Pattern recognition receptor-mediated cytokine response in infants across 4 continents☆

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Background: 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. Because innate immunity instructs adaptive immunity, we hypothesized that differences between populations in innate immune responses may represent a mechanistic link to variation in susceptibility to infection or response to vaccination.

Objective: Determine whether differences in innate immune responses exist among infants from different continents of the world.

Methods: We determined the innate cytokine response following pattern recognition receptor (PRR) stimulation of whole blood from 2-year-old infants across 4 continents (Africa, North America, South America, and Europe).

Results: We found that despite the many possible genetic and environmental exposure differences in infants across 4 continents, innate cytokine responses were similar for infants from North America, South America, and Europe. However, cells from South African infants secreted significantly lower levels of cytokines than did cells from infants from the 3 other sites, and did so following stimulation of extracellular and endosomal but not cytosolic PRRs.

Conclusions: Substantial differences in innate cytokine responses to PRR stimulation exist among different populations of infants that could not have been predicted. Delineating the underlying mechanism(s) for these differences will not only aid in improving vaccine-mediated protection but possibly also provide clues for the susceptibility to infection in different regions of the world. (J Allergy Clin Immunol 2014;133:818-26.)

Key words: *Innate immunity, immune development, infectious disease, global*

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.[1,4,5] Around the world, the Expanded Program on Immunization and similar regional or national programs direct the immunization of infants.[6,7] These public health programs have greatly contributed to diminishing infectious mortality and morbidity in early life.[8] Because the formulations and schedules of vaccination do not vary considerably among countries, these vaccination strategies rely on the notion that responses to vaccination would be similar among infants living in different regions of the world.[7,9,10] However, it has become apparent that vaccine responses differ in infants from varying geographic regions.[11] The underlying mechanisms leading to different vaccine responses in different populations remain largely unknown. This lack of understanding prevents optimization of infant vaccine responses. Because innate immunity directs adaptive immunity, we reasoned that the first step in identifying the mechanistic cause leading to variation in vaccine responses in infants from diverse regions of the world would be to determine whether 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.[5] We set out to contrast the PRR response to stimulation of infants across 4 continents (Africa, North America, South America, and Europe) by using a highly standardized, stringently controlled innate immune phenotyping platform, ensuring the same experimental setup for all sites. We found significant differences in innate immune responses to PRR stimulation among infants from different populations.

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Abbreviations used
LPS: Lipopolysaccharide
MDP: Muramyl dipeptide
NOD: Nucleotide-binding oligomerization domain-containing protein
PCA: Principal-component analysis
PGN: Peptidoglycan
Poly I:C: Polyinosinic-polycytidylic acid
PRR: Pattern-recognition receptor
R848: Resiquimod
TLR: Toll-like receptor

METHODS

Ethics statement

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

Participant recruitment and enrollment

This study compared infants aged approximately 2 years from 4 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.[12] 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. Infants from Ecuador were recruited within a rural-based population cohort study.[13] South African infants had been enrolled in an urban-based birth cohort established at Stellenbosch University.[14] A subject was included in the study if the infant was considered healthy on the basis of a history-driven health assessment. Subjects were excluded from the study if they had met 1 or more of the following criteria: significant chronic medical condition, immune deficiency, immunosuppression by disease or medication, cancer, bone marrow or organ transplantation, receipt of blood products within 3 months, bleeding disorder or major congenital malformation, or genetic disorder. Infants born to HIV-positive mothers were also excluded.

Blood collection

Given that one of the major roles of the innate immune system is sensing environmental changes,[15,16] technical artifacts can easily plague innate immune assessment.[17] 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 sites. All materials and reagents from blood draw to final analysis were tested to ensure absence of innate immune activation substances as previous work had shown lot-dependent variation.[17,18] 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, San Jose, Calif, catalog no. 8019839). Blood samples were kept at room temperature and processed within hours of blood draw as described previously.[17,18]

Toll-like receptor stimulation and blood culture

Master mixes of all reagents were made in quantities adequate for the entire study, frozen, and shipped under monitored conditions to all the 4 sites. The same person (K.S.) performed all aspects of the experiments at all sites by using our well-established robust, validated, and quality-controlled innate immune phenotyping protocol.[12,17-20] In brief, deep 96-well (VWR, Mississauga, Ontario, Canada) source plates with each well containing 1.3 mL of a specific Toll-like receptor (TLR) ligand, were prepared by using sterile procedures under a laminar airflow hood. A total of 22 μL from each well of the source plate was dispensed into each well of recipient 96-well round-bottom polystyrene plates (Corning, Corning, NY) by using the Evolution P3 Precision Pipetting Platform (Perkin Elmer, Waltham, Mass). 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, San Diego, Calif) at 1 μg/mL; polyinosinic-polycytidylic acid (Poly I:C; TLR3; GE Healthcare, Fairfield, Conn) at 100 μg/mL; lipopolysaccharide (LPS; TLR4, InvivoGen) at 10 ng/mL; resiquimod (R848; TLR7/8, InvivoGen) at 10 μM; peptidoglycan (PGN; nucleotide-binding oligomerization domain-containing protein 1/2 [NOD1/2], InvivoGen) at 10 μg/mL; muramyl dipeptide (MDP; NOD 2, InvivoGen) at 0.1 μg/mL; and media alone. All TLR ligands were diluted in RPMI medium to obtain the desired concentration.

Whole blood was diluted 1:1 with sterile prewarmed RPMI 1640, and 200 μL of the diluted blood was added to each well of the premade plates containing the specific TLR ligands. Blood was incubated for 24 hours, after which plates were centrifuged at 600*g* and subsequently 100 μL of the supernatant was removed and frozen at −80°C for multiplex assay analysis later. Samples were shipped on dry ice via World Courier, Inc, with a temperature monitor in each shipment ensuring maintenance of the desired temperature (−80°C). Samples were stored at −80°C in the central analysis site (Vancouver, Canada), and were all run within 12 months of collection.

Cytokine measurement

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

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, San Diego, Calif), and assays were performed according to the manufacturer's specifications. Plates were read at 450 nm with 570 nm subtraction on a SPECTRAmax Plus. A 4-parameter sigmoid logistic curve was used to generate the standard curve.

Statistical analysis

Kruskal-Wallis analysis was performed to compare the 4 sites for significant variance among the median cytokine concentrations. Bonferroni test was applied to correct for multiple comparisons. Dunn's posttest was used to determine which of the sites contributed to the significant differences. Statistical analysis was conducted in Prism Version 6 (GraphPad Software).

Principal-component analysis

To visualize the data in an intuitive fashion, we plotted the data by using principal-component analysis (PCA). The cytokine data were log-transformed and then subjected to PCA by using GINKGO: Multivariate Analysis System.[21,22] The data were plotted by using Tableau visualization software