ensure comparable results from each study run, one machine was used and calibrated. Application settings were used to ensure continuity of the analysis. All of the reagents used for the analysis were calculated and bulk ordered to minimize variation between reagent lots. The samples were all run within a limited amount of time (2 weeks) to limit machine variation over time. And lastly, samples from different sites were scrambled across different runs to avoid batch artifacts influencing results.

One of the major limitations I found when comparing my data with previously published studies from our lab, we have previously focused on PBMC instead of WB. There are profound differences between these components, as whole blood contains all of the cell types and proteins found in blood, while PBMCs, contain white blood cells and do not contain plasma, and within the cellular compartment also lacks platelets and neutrophils. Components of plasma such as adenosine derivatives exert significant regulatory function on innate immune responses to TLR stimulation and do so in an age-dependent manner[120]. Furthermore, platelets as well as neutrophils are key cellular compartments of innate immunity. The advantage then of a WB-based assay was thus several fold, namely reduced handling (thus reducing the chance of introducing technical artifacts) as well as inclusion of all cell types and soluble factors found in blood. However, in my hands whole blood appears more sensitive to variation in time between blood collection prior to stimulation of samples as compared to PBMCs, as well as changes in temperature.

Another hurdle limiting our ability to compare the data from the 4 sites arose from variation between subjects within each cohort. For instance, the immune system changes rapidly with age [94]. The fetus develops in stages, and at the time of birth the immune system is functional but not equivalent to the adult immune response. In the first years of life there are a number of immunological changes that occur. For instance, innate effector cells, although detectable in emerging immune organs and fetal circulation in the first trimester are limited in function during infancy due to limited expansion capacity[54]. Additionally certain cell types such as tissue macrophages, natural killer cells and their cytolytic activation do not reach adult levels until later in childhood [54]. Studies that included larger age ranges could thus potentially skew or distort the immunological landscape making it difficult to compare groups if age differences between groups existed. For example, differences presumed to be due to distinct geographically variables could have simply been due to age dependent differences [121]. Most importantly, in my pilot experiments, I found that the highly sensitive sentinel nature of the innate immune system dictates the use of the exact same reagents (right down to the lot number). I conclude based on my pilot data that lack of standardization in all previously published studies could have had such a profound impact on the experimental results that cross-study comparisons can simply not be made [119].

I thus strove to apply this stringently controlled, robust, high-throughput innate immune phenotyping platform I developed to systematically examine innate immune response to TLRs and NLRs in North American, South American, European, and African children. The results of this global cohort study reveal that while differences between populations of 2 year old children in innate immunity exist, these differences are relatively small compared to our previous findings regarding age-dependent changes over the first two years of life. While this conclusion applies to all children studied from Canada, Ecuadaor, and Belgium, study subjects from South Africa responded in an entirely different pattern that was significantly different from subjects from the other 3 sites. Specifically, we found that the innate cytokine response to PRR stimulation in South African children was lower for nearly all parameters tested from the other three sites, except for the NLR ligands. This suggests that it was not an overall inability of South African children to respond with cytokine production to PRR stimulation, but that the pattern of innate cytokine production following PRR stimulation in

infancy varied by geographic region in response to the particular type of PRR stimulation. I further confirmed this difference in global cytokine secretion between South African and other children at the single-cell level. This allowed me to determine if differences in global cytokine production were due to differences in blood cell composition, cell subset differences in response to PRR stimulation, or both. Of note, not only was the production of cytokines different at the single cell level, but the composition of the peripheral white blood cell compartment varied between children across all four continents, with the peripheral blood from South African children harbouring the lowest fraction of the main PRR responder cell types, namely monocytes, cDC and pDC. My data supports the notion that differences in cellular composition could contribute to differences in cytokine secretion following PRR stimulation that I previously detected. However, as South African whole blood contained as many monocytes, cDC and pDC as the whole blood of children from at least one of the other sites, thus appears unlikely that differences in cellular composition alone accounted for the consistently lower secreted cytokine response of South African children's whole blood. My research suggests that differences in regulation of cytokine production following PRR stimulation exist at the single cell level.

Together, the main findings of my thesis strongly suggest that innate immune ontogeny proceeds along similar paths for at least ¾ of the populations that I examined, which in turn indicates that, despite differences in host genetics, age-dependent demands on innate immune ontogeny drive an overall similar developmental program. However, my findings also show that variations in environment (such as between South Africa and other sites) can override this developmental program. This, in turn, indicates that the functional demands on children growing up in South Africa differ from those at the other sites.

The ability of the platform I developed to discriminate between populations on a total immune response- as well as individual cell-level now allows for the identification of the role played by environmental vs. host genetics. Being able to determine the difference between individuals at the cellular level opens the door to understanding molecular details of the immune regulation. I hypothesize that such regulatory components include epigenetic

changes leading to innate immune memory ('trained' innate memory) as well as humoral aspects (e.g. adenosine) and cellular aspects. Identification of the relevant cellular and molecular mechanisms will greatly help to further understand the influences of environment on the developing immune system.

By successfully establishing a highly standardized method, I have shown that implementing a standard protocol to various studies is feasible and allows for a comparative and comprehensive look at the innate immune responses of populations in different geographic locations.

Although this research is still unable to conclude whether these differences are host-genetic or environmentally derived it does provide a great deal of insight into the specific innate immune response of the four sites, and provides further evidence for greater environmental considerations. For instance, the four cohorts within this study had a rather diverse genetic makeup, based on the mixed ethnicity of the children enrolled at each site one could speculate that although genetics cannot be dismissed, it appears that differences in environment play a larger role in the variation of innate immunity between the populations in question than differences in host genetics.

Although this study does not support the northern- vs. southern- hemisphere, or resource-rich vs. –poor, or hygiene hypothesis it does show that perhaps there are more narrowly defined pockets of environmental variations that have a profound effect on the immune system. Specifically, although the mechanism is yet to be determined, my data study suggests that there are specific environmental factors peculiar to the area within South Africa, from which we recruited our subjects. A more fine tuned mapping of innate immune status across South Africa would help in narrowing down possibly responsible factors. There are a number of environmental factors that are known to affect the innate immunity. South Africa, and Cape Town specifically, has some highly peculiar environmental factors compared to the rest of the regions that I studied. First and foremost, South Africa has a largely diverse flora. The flora kingdom of Capensis is situated on the Western Cape's coastal region. Additionally, South Africa is known for its wine but perhaps less known for the heavy pesticide usage

specifically in the Cape Town region, which have been shown to have many negative health outcomes [122,123].

As with any study, this thesis possesses several strengths but also some weaknesses. A major strength of this work is the highly standardized experimental set up of the study. Further, this is among the first studies to look at various populations, in distinct geographic locations, and systematically compare them in a controlled fashion. This information is invaluable to advance our fundamental insight into variations in immunity in relation to protection from infection as well as response to vaccination.

The major limitation of this thesis is the small sample size per site. These finding will need to be repeated with a larger number of subjects per site, as well as potentially a larger number of sites to really determine the details of the differences observed. With this, a more complex analysis can be applied allowing for an in-depth interpretation of the data.

Furthermore, the cohort recruitment was not representative of the entire population at each site. This limits the findings to the specific location and does not allow extrapolation to the greater region or country. Additionally the information collected on the individuals was retrospective which introduces an element of certain 'recall' bias as the parents or guardian of the children may not have recalled every relevant event in specific detail.

The potential application of these research finding go far beyond the laboratory. Subjects with reduced ability to respond with innate cytokines are at risk for infectious disease as well as suboptimal vaccine responses. Cape Town and the surrounding area has long been known to have an exceptionally high rate of TB [124], as well as a particular fungal infection, caused by *Emmonsia* species, recently described by Kenyon C. et al [114]. These isolates of the *Emmonsia* species represent a new species of dimorphic fungus that is pathogenic to humans, especially those who are severely immunocompromised. Additionally, lower vaccine responses have also been noted in Cape Town and the areas around it, specifically to novel TB vaccines. Despite highly promising results elsewhere, the MVA85A TB vaccine trial, designed to enhance the protective efficacy of the BCG vaccine, failed to protect subjects

living in and around the Cape Town area, providing only a modest cellular response [125]. Given the lower response rates, increased suseptibilty to infection, decreased vaccine response, as well as the uniquness of the the geographical location of Cape Town in terms of the floral kingdom of Capensis, one can speculate that all of these factors are related and perhaps have a compounding cause-and-effect relationship.

By understanding the mechanisms of this immune alteration in our subjects from South Africa, one should be able to extract information regarding targeted therapies to improve immune response within immune-suppressed cases or perhaps dampen the immune response in children with an over-active immune response, for such patients as those with hyper-inflammatory conditions such as allergies and autoimmune diseases. In short, my findings are likely going to impact the world far beyond Cape Town and even South Africa.

Future directions: Several exciting avenues for future research can be built on the work of my thesis:

1. Mechanisms responsible for variation in innate immune ontogeny:
    a) now that we know differences can be found at the cell subset level we can proceed to identify the intracellular molecular mechanisms
    b) now that we know that there are aspects of variation in whole blood cell composition between children from different sites, we will be able to assess the role of cell-cell interactions and/or soluble factors in variation of innate immunity between sites

With mechanisms identified, we are more likely to be able to proceed to identify upstream cause and downstream (clinical) effect.

To advance these important aspects, a large, longitudinal prospective multi-site study will likely be necessary. This study would require a large enrollment of age- and ethnicity-defined subjects, at each central site as well as satellite sites within each populations of interest. The multi-site sampling will allow for a complete assessment of each population and will also

allow for the variations in the responses to be mapped out. To fully understand the genetic influences extensive genotyping and SNP identification would possibly be helpful.

To understand the environmental influences, a prospective longitudinal design would be essential. Collecting an in depth medical history in real time, as well as screening the environment for influences of socio-economic factors (such as education and income), physical location (such as urban vs. rural as well as developed vs. developing), infectious agents (with a focus on fungal and parasitic agents), toxins (naturally occurring as well as industry related), pollution, water contamination, household ovens, etc. would allow narrowing down possibly responsible environmental factors.

The data generated through my work now allow the precise power calculations to be conducted for the necessary larger follow-up studies. And by establishing a standardized method, I have now shown that implementing a standard protocol around the world is a reasonable and feasible approach to cohort studies. This standard can be applied to various studies, populations, and age groups to form the baseline understanding of the innate immune response across, different periods of life, for example.

Understanding the baseline response of populations might allow for a greater understanding of specific immune responses within a certain region that might be governed by its environment. A complete understanding of the various components that influence the immune response and what the baseline responses are, might allow for targeted treatments and appropriate adjuvant use within vaccinations, allowing for personalized medicine.

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Appendix A: Age of recipient and number of doses differentially impact human B and T cell immune memory responses to HPV vaccination.

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