# Antibody and T-cell responses in rotavirus vaccinated Zambian infants: impact of human cytomegalovirus infection.

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**Declaration**

I, Natasha Makabilo Laban, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed Date 30th November 2024

**Abstract**

Oral rotavirus vaccines (ORV) demonstrate lower seroconversion rates in low-income compared to high-income settings, a phenomenon that is not fully understood. There is also limited knowledge on the T-cell immune responses in vaccinated infants creating a gap in understanding rotavirus immunology.

I used plasma and peripheral blood mononuclear cells collected from infants under a rotavirus vaccine trial in Zambia to measure the rotavirus specific immunoglobulin IgA (RV-IgA) antibody responses comparing two and three doses of an ORV (Rotarix) and the T cell responses associated with vaccination. I also investigated the influence of human cytomegalovirus immunoglobulin M (HCMV-IgM) seropositivity on vaccine immunogenicity. To contribute to coronavirus research, post the COVID-19 pandemic, I explored antibody responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and common cold coronaviruses among this mother-infant rotavirus trial study cohort.

A low seroconversion rate of 27.8% was observed one month after two dose ORV administration and there was no significant boosting of RV-IgA three months after a third ORV dose administered at 9 months (p=0.223). HCMV-IgM seropositivity did not affect RV-IgA responses among overall infants but significantly reduced RV-IgA responses by 63% in HIV-exposed-uninfected infants (p=0.008). Limited and very low frequency rotavirus VP6-specific T-cell responses were detected in vaccinated infants but enriched rotavirus VP6-specific CD4+ T-cell responses were observed among vaccine seroconverters.

Overall, the thesis provided evidence that a booster ORV dose at 9 months did not improve vaccine immunogenicity by 12 months suggesting alternate rotavirus vaccine strategies or formulations may be necessary to improve vaccine immunogenicity in Zambia, and that HCMV-IgM seropositive HIV-exposed-uninfected infants were sub-populations vulnerable to reduced rotavirus immunity. The limited rotavirus-specific T-cell responses suggested that infants mount short-lived memory T-cell responses to ORV but also showed evidence of VP6-targeted CD4 T-cell dependent antibody response to rotavirus vaccination.

**COVID impact statement**

I completed my field work processing of plasma and PBMC samples in Zambia in October 2019 and successfully upgraded in February 2020. However, due to COVID-19 pandemic declared in March 2020, I had to abruptly cut short my time at the school and travel back to Zambia thus interrupting my plans to use the LSHTM Flow Cytometry facilities. Subsequent flight restrictions and travel ban on Zambia prevented my return to the UK.

My original testing plan included ex-vivo and in-vitro stimulation flow cytometry analysis of various innate cells inclusive of innate lymphoid cell types and rotavirus specific adaptive immune cells comprised of memory, Th1/Th2 and homing phenotypes using the LSHTM BD LSRII Flow Cytometer with capacity to measure up to 13 parameters. I also planned parallel evaluation of the interferon secreting PBMC responses using Enzyme-Linked Immunospot (ELISpot) assay. Due to the uncertainty of international travel, I adapted my testing strategy and re-designed the staining panels for detection of (i) general rotavirus specific activated CD4 and CD8 T cells (ii) and key innate cell and unconventional T cells to make use of a BD FACSVerse Flow Cytometer with 6 fluorescent parameters that was available in Lusaka, Zambia.

To contribute to the coronavirus research efforts in Zambia post the COVID-19 pandemic, I investigated antibody responses to circulating human coronaviruses including SARS-CoV-2 in my infant study cohort.

## Statement of contribution

This PhD study was nested under a rotavirus vaccine clinical trial. My contributions and the contribution made by others are detailed below with reference made to the relevant thesis Chapters.

I conceived the research topic addressed in the systematic review in Chapter 2. I formulated and refined the research question in consultation with my Supervisors Martin Goodier and Roma Chilengi. I identified the key words which I used to conduct the literature search strategy in electronic databases. I conducted the study selection and study data extraction. I performed the quality assessment of included studies. During the entire process, I consulted and received feedback and contribution from co-authors. I wrote the first manuscript draft and implemented the contributions to the manuscript from all co-authors and journal reviewers up to publication.

The primary research hypothesis and methodology for the rotavirus clinical trial in Chapter 4 was conceived by my Supervisor Roma Chilengi who also obtained funding for the study. I contributed to writing of the research proposal during the application for funding and ethics approval and developing the laboratory standard operating procedures for the study objectives with other study contributors. Other study staff conducted the participant recruitment, follow up and sample collection procedures. I coordinated the laboratory aspects of the trial which involved processing, storing and testing of study samples (See also Chapter 3). I conducted the processing of plasma from whole blood samples, calibrated and performed the ELISA assay for rotavirus IgA, generated the data on rotavirus IgA titres from the ELISA outputs and compiled the data for statistical analysis assisted by the study MSc student who I supervised. Other study contributors conducted the formal statistical analysis. I wrote the first draft of the manuscript and implemented revisions from all co-authors and journal reviewers through to final publication. I contributed to writing of all the study technical reports to the funder over the four year study period.

I formulated the hypothesis for the HCMV work reported in Chapter 5 and worked with my Supervisor Martin Goodier to define the experimental strategy. I performed the ELISA testing for HCMV IgM assisted by two laboratory staff. I conducted the data compilation and data cleaning for analysis. I performed the statistical analysis and created the graphs for data presentation in consultation with Samuel Bosomprah. I wrote the first draft of the manuscript and incoporated edits from reviews by co-authors and journal reviewers up to the publication of the manuscript.

I formulated the hypothesis, experimental strategy and refined the laboratory methods for the T-cell study in Chapter 6 in consultation with my Supervisors Martin Goodier and Roma Chilengi. I acquired the funding for the T-cell work. I conducted the isolation and cryopreservation of all PBMC from whole blood samples included. I designed the Flow Cytometry panel appropriate for the cytometer used for testing jointly with my Supervisor Martin Goodier. I conducted the PBMC recovery from cryopreservation, in-vitro stimulation, culture and phenotyping, ex-vivo staining, flow cytometry acquisition and post-acquisition analysis of the generated data in FlowJo. I compiled and cleaned the data for statistical analysis. I performed the formal statistical analysis under guidance from Samuel Bosomprah. I created the graphs for data presentation. I wrote the first draft of the manuscript and included edits made by co-authors up to the final version included in the thesis in preparation for publication.

I formulated the research objective and hypothesis for the coronavirus work presented in Chapter 7. I set up and optimised the in-house human coronavirus ELISA in the laboratory. I conducted the testing of plasma samples for human coronavirus IgG assisted by two laboratory staff. I generated antibody concentration data from ELISA outputs, compiled and cleaned the data for statistical analysis. I performed the formal statistical analysis under guidance from with Samuel Bosomprah. I wrote the first draft of the manuscript and included edits from all reviews made by co-authors up to the final version included in the thesis that was submitted for publication.

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Firstly, I would like to express my immense gratitude to Martin Goodier who has been my primary supervisor from the start of my PhD journey and has been a constant source of motivation. Thank you for cheering me on throughout both the good times and the difficult times, especially during the turbulent COVID-19 period that brought with it a lot of disruptions to my laboratory work. Your moral and intellectual support has been invaluable throughout this journey for which I am ever grateful.

Next, I would like to thank my secondary supervisor Roma Chilengi for his support of my growth as a research scientist from before and throughout my PhD research. I am grateful for the mentorship and the platform you provided for me to learn about rotavirus research and vaccine trials. I would also like to thank Samuel Bosomprah for his guidance on the statistical analysis I conducted in my PhD research. Thank you for supporting my navigation into the world of STATA as I developed my competencies in running statistical analysis.

Many thanks to my colleagues at the Centre for Infectious Disease Research in Zambia (CIDRZ) Enteric Diseases and Vaccines Research Unit (EDVRU) who were ready to offer an extra helping hand or advice when needed. I am grateful to the EDVRU lead Scientists Michelo Simuyandi and Caroline Chisenga and the laboratory, clinical research site and administrative staff under the rotavirus trial. Special thanks also go to CIDRZ as an institution for providing me with the opportunity and conducive environment to pursue this research.

I would like to thank Dr. William Kilembe, Dr. Clive Michelo and Mr. Vernon Musale at the Centre for Family Health Research in Zambia for their hospitality during my work on PBMC processing in their laboratory. I also acknowledge the support offered by Professor Paul Kelly and his laboratory team at the University Teaching Hospital Tropical and Gastroenterology and Nutrition Group for access to their FACSVerse flow cytometer.

Last but not the least, I would like to thank and dedicate this thesis to my partner and family. I thank you for your unwavering support and belief in me throughout this PhD journey. I would not have done this without you.

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**List of Abbreviations**

| | |
|---|---|
| AIM | Activation Induced Marker |
| APC | Antigen Presenting Cell |
| ASC | Antibody Secreting Cell |
| BCG | Bacille Calmette-Guérin |
| BSA | Bovine Serum Albumin |
| CD | Cluster of Differentiation |
| CIDRZ | Centre for Infectious Disease Research in Zambia |
| COVID-19 | Coronavirus Disease 2019 |
| CRS | Clinical Research Site |
| DC | Dendritic Cell |
| DLP | Double Layered Particle |
| DNA | Deoxyribonucleic Acid |
| DPT-HepB-HiB | Diphtheria-Pertussis-Tetanus-Hepatitis B-Haemophylus Influenza |
| DSMB | Data Safety and Monitoring Board |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ELISpot | Enzyme Linked Immuno Spot |
| EPI | Expanded Program on Immunisation |
| FCS | Fetal Calf Serum |
| GALT | Gut Associated Lymphoid Tissue |
| GMT | Geometric Mean Titre |
| HCMV | Human Cytomegalovirus |

| | |
|---|---|
| HCMV-IgM | Human Cytomegalovirus Immunoglobulin M |
| HCoV | Human Coronavirus |
| HEU | HIV Exposed Uninfected. |
| HIV | Human Immunodeficiency Virus |
| HU | HIV-Unexposed |
| IFN | Interferon |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| LMICs | Low- and Middle- Income Countries |
| MAIT | Mucosal Associated Invariant T-cell |
| MHC | Major Histocompatibility Complex |
| MR | Measles-Rubella |
| NK | Natural Killer |
| NKT | Natural Killer T-cell |
| OPD | o-Phenylenediamine Dihydrochloride |
| OPV | Oral Polio Vaccine |
| ORV | Oral Rotavirus Vaccines |
| PAMP | Pathogen Associated Molecular Patterns |
| PBMC | Peripheral Blood Mononuclear Cells |
| PCV | Pneumococcal Vaccine |
| PHA | Phytohemagglutinin |
| PP65 | Phosphoprotein 65 |
| PRR | Pattern Recognition Receptor |
| PSG | Penicillin Streptomycin L-Glutamine |

| | |
|---|---|
| rAU | relative Absorbance Units |
| RCT | Randomised Controlled Trial |
| RNA | Ribonucleic Acid |
| ROVAS-2 | Rotavirus Vaccine Study-2 |
| RPMI | Roswell Park Memorial Institute |
| RTI | Respiratory Tract Illness |
| RV-IgA | Rotavirus Immunoglobulin A |
| RV-SIg | Rotavirus Secretory Immunoglobulins |
| SARS-CoV | Severe Acute Respiratory Syndrome Coronavirus |
| SARS-CoV-2 | Severe Acute Respiratory Syndrome Coronavirus 2 |
| SIgA | Secretory Immunoglobulin A |
| SLP | Single Layered Particle |
| TCR | T Cell Receptor |
| TLP | Triple Layered Particle |
| VP | Viral Protein |
| WASH | Water Sanitation and Hygiene |
| WHO | World Health organisation |

## Chapter 1: Introduction

### 1.1 Background

Rotavirus is the most common cause of diarrhoea in children aged below five years old (1, 2) responsible for high mortality in this population especially in sub-Saharan Africa (3). Fortunately, live attenuated, oral rotavirus vaccines (ORV) are available and since introduction have significantly reduced the disease burden. However, ORV exhibit diminished seroconversion rates in low-income compared to high-income settings, a phenomenon attributed to several factors but that remains incompletely understood (4-6). This is of particular relevance to Zambia where rotavirus remains a significant cause of diarrhoea (7) and work from our laboratory has shown moderate vaccine seroconversion rate of approximately 60% (8). This under performance hinders further reduction of rotavirus diarrhoea burden.

Alternative vaccine schedules (9) such as additional doses to the primary series vaccination or booster doses later in infancy (10-12) coupled with a better understanding of vaccine induced immunity can contribute towards improving vaccine performance. By the time of this thesis only two studies had investigated the benefit of a booster rotavirus vaccine dose during later infancy in Africa (10). T cell immunity to rotavirus is understudied (13) despite being critical for generation of antibody immunity (14) that is associated with protection against rotavirus (15). It is important to determine vaccine induced T-cell immunity to provide insights that may be crucial for developing more effective next-generation vaccines.

Studies elsewhere and in our laboratory have in the past investigated the effect of diverse maternal and infant factors on ORV immunogenicity (6, 8, 16-18) but none have assessed the influence of herpesviruses such as human cytomegalovirus (HCMV). Whilst prevailing evidence on the impact of HCMV on immunogenicity of other childhood vaccines such measles, diphtheria-pertussis-tetanus (DPT), Haemophilus influenzae type B (Hib), meningococcal, polio, Hepatitis B and Bacille Calmette-Guérin (BCG) vaccines in Africa is conflicting (19-24), the potential role that HCMV may play in ORV immunogenicity needs to

be understood as it is highly prevalent in sub-Saharan Africa and known to profoundly modulate infant T-cell immunity in early life (19, 25-30).

This thesis therefore addressed the issue of the ORV underperformance by contributing knowledge on immune benefits of booster dose ORV, vaccine-induced T cell immunity and the influence of HCMV with the potential to impact local ORV policy and future vaccine design.

## 1.2 Rotavirus

### 1.2.1 Rotavirus biology

Rotaviruses, non-enveloped double stranded ribonucleic acid (dsRNA) viruses belonging to the Reoviridae family and Rotavirus genus, infect a range of animals including humans. Rotavirus has an 11-segmented dsRNA genome that encodes structural and non-structural proteins (NSP) for viral attachment, replication, pathogenesis, and induction of immune response in the host during infection (31-33). As shown in Figure 1-1, structural viral proteins (VP) make up three different layers of the rotavirus particle surrounding the dsRNA genome when observed under electron microscopy (31). The innermost capsid layer is made up of VP2 core shell which encases the dsRNA genome including VP1 RNA polymerase and VP3 RNA capping enzymes which are intimately associated with the dsRNA and together form the single layer particle (SLP). VP6 surrounds the core shell as the intermediate capsid layer forming the double layered particle (DLP) structure. VP4 (composed of VP5* and VP8* subunits) and VP7 constitutes the outer capsid layer forming the triple layer particle (TLP) structure which is the fully infectious rotavirus particle or virion (31, 33).

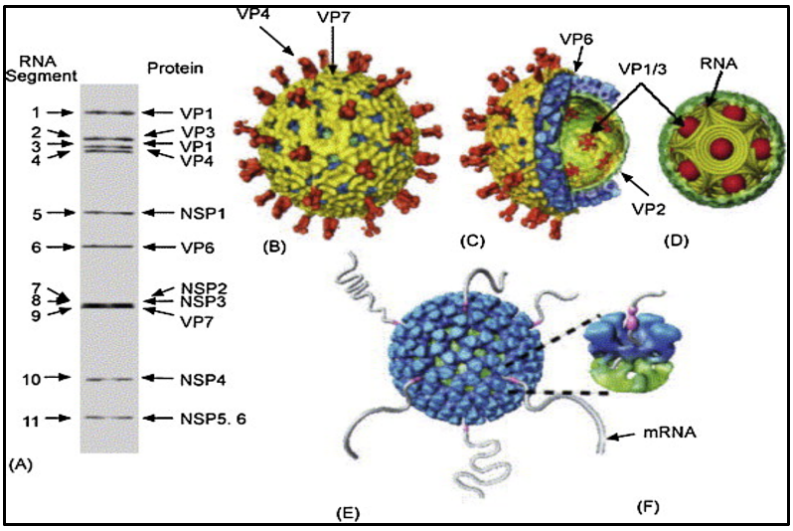


**Figure 1-1. Structure of rotavirus particle**

Illustration of rotavirus particle depicting the segmented double stranded ribonucleic acid (dsRNA) genome with associated translated proteins (A); the viral proteins (VP) that make up the outer capsid layer in the triple layer particle (TLP) form (B and C); the intermediate capsid layer in the double layer particle (DLP) form (C and E); and the core shell in the single layer particle (SLP) form (C and D). Image obtained from and copyright of Desselberger et al. (2014) (31)

Based on shared cross-reactivity (or nucleotide sequences) of epitopes on the highly immunogenic inner layer VP6 and differences in neutralising epitopes of the outer layer protease sensitive (P) VP4 and glycoprotein (G) VP7, rotaviruses can be classified into serogroups (or genogroups) and P and G serotypes (or genotypes) respectively (32, 33) using serological or molecular based methods (34). Distinct serogroups of rotavirus (designated RVA, RVB, RVC, RVD, RVE, RVF, RVG and so forth) infectious to humans and animals have been identified (31-33). Rotavirus serogroup RVA, RVB and RVC can cause infection in humans but RVA is the most significant cause of diarrhoea in children (33). Although rotavirus strains are diverse, several distinct VP7 (G) serotypes and VP4 (P) serotypes of RVA infect humans and have been isolated, with a close interspecies relationship observed between human and some animal (for instance porcine and bovine) RVA strains (32, 33). G1 to G4 types have been the most detected globally with G5, G6 and G8 also dominant in some regions. Among the G and P type combinations G1P[8], G2P[4], G3P[8] and G4P[8] are among those that predominate (31, 33)

The natural host cell target for rotavirus is primarily the mature enterocytes at the apex of villi of the small intestines and infection involves multi-step processes of attachment, penetration

and viral uncoating, viral RNA synthesis, viral assembly and egress (31, 33). Current collective evidence shows that VP4 and more specifically the VP8* subunit mediates the initial attachment to glycoprotein cell membrane receptors followed by conformational change in the VP5* subunit and post-attachment interactions between VP5* subunit and VP7 with other co-receptors resulting in membrane penetration directly or via endocytosis (31, 33). Penetration into cell cytoplasm is followed by solubilisation of the outer VP4 and VP7 coating into a transcriptionally active DLP transcribing viral mRNA via VP1/VP3 transcription complex (31, 33). DLP formed in viroplasms are directed to the endoplasmic reticulum for encapsidation with VP4 and VP7 and maturation into TLP. Matured TLP are released from the cell via cell lysis or through transportation in vesicles to continue the infection cycle (31, 33).

Rotavirus generally leads to acute diarrhoea within 1-4 days post infection through the cytocidal effect on enterocytes and functional impairment of the intestinal villi resulting in malabsorption and large water and electrolyte secretion into gut lumen. No specific anti-rotavirus therapy exists and management usually involves replenishment of fluids and electrolytes thus deaths may happen without timely rehydration (33). Rotavirus particles are environmentally stable and shed in large amounts (estimated at up to $10^{11}$ particles/ml) in stool or vomitus of infected individuals which facilitates their faecal-oral transmission (31, 33).

### 1.2.2 Rotavirus diarrhoea burden

Rotavirus infections occur in both resource-rich and resource-poor settings and are among the leading causes of moderate to severe and less severe diarrhoea in young children particularly those aged below five years old (1, 2). However, the burden of serious diarrhoeal illness and mortality from rotavirus disproportionately occurs in poor resource areas in which infants have the worst outcomes (33). Rotavirus diarrhoea is responsible for substantial diarrhoea associated mortality in this population with a high burden in sub-Saharan Africa (3). Large, multicentre diarrhoea aetiology studies conducted in Africa and Asia revealed rotavirus as among the top four most significant causes of moderate to severe diarrhoea at the population level, with the highest attributable fraction in infants aged < 1 years old (1, 35). In these studies, rotavirus dominated moderate to severe diarrhoea incidence rates in the first two years of life, was frequently detected as the sole or primary diarrhoea associated pathogen in these cases and at more than double the incidence rate of other enteric pathogens (1, 35) (Figure 1-2).

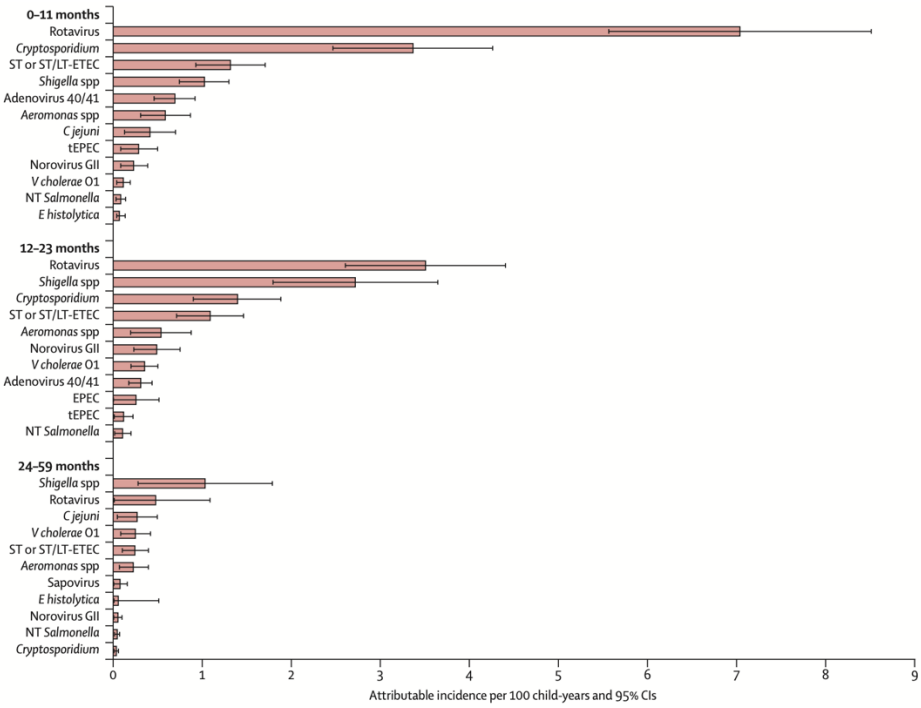


**Figure 1-2. Enteropathogen attributable incidence in children aged <5 years old with moderate to severe diarrhoea.**

Attributable incidence of various viral, bacterial, and parasitic enteropathogens towards moderate to severe diarrhoea in children by age groups 0-11 months, 12-23 months and 24-59 months in Africa and Asia. Image obtained from and copyright of Kotloff et al. (2013) (1)

Other than moderate to severe diarrhoea illness, rotavirus also had the highest attributable incidence for less severe diarrhoea which can constitute >70% of acute diarrhoea cases presenting to health facilities in infants and toddlers (2). Among diarrhoea cases occurring within the communities, rotavirus had the second and third highest attributable fraction in the first and second year of life respectively and most associated with acute and higher diarrhoea severity score, worse clinical presentation, and hospital admission (36).

Recent global estimates place rotavirus as the top enteric pathogen attributed to an estimated 128,515 deaths in a single year in children < 5 years old with child wasting, unsafe water, and unsafe sanitation as leading risk factors (3).

### 1.2.3 Rotavirus vaccines

Rotavirus was recognised as a vaccine preventable disease through observations that i) the occurrence and severity of diarrhoea was reduced in previously infected children who had rotavirus re-infections even if the re-infecting strain differed from the original; ii) this protection was associated with rotavirus specific antibodies iii) and that naturally attenuated rotavirus

strains causing asymptomatic infections or mild diarrhoea were still able to induce immunity and protect against severe diarrhoea in re-infections. Therefore live, attenuated vaccines could be used to mimic repeated infections and provide protection against frequency and severity of disease (31, 33).

Vaccine research and development over the years has resulted in four live, attenuated, oral rotavirus vaccines licensed and pre-qualified by the World Health organisation (WHO) between 2008 and 2018 (5). As shown in Table 1-1, Rotarix (GlaxoSmithKline Biologicals, Belgium, prequalified in 2009) is a liquid monovalent G1P[8] human strain isolated from infant with diarrhoea. RotaTeq (Merck & Co. Inc, USA, prequalified in 2008) is a liquid pentavalent G6P[5] bovine-human reassortant strain with five reassortant rotaviruses each expressing one of the VP7 types (G1, G2, G3, G4), or VP4 type P1A [8]) from human rotavirus strains. Rotavac (Bharat Biotec International Ltd, India, prequalified in 2018) is a liquid-frozen monovalent G9P[11] natural bovine-human reassortant strain isolated from a neonate. Rotasiil (Serum Institute of India, India, prequalified in 2018) is a lyophilised and liquid pentavalent G6P7[5] bovine-human reassortant strain containing human G1, G2, G3, G4 and G9 types (5, 6, 31, 33, 37). Rotarix is recommended as two-doses at one month apart from 6 weeks of age and completed by 24 weeks old. RotaTeq, Rotavac and Rotasiil are all recommended as three-doses at a minimum of one month apart from 6 weeks of age and completed by 32 weeks, 8 months and by 12 months of age respectively (5, 6). Two other vaccines are available nationally. A liquid G10P[15] Lanzhou lamb rotavirus vaccine (Lanzhou Institute of Biomedical Product, China) isolated from lamb is available since 2000 in China and administered annually between 2 months and 3 years of age. Another liquid-frozen G1P[8] Rotavin M-1 vaccine (Centre for Research and Production of Vaccines and Biologicals PolyVac, Vietnam, licensed nationally since 2012) isolated from a child hospitalised with diarrhoea is available in Vietnam and administered as two doses at 2 and 4 months of age (5, 6, 33, 37).

As of 2018, more than 90 countries worldwide had Rotarix or RotaTeq in their immunisation schedules (5, 33, 37). Several other rotavirus vaccines are in the development pipeline with some live naturally attenuated oral neonatal strains and parenteral non-replicating subunit types at advanced stages (6, 37). In Zambia, the Rotarix vaccine was introduced in 2012 through a pilot programme conducted in Lusaka the success of which led way for nation-wide rollout of the vaccine in November 2013 (38).

**Table 1-1. Live attenuated oral rotavirus vaccines available.**

| Vaccine name | Strain | Species | Valency | Standard EPI schedule; ages |
|---|---|---|---|---|
| Rotarix | G1P[8] | human | monovalent | two doses, 6 and 10 weeks |
| RotaTeq | G6P[5] containing human G1, G2, G3, G4), or VP4 type P1A [8] reassortants | bovine-human | pentavalent | three doses; 6, 10 and 14 weeks |
| Rotavac | G9P[11] | natural bovine-human | monovalent | three doses; 6, 10 and 14 weeks |
| Rotasiil | G6P7[5] containing human G1, G2, G3, G4 and G9 reassortants | bovine-human | pentavalent | three doses; 6, 10 and 14 weeks |
| Lanzhou lamb rotavirus | G10P[15] | lamb | monovalent | Annually, 2months to 3 years |
| Rotavin M-1 | G1P[8] | human | monovalent | Two doses; 2 and 4 months |

Rotavirus vaccines have significantly reduced diarrhoea burden but demonstrate lower seroresponses, efficacy and effectiveness in high burden settings.

Rotavirus vaccines have shown demonstrable reduction of rotavirus and overall diarrhoea burden in children <5 years old. Globally, between 2006 and 2019, vaccine introduction reduced rotavirus diarrhoea hospitalisation and all cause diarrhoea hospitalisation and deaths by an estimated median 59% and 36% respectively (4). Among infants aged less than a year old and who were age eligible for vaccination, a median 97% and 62% reduction in rotavirus and all cause diarrhoea hospitalisations respectively occurred within seven years post Rotarix and RotaTeq vaccine introduction with larger reductions associated to higher rotavirus vaccine coverage (4). Reductions have also been evident in ages 12-23 months and 24-59 months

within the first year of vaccine introduction despite these age groups not being age eligible for vaccination at that time therefore attesting to a level of herd immune protection (4). Annual rotavirus positivity in stool among children below 5 years old hospitalised with diarrhoea was halved from 40% to 20% within four years after vaccine introduction (4).

In Zambia, two years after Rotarix vaccine introduction in the country and a vaccine coverage of about 77%, annual rotavirus positivity among children hospitalised with acute gastroenteritis in a large referral hospital declined from 40.1% in 2009 to 2011 pre-vaccine era to 24.7% in 2014 in those aged <5 years old(39). This reduction was mostly observed among those aged <1 year old in which a 51% reduction was observed with a significant reduction also in children aged 1 year old but no reduction observed in those aged 2 to 4 years old (39). Furthermore, seasonal peaks of rotavirus diarrhoea against a backdrop of year round transmission were dwarfed and in <1 year olds, a decline of 18% for all cause diarrhoea hospitalisations and a 33% drop in all-cause diarrhoea in-hospital deaths occurred by 2014 compared to pre-vaccine era (39). These declining trends remained generally sustained four years post vaccine introduction. In 2016, with vaccine coverage increased to 94% among <1 year old children, a median 52% and 56% reduction in rotavirus positivity was observed from 2013 to 2016 among children aged <5 years old and those aged <1 year old respectively speaking towards a sustained gradual disease burden reduction (40).

Although these trends in disease burden reduction are consistent across low, medium, and high mortality regions, lower reductions are seen within high mortality compared to low mortality regions (4). Aligned with this observation, vaccine efficacy and real-world effectiveness estimates are markedly reduced in high mortality regions. In a recent Cochrane review of mostly placebo-controlled trials conducted from 1974 to 2020, efficacies of all four currently available vaccines ranged from 48% to 58% against severe rotavirus diarrhoea in high mortality regions but were >90% in low mortality regions in the first year of life (41). While percent reductions are at a lower level, similar vaccine efficacy trends are seen in the second year of life and against rotavirus diarrhoea of any severity (41). Furthermore, a greater reduction in vaccine efficacies between the first and second year of life occurs in high mortality compared to low mortality settings (41). In the same fashion, a gradient of vaccine effectiveness of Rotarix and RotaTeq against rotavirus diarrhoea hospitalisation is observed by mortality settings. In the first year of life a higher vaccine effectiveness estimate of 86% occurs in low mortality settings for both vaccines while vaccine effectiveness of 66% and 63% is seen for Rotarix and RotaTeq respectively in high mortality setting (42) resulting in up to 47% relative difference in effectiveness between these low and high mortality settings. Similar

to efficacy trends, lower vaccine effectiveness is observed in the second year of life compared to the first (42). Rotavirus vaccine induced seroresponses are also inversely related to mortality. Rotavirus seroresponse concentrations and seroconversion rates post vaccination are lower in high mortality settings compared to low mortality settings for both Rotarix and RotaTeq (43). The average vaccine induced seroresponses are four-fold and eight-fold lower in high mortality settings compared to low mortality settings for Rotarix and RotaTeq respectively (43). These lower seroresponses in high mortality settings have been associated with the observed lower vaccine efficacy and greater waning of immunity in the second year of life (43).

For instance, in Zambia, the effectiveness of Rotarix vaccine not long after introduction was low. In children aged ≥ 6 months old, despite a 70% and 58% vaccine coverage for at least one dose and two dose Rotarix respectively, and timely vaccination, vaccine effectiveness for at least one dose ranged from 17% to 60% across all severity, mild and very severe diarrhoea and diarrhoea requiring hospitalisation (44). Also, in an immunogenicity study conducted by our group shortly after the national introduction of Rotarix a modest seroconversion of 60.2 % post two-dose vaccination at 6 and 10 weeks of age was observed (8).

Reasons behind the observed disparity in oral rotavirus vaccine performance are unclear.

The observed phenomenon of underperformance of the existing oral rotavirus vaccines in high burden and typically poor-resource regions is attributed to several factors but remains incompletely understood (4-6). These factors are categorised broadly into those that lower the vaccine virus infectivity titre and those that impair the infant immune responses. In the former category, maternal breastmilk rotavirus specific antibodies and non-antibody antiviral immune components acquired by the infant through breastfeeding are thought to inhibit infectivity and neutralise effective replication of the vaccine virus in the gut and are associated with reduced seroresponses (6, 8). High levels of transplacental maternal IgG in infants are associated with decreased likelihood of seroconversion when vaccines are administered early in life (6, 8). Also, co-infection with non-polio enteroviruses at vaccination and concomitant administration of oral polio vaccines (OPV) thought to outcompete rotavirus replication in the gut are associated with lower vaccine seroresponses and higher vaccine failure (6). In the latter category, poor household-level water sanitation and hygiene (WASH) resulting in an environment of early and high enteric pathogen exposure, alterations in infant intestinal or maternal breastmilk microbiome, and environmental enteric dysfunction (EED, a gut disorder of blunted villi, impaired gut barrier function and malabsorption) are known to influence vaccine immunogenicity (6). Deficiencies in Zinc and Vitamin A are other factors linked to diminished vaccine immunogenicity (6). Infant host genetics controlling secretion of sialic acid receptors

utilised for rotavirus attachment and therefore susceptibility to rotavirus infection have also been linked to the variation in vaccine performance (6). Waning vaccine immunity is another reason proposed for reduced vaccine efficacy and effectiveness observed in the infants' second year of life (6, 41, 42). It is most likely the reasons for underperformance are a combination of these multiple factors. However, as rotavirus burden remains a significant problem, research on understanding the reasons behind variable vaccine immunogenicity must continue to improve the performance of existing vaccines or inform the design of more effective ones for an even greater impact in high burden settings.

In the Zambian setting, our group has shown that seroconversion frequencies are inversely related with infant rotavirus seropositivity prior to vaccination, high levels of rotavirus neutralising breastmilk antibodies and transplacental antibodies (8, 17). We have also found that these vaccine seroconversion rates in Zambian infants are negatively impacted by increased levels of maternal breastmilk antiviral non-antibody components (18) and associated with markers of EED (16). More recently, a phase 2b comparative study of the safety, reactogenicity and immunogenicity of two Rotavac vaccine formulations, Rotavac and Rotavac 5D, administered at 6, 10 and 14 weeks of age similarly conducted by our group found modest seroconversion rates of 33.1% and 40.6% for these oral rotavirus vaccines respectively (45). In the same study, Rotarix vaccinated infants used as reference population had seroconversion rates of 52% (45). Important to note from these studies in our setting was that use of rotavirus strains heterologous to the administered vaccine strain in the laboratory measurement of antibody responses showed lower seroresponses rates than using vaccine homologous strains (8, 45).

Additional oral rotavirus vaccines doses have potential to improve vaccine performance.

Strategies to improve the performance of existing oral rotavirus vaccines in high burden settings are necessary to further reduce the rotavirus diarrhoeal disease burden. Potential interventions have been comprehensively reviewed elsewhere and included micronutrient and probiotic supplementations, anthelminthic and antibiotic therapies, withholding of breastfeeding at vaccination, and changes in vaccine formulations, inoculum concentrations, number, and timing of doses (9). Overall, for rotavirus vaccines, no significant impact on vaccine efficacy or immunogenicity has been observed for Zinc supplementation, withholding of breastfeeding or narrower intervals between vaccine doses (9). However, there is evidence of improved immunogenicity with additional rotavirus vaccine doses, delayed administration of the first dose, and staggered administration with OPV where rotavirus vaccine is given separately from OPV rather than concomitantly (9). In a meta-analysis of studies evaluating

additional rotavirus vaccine doses in high mortality settings, an increased proportion of children aged <2 years old have seroresponses when administered an additional rotavirus vaccine dose in either the first or second six months of life (10). However, the increase in seroresponses was seen to be greater for booster doses given in later infancy (around 9 to 11 months old) than additional doses administered early or later in the first six months of life as an extension of the primary series (between 6 and 14 weeks old) (10) likely due to declined interference from maternal antibody and maturation of the infant immune system. These increments in seroresponses by additional doses are also more pronounced in infants seronegative for rotavirus antibodies or with low antibody concentrations at baseline (10-12). Co-administration of booster rotavirus doses with measles-rubella, yellow fever or meningococcal vaccines in later infancy does not impair immunogenicity of these other childhood vaccinations (11, 12). Also, though data is sparse, lower proportion of children experience severe rotavirus diarrhoea if they receive an additional dose in the first year of life compared to those who receive standard doses and evidence indicates that these additional doses are well tolerated without an increased risk of intussusception (10-12). The improved performance and lifesaving potential of booster doses has been modelled whereby the enhanced anti-rotavirus immunity from booster dose administration at 9 or 12 months of age is predicted to prevent up to 19,600 additional rotavirus-associated deaths in the second year of life annually (46). This statistical modeling of the effect of booster rotavirus vaccine doses on the waning immunity has predicted a substantial number of deaths from rotavirus diarrhoea that could be averted. An estimated median 1,200 to 9,800, 4000 to 19,600, and 6,100 to 29,400 deaths from rotavirus diarrhoea could be avoided in scenarios where the booster dose reduced waning in the second year of life by 50%, reestablished vaccine efficacy in the second year of life to that of the first year, and boosted vaccine efficacy by 50% of that in the first year of life within the second year of life respectively with the largest impact seen Africa (46). This underscores the potentially huge impact a booster dose may have on rotavirus diarrhoea burden necessitating evaluations within local settings having high disease burden. In fact based on such data the WHO expanded its recommendations for rotavirus vaccines to be given up to 2 years of age (5).

While significant progress in reducing the rotavirus diarrhoea burden has been achieved by introduction and good coverage of oral rotavirus vaccines, rotavirus infections still occur even among vaccinated children. For instance, in Zambia among infants aged between 2 and 12 months old with acute diarrhoea of which >70% were fully vaccinated with Rotarix, 36.1% were infected with rotavirus of diverse genotypes with the vaccine strain G1P8 being among the predominant type (7). These residual and breakthrough rotavirus infections further emphasize

the need for improving the effectiveness of these vaccines within our local setting and other resource-poor regions with similar realities. While newer rotavirus vaccines are in development, it is necessary to evaluate effective strategies like these booster doses to improve the performance of existing vaccines in routine use within local settings especially as these vaccines continue to be introduced in many other high burden countries.

### 1.2.4 Immune responses to rotavirus

#### Immune responses to viruses

The human immune response to viruses involves both innate and adaptive immunity. For enteric viruses innate immunity is activated via detection ofconserved pathogen-associated molecular patterns such as viral nucleotides, proteins or stress signals by host cell pattern recognition receptors (PRR) expressed on intestinal epithelial cells and innate immune cells such as dendritic cells (DC) and macrophages within the gut associated lymphoid tissue (GALT) (47). Innate immune activation by enteric RNA viruses like rotavirus, occurs through recognition of their viral single stranded RNA (ssRNA) and dsRNA nucleotides via key cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), namely RIG-1 and melanoma differentiation associated 5 (MDA-5), but also endosomal Toll-like receptors (TLR), TLR3, TLR7 and TLR8 (47, 48). Viral antigen recognition activates a signal cascade that results in secretion of interferons (IFN) and proinflammatory cytokines by enterocytes and innate immune cells, and recruitment and activation of other innate immune cells like natural killer (NK) cells that can limit viral replication by degrading viral RNA, suppressing viral protein translation, inhibiting entry of viral genome into cytoplasm and direct cytolysis (47-49) within hours after infection to provide non-specific immune protection (50). For example, children with rotavirus diarrhoea have elevated levels of pro-inflammatory cytokines such as interleukin (IL) 1$\beta$, TNF-$\alpha$, IL-2, IL-6, IL-8, IFN-$\gamma$ during acute stage of infection some of which are correlated with disease symptoms and presentation (51, 52). This anti-viral innate immunity is important in slowing or limiting virus infection before the adaptive immune response and is critical for primary enteric viral infections after physical mucus and epithelial membranes barriers are overcome by the virus (47).

In contrast to innate immunity, the adaptive immune response to viral infection is mediated by antigen specific T cells and B-cell generated antibodies and occurs later in the course of infection generally within one to a few weeks after initial infection. Also, a distinct feature of adaptive immunity is that a proportion of activated T and B cells develop into antigen-experienced memory cells that can respond rapidly within days upon re-infection with the

same pathogen. This immunological memory is key for the development of protective immunity and underlies goals of vaccination (14). Virus specific antibodies can bind viral antigens to block infection or re-infection whereas T cell mediated immunity destroys virus infected cells to combat cell to cell spread and also provides B-cell help for antibody generation (49).

Adaptive immunity is activated mainly through specific recognition by T-cell receptor (TCR) of viral antigens presented by antigen presenting cells (APC) via the major histocompatibility complex (MHC). MHC are transmembrane glycoproteins with peptide binding grooves that are expressed on different type of human cells (14). For enteric viruses, viral antigens are transported across the intestinal epithelium via specialised epithelial microfold cells (M-cells) and are presented by professional APC such as the innate DC and macrophages to the adaptive T and B cells within organised inductive sites of the GALT (49). These inductive sites include Peyer's Patches and intestinal draining mesenteric lymph nodes and contain B cell follicles and germinal centres surrounded by intervening T cell zones (49). The APC with viral antigen travel to these inductive sites via gut draining lymphatics and present these antigens to naïve antigen specific T cells as processed MHC-bound peptide fragments on their cell surface (47). MHC class I (MHC I) binds shorter antigen peptides and presents to CD8 T-cells while MHC class II (MHC II) binds and presents comparatively longer peptides to CD4 T-cells (14). Both CD4 and CD8 T-cell activation is augmented by co-stimulatory molecules mainly CD28 but also others such as CD137 (4-1BB) and CD134 (OX40) (53-55). Activation of naïve T-cells leads to their proliferation and differentiation into two distinct effector types broadly defined as Helper T-cells (Th) and cytotoxic T-cells with varying effector functions (53) and also the development of T-cell memory subsets (56, 57). In viral infections CD4 T-cells mainly differentiate into Th type 1 effector cells that secrete IFN-$\gamma$ and help the viral lysis of infected cells by cytotoxic T-cells and into T follicular helper (Tfh) that support B-cell antibody production (53). Effector CD8 T-cells in viral infections function to kill cells infected with virus through production of anti-viral cytokines and cytotoxic molecules (55, 58).

### 1.2.5 Rotavirus-specific antibody responses

Intestinal secretory IgA is most importantly correlated with protection against enteric viruses however protection is also correlated with circulating serum virus specific antibodies despite an unclear mechanism of action (47). For rotavirus, adult human challenge studies and observation of natural infection in children have shown that serum rotavirus specific IgA (RV-IgA) or IgG (RV-IgG), serum or intestinal fluid neutralising antibodies and intestinal fluid or faecal RV-IgA or RV-IgG are important protective antibody responses but findings are conflicting and correlation with protection is not always consistent (33, 59, 60). Intestinal

antibodies acting at the site of infection are the likely immune mediators but are impractical to measure which has hindered their wider investigation (60). The ease of measurement of serum RV-IgA and RV-IgG antibodies and their observed high predictive accuracy for intestinal RV-IgA after natural rotavirus infection has made these responses to be widely accepted as possible surrogates of protection and markers of vaccine immunogenicity (60). Naturally infected children have increased serum RV-IgA and RV-IgG after every consecutive asymptomatic or symptomatic rotavirus infection (15). At the individual level, RV-IgG > 1:6400 and RV-IgA > 1:800 achieved after two consecutive natural infections is strongly predictive of reduced risk of rotavirus infection and rotavirus diarrhoea respectively with complete protection against moderate to severe diarrhoea for RV-IgA (15). Other thresholds of >1:200 for RV-IgA and 100 – 199U/mL for RV-IgG have also been observed to be predictive of protection from rotavirus infection and diarrhoea respectively (60).

Due to the induction of RV-IgA in naturally infected children and its association with protection, serum RV-IgA is routinely measured in vaccine trials to assess vaccine immunogenicity among infants (60). These seroresponses are defined at a ≥ 20U/mL threshold based on its use to determine natural infection and as increments in concentration between pre-vaccination sera and sera taken after the last dose using standardised immunoassays (60). Vaccinated infants with RV-IgA seroconversion have reduced risk of rotavirus infection and diarrhoea and so RV-IgA responses have been thought to reflect vaccine efficacy (60, 61). For example, having a RV-IgA seroresponse post vaccination has been linked to protection against rotavirus diarrhoea of any severity while seronegativity in infants has been associated with the highest incidence of severe and any severity rotavirus diarrhoea (62). Also, an increase in post-vaccination RV-IgA from ≥20U/mL up to ≥2560 U/mL has been associated with a greater reduction in risk of rotavirus diarrhoea in both low and high mortality settings (62). However the level of protection offered by these RV-IgA thresholds varies by mortality setting where antibody thresholds show less protective effect in high mortality settings compared to low mortality settings limiting their generalisability and thus no standard individual protective threshold exists (62). At the population level, post-vaccination RV-IgA <90 U/mL is correlated with suboptimal vaccine efficacy (defined as less than 50%), significantly lower efficacy in the second year of life and greater relative decline in efficacy by the second year (43). This threshold has been proposed as a predictor of vaccine performance in lieu of a defined immune surrogate endpoint at this level (43). Overall, there is no consensus for a seroprotective threshold against rotavirus infection or post vaccination.

While serum RV-IgA is highly predictive of vaccine “take” and protection this is not always the case. Children with RV-IgA seroconversion post vaccination can still have rotavirus diarrhoea and a considerable proportion of children that do not seroconvert can be protected against rotavirus diarrhoea (61). Such observations imply that circulating RV-IgA, while predictive of protection, is not the only immune effector at play against rotavirus. Other immune responses including rotavirus specific secretory antibodies (RV-SIg), antibody secreting cells (ASC), memory B cells and T cells are found to be involved in the human immune responses to rotavirus and proposed for further investigation as alternative or complementary markers of vaccine immunogenicity to RV-IgA and potential surrogate CoP for vaccines (60). RV-SIg are mounted and detectable in serum or plasma in children with rotavirus infection and after rotavirus vaccination but compared to serum RV-IgA are transiently detected within a week and disappear by a month later which has limited their measurement as an alternative surrogate (63-65). Nevertheless, RV-SIg is observed to have immune protective roles. Serum RV-SIg correlates with protective intestinal RV-SIg responses in natural rotavirus infections (66, 67). In vaccine trials, plasma RV-SIg seropositivity in both placebo and vaccinees and increase in plasma RV-SIg titres post two doses among vaccinees is correlated with greater protection against rotavirus diarrhoea in children (65). Rotavirus vaccinated children that are RV-IgA seropositive also have higher plasma RV-SIg responses indicating that these two responses overlap but poor overall correlation between RV-IgA and RV-SIg is observed (65). Rotavirus specific IgA and IgG ASC are induced after vaccination in infants and in one study an increased frequency of rotavirus specific total and gut homing IgA and IgG ASC is observed after two doses of Rotarix (68). These ASC responses were more likely observed among RV-IgA seropositive infants but RV-IgA negative infants also mounted these responses (68). Like RV-SIg, measurement of ASC responses is limited by the small window of detection which is usually up to a week after vaccination (60).

### 1.2.6 Rotavirus-specific T-cell responses

The literature review on T-cell immune response to rotavirus in children is provided in Chapter 2 and summarised here. The T cell immune response to rotavirus has been mainly garnered using animal models and to a lesser extent in humans after infection and vaccination but has revealed an importance of both CD4 and CD8 T cells for protective immunity (13). Rotavirus specific T cell immunity is absent at birth but becomes more readily detectable with increasing age (69-73). This age-related development of T cell immunity is most likely through initial and repeated exposure to natural infection as these proliferative T cell responses are more frequently detected in rotavirus IgA or IgG seropositive children than in the seronegative and more in secondary than primary rotavirus infections (70, 71, 74). There is also an evident close

association between the rotavirus T cell and antibody response. Higher magnitude T cell proliferation in children are observed to coincide with higher antibody responses (71). Additionally higher frequency of total and rotavirus specific CD4 T cells are linked to larger rotavirus specific neutralising IgG antibody titres (75) and rotavirus IgA seropositivity after vaccination (74) respectively. The close association of T cells with the antibody responses and the importance of these T cells in protection against rotavirus in children is exemplified by studies where T cell deficiency results in the inability of infants to mount any antibody response and clear rotavirus infection leading to persistent infection and death (76). However unlike the antibody responses which persist longer, rotavirus antigen induced T cells in infants are transient as although they can be detected as early as two weeks after natural infection or vaccination these T cell responses disappear from circulation within shorter periods of time (71, 74, 77).

When detected using assays based on CD4 and CD8 T cell frequency, secretion of specific cytokines or rotavirus tetramer staining, these T cell responses in children are low in frequency after natural rotavirus infection (78) or vaccination (74). Nevertheless, in studies where these responses have been sufficiently detected in children, the IFN-γ secreting CD4 Th1 and CD8 T cell responses dominate (79, 80) suggesting an importance of these subsets as T cell effectors of protection. Majority of the detected T cell responses to rotavirus against infection and post vaccination express the gut homing markers integrin α4β7 and/or chemokine receptor CCR9 in both adults and children (74, 80, 81). This preferential movement to intestinal sites is therefore important for T cell protective effector functions against rotavirus and thus their induction by rotavirus vaccination would be important for vaccine effectiveness. The studies on the T cell immune responses to rotavirus in children have mostly been done within the context of rotavirus infection with very few done among vaccinated infants especially in Africa (82). These T cell responses are potential biomarkers proposed for assessing immunogenicity of new vaccine candidates (60).

Measurement of rotavirus specific T cell responses in infants among studies done thus far have utilised diverse immunophenotyping methods including enzyme-linked immunosorbent spot (ELISpot), proliferation and flow cytometry surface marker phenotyping and intracellular cytokine staining assays reporting heterogenous T cell subsets mainly on the basis of pre-specified cytokine expression as end points which may underestimate the total antigen specific T cell response (82). Newer assays are available to improve sensitivity of detecting antigen

specific T cell responses such as activation induced marker (AIM) T cell assay that gives a broader view of overall antigen specific T cell responses and which is useful in detecting low frequency or rare T cell responses induced by vaccination (83). As rotavirus burden is highest within the African region (3) it is imperative that T cell immunity induced by these vaccines be investigated in parallel to the antibody responses in this region. Understanding these T cell responses may offer important insights for improvement of the performance of these vaccines.

## 1.3 Human cytomegalovirus

### 1.3.1 HCMV epidemiology and immune response

Human cytomegalovirus (HCMV), a member of the Herpesviridae family of double stranded deoxyribonucleic acid (dsDNA) genome viruses, infects humans and a range of other animal hosts (84, 85). HCMV exists as a triple layered particle with a dsDNA genome core contained in an inner protein nucleocapsid layer, surrounded by anamorphous middle phosphoprotein (pp)-rich layer (tegument), and an outer lipid-glycoprotein bilayer envelope (84, 85). HCMV establishes lifelong infection and involves lytic, latent and reactivation stages within its lifecycle (85, 86). Predominant permissive cells for disseminated HCMV infection include endothelial cells, epithelial cells, fibroblasts, smooth muscle cells and myeloid DC and monocytes (87). Hematopoietic progenitor $CD34^+$ cells in bone marrow and polymorphonuclear leukocytes, such as granulocytes and monocytes which are permissive to the virus but do not support lytic infection are important latent infection reservoirs (88). Latent HCMV can reactivate due to the changes in the infected cell environment conditions such as differentiation, inflammatory mediators, DNA damage and cellular injury, sepsis, or infection with other pathogens (86, 88).

#### HCMV infection is highly prevalent and acquired rapidly during early infancy in Africa.

In infants, HCMV can be transmitted congenitally in-utero and acquired during early childhood through direct contact with bodily secretions such as saliva, urine, breastmilk from an infected host (86, 89). With exception of congenital transmission which occurs in the placental cells, primary HCMV infection is typically initiated in mucosal epithelial cells where viral replication and shedding occurs while systemic dissemination and seeding into diverse body tissues and organs is facilitated through infection of myeloid cells and HPC (86). Globally, HCMV seroprevalence has been estimated at 83% (95% UI: 78%-88%) in the general population and 86% (95% UI: 83%-89%) among women of childbearing age and associated with lower socioeconomic status (90). In Africa, HCMV is highly seroprevalent estimated at 88% (95%

UI: 80%- 93%) in the general population and 90% (95% UI: 80%-93%) in women of reproductive age (90). Infants in high prevalence settings mount robust IgG and IgM responses against HCMV proteins that can be used to monitor exposure (75). A high pooled seroprevalence of 88.1% (range 80% to 100%) is reported in African children and infection is acquired early with seroprevalence estimates of 86.4 % in the first 12 months of life (91). Congenital HCMV has been reported in up to 14% of live births in sub-Saharan Africa (26, 92) and non-congenital primary HCMV infection is rapidly acquired in African children (26) and is nearly universal by the child's second birthday (27). Therapeutic drugs for treatment of active HCMV infection in immunocompromised individuals and for prophylactic administration in transplantation recipients are available but in limited use (93). There is currently no licensed HCMV vaccine available but clinical trials of candidates are underway (94).

HCMV takes up a large proportion of the infant CD8 T cell "immunological space".

HCMV specific T cells can be directed against 70% of the virion antigens (95). A median ~3% and up to 32% $CD8^+$ T cells out of the total $CD8^+$ T cell population can respond to a single HCMV peptide among infected infants in their first year of life while the total population of circulating $CD8^+$ T cells remain unchanged (26). Actually, these HCMV specific responses are underestimated as HCMV has a large proteome (96). For example, congenitally HCMV infected new-borns can have detectable high frequencies of HCMV $tetramer^+$ CD8 T cells as high as 7.5%; and even in-utero ( as early as 28 week of gestation) 2% of total $CD8^+$ T-cells can be HCMV $tetramer^+$ (30). In prospective studies up to 8.5% of the infant $CD8^+$ T cell population is directed against HCMV two years after initial infection (27). HCMV infected infants are also observed to have fifty percent more circulating $CD8^+$ T cells than uninfected infants associated with a lower $CD4^+$:$CD8^+$ T cell ratio (19) indicating that a large proportion of the infant $CD8^+$ T cell population can be committed to HCMV in early life.

HCMV inflates terminally differentiated memory and TCR restricted T cell phenotypes and potentiates an inflammatory environment.

a) Conventional CD4 and CD8 T cells

Majority of $CD8^+$ T cells responding to HCMV in early infancy are of terminally differentiated effector memory (TEMRA) $CD95^+$ $CD62L^-$ $Bcl\text{-}2^{low}$, $CD27^{-/low}$ , $CD28^{-/low}$ , $CCR7^-$ , $CD57^+$, $perforin^+$ /granzyme $A^+$ and $CD45RA^{bright}$ phenotypes and which remain elevated for as long as 12 months post primary infection both in the HCMV specific $CD8^+$ T cell response and the overall $CD8^+$ T cell population (19, 26, 27, 30, 97). These TEMRA HCMV $CD8^+$ T cells also tend to be highly cytotoxic (26, 29, 30). HCMV infected infants tend to have higher $CD27^{+/-}$ granzyme $A^+$ $CD8^+$ T cell subsets relative to the uninfected (26) and HCMV pp65 tetramer+

$CD8^+$ T cells frequently co-stain with perforin and granzyme A (29). Higher proportions of granzyme $A^+$ and higher expression of perforin in HCMV specific $CD8^+$ T cells are also observed in asymptomatic new-borns with congenital HCMV infection than in uninfected new-borns and interestingly this observation is the same for the total $CD8^+$ T cell population (30). Majority of these HCMV specific $CD8^+$ T cells are associated with expression of antiviral proinflammatory IFN-$\gamma$, CCL3, and TNF-$\alpha$ cytokines (30).

HCMV infected infants also accumulate TCR repertoire restricted CD8 T cell clones associated with reduced immune responsiveness (immune senescence) (30, 95). This restriction arises from alterations in the distribution and repertoire of the beta-chain variable gene (BV) of $CD8^+$ T cells resulting in reduced TCR diversity (30). The effect of HCMV on the $CD4^+$ T cell response is less profound than that for $CD8^+$ T cell response with lower responding frequencies in infants, however, HCMV responding $CD4^+$ T cells in infants also tend to be differentiated ($CD27^{neg}$) and of memory ($CCR7^{low}$) phenotype (19, 25, 27). HCMV infected infants tend to have slightly reduced proportions of central memory $CD4^+$ T cells and a higher median IFN-$\gamma$ secretion by $CD4^+$ T cells (19).
The large T cell "immunological space" taken up by HCMV, memory inflation and accumulation of HCMV TCR restricted clones may skew the infant naïve-memory T cell ratios and impair T cell responses to other viral antigens. Inflammatory effects and bystander production of cytokines resulting from HCMV infection could potentially impact on infant specific immune responses to other antigens.

b) Unconventional T cell subsets

HCMV also alters the profiles of unconventional T cell subsets including gamma delta ($\gamma\delta^+$) T cells. The $\gamma\delta^+$ T cells, comprising $V\delta1^+$, $V\delta2^+$ and $V\delta3^+$ subsets, represent ~<6% of the overall T cell population and increased frequency of circulating $\gamma\delta^+$ T cells coincide with active HCMV infection (95, 98). These $\gamma\delta^+$ T cells are unconventional in that (i) they utilise $\gamma$ and $\delta$ TCR chains, (ii) they are not MHC restricted for their antigen recognition or activation, making use of other diverse ligands, and (iii) differ in their effector cell development compared to conventional $\alpha\beta^+$ TCR chain $CD4^+$ and $CD8^+$ T cells (98). However these $\gamma\delta^+$ T cells share similarities with $\alpha\beta^+$ $CD8^+$ T cells including the observed $\gamma\delta^+$ T cell expansion and persistence in response to HCMV, restriction of $\gamma\delta^+$ TCR repertoire, and accumulation of effector TEMRA phenotypes (98). Significantly higher proportions of $\gamma\delta^+$ T cells relative to total $CD3^+$ population and absolute numbers per volume of blood are observed in congenitally HCMV infected new-borns than in those uninfected (99). In infants, HCMV causes expansion and differentiation of total $\gamma\delta^+$ T cells preferentially increasing the frequency of $V\delta1^+$ and $V\delta3^+$ subsets from birth to

12 months of age with a highly differentiated ($CD27^-$ $CD28^-$) and cytotoxic (granzyme family A, B, H, M, perforin and granulysin) phenotype that can be detected even in utero as early as 20 weeks to 29 weeks of gestation (99, 100). These HCMV induced $\gamma\delta^+$ T cells phenotypes are predominantly producing IFN-$\gamma$ with increased expression of chemokines (CCL3, CCL4, CCL5) and chemokine receptors (CCR5, CX3CR1) (99, 100). Most of these detected $\gamma\delta$ T cells express $V\delta1^+$, $V\delta2^+$ or $V\delta3^+$ $\delta$TCR chains and predominantly $V\gamma9^{neg}$ (expanding preferentially $V\gamma4^{+}$ and $V\gamma8^+$) $\gamma$TCR chains (99). HCMV infected infants particularly show an increase in a unique clustering of $V\gamma8/9^{neg}$ $V\delta1^+$ cells with HLA-$DR^+$ $CD27^-$ $CD28^-$ $CD8\alpha^+$ $CD57^+$ $CX3CR1^+$ granzyme $A^+$ phenotype compared to uninfected emphasising the important role of this particular $V\delta1^+$ $\gamma\delta^+$T cell subset in immune response to HCMV (100).

As observed for $\alpha\beta^+$ $CD8^+$ T cells (30), $\gamma\delta^+$ T cells in HCMV infected infants show restricted TCR repertoire clones (99, 100) which are highly cytotoxic (99) and commonly detected in infected infants (100). This oligoclonality in HCMV infected new-borns has been observed for complementary determining regions of the $\delta$TCR for both $V\delta1^+$, $V\delta2^+$ subsets and the $V\gamma8^+$ preferential chain of $V\delta1^+$ subset. These $V\delta1^+$ $V\gamma8^+$ oligoclonal $\gamma\delta^+$ T cells are experimentally shown to be highly cytotoxic and limiting viral replication and potently expressing IFN-$\gamma$ ex-vivo (99). These public $V\delta1^+$ $V\gamma8^+$ clones are absent in uninfected infants but have been detected in an estimated 22% of infants, including post-natally and congenitally infected (100). In post-natal HCMV infections these clones are detectable before four months of infant age, appearing close to the timing of primary infection but rapidly declining within two months and inversely associated with age (100).

Natural Killer T cells (NKT) are another unconventional T cell subset that express CD3, NK cell receptors, and are either CD4, CD8 positive or double negative that respond to HCMV. HCMV infection increases NKT frequencies expressing NK cell activating markers (101, 102). HCMV infected infants tend to have significantly higher proportions and absolute numbers of $CD3^+$ $CD56^{+/-}$ $NKG2C^+$ $LILRB1^+$ $CD161^+$ NKT cells (101) and more frequently in HCMV seropositive infants below 2 years old (102). These unconventional $\gamma\delta^+$ and NKT T cell subsets and other atypical T cells such as mucosal associated invariant T cells (MAIT) are enriched within mucosal tissues and shown to be important in immunity against intestinal viral infections at the interface between innate and adaptive immunity (103). It is unclear how their modulation by HCMV may be associated with the infant's net immune response to other intestinal pathogens.

Infants in high prevalence settings mount robust HCMV IgG and IgM responses in early life.

HCMV infection also induces humoral immune responses that can be used to determine exposure. HCMV antibodies are associated with lower HCMV mortality (104) and protect against infection and reduce viral replication (86, 94, 95). Nearly all individuals that are exposed to HCMV mount virus specific antibodies targeting envelope glycoproteins, tegument and non-structural proteins (86). Primary HCMV infection causes rapid appearance of HCMV tegument protein specific antibodies followed by envelope glycoprotein specific antibodies (104). These antibodies limit transmission by targeting HCMV while in its “cell free” state within bodily secretions (86).

Majority of women in Africa have high levels of anti-HCMV IgG (91), but intense HCMV transmissions in this region suggests these maternal antibodies are inadequate in preventing transmission (104). Nevertheless, high avidity maternal IgG antibodies are inversely related to infant HCMV viral load (104) and pre-conception humoral immunity associated with reduced likelihood of congenital transmission to their infants (95). The contribution of infant HCMV IgG response towards protection is limited by the interference of transplacental maternal IgG antibodies, however HCMV IgM is commonly detected in congenitally infected new-borns (75). Infants can mount high titre HCMV IgG and IgM responses. In Zambia for instance, about 83% of infants are HCMV IgG seropositive by 18 months of age (24, 105) and HCMV IgM is also detectable in approximately 1% congenitally infected neonates (106). Similarly, elsewhere in The Gambia for example, about 65% to 68% infants are HCMV IgG and/or IgM positive by 9 months of age, 66% to 88% between 10 to 13 months old and 85% by 18 months (19-21, 105).

HCMV downregulates immune recognition by host T cell immunity and causes alterations in intestinal integrity and microbiome.

HCMV infected cells employ key mechanisms to evade host T cell recognition. HCMV tegument phosphoprotein pp65 can phosphorylate early viral proteins produced during infection which prevents MHC I presentation, degrades $\alpha$-chain of the MHC II receptor HLA-DR, a TCR ligand, and decreases expression of MHC II molecules (107). Similarly, HCMV pp71 can cause decreased cell surface expression of MHC I molecules thereby affecting antigen presentation to cytotoxic $CD8^+$ T cells (108). Certain HCMV gene products (US2, US3, US6 and US11) can block translocation and cause degradation of MHC I molecules and (US2) can redirect HLA-DR and HLA-DM alpha chains of MHC II molecules for degradation in the

cell cytosol (109, 110). Other viral gene products (UL148) can suppress expression of CD58, a costimulatory molecule important for T cell activation (111). HCMV also expresses an immunosuppressive viral IL-10 homolog (UL111A) (109, 110). Altogether, these evasion strategies can have an effect of disrupting the activation of host cytotoxic HCMV $CD8^+$ and helper $CD4^+$ T cells and downregulating the adaptive immune responses to HCMV infected cells.

Aside from these effects on immune differentiation and evasion, HCMV has been linked to changes in the human intestinal environment. Human intestinal epithelial cells are susceptible to HCMV causing increased inflammation and intestinal barrier disruption (112, 113) which may influence infectivity by intestinal pathogens. Evidence of an increased abundance of Bacteroidetes in the intestinal microbe composition has been reported during HCMV infection (114) and infants who acquire HCMV in early life have decreased microbiome alpha diversity associated with a heightened inflammation (115). The intestinal abundance of Bacteroidetes has been significantly correlated with a lack of seroresponses to Rotarix vaccine in Ghanian infants (116). Human microbiome colonization and immune development are intimately related and influence infant immune responses to vaccines (117), suggesting that HCMV may influence infant vaccine responses via its effect on the intestinal microbiome.

### 1.3.2 HCMV and childhood vaccines

HCMV infection exerts variable influence on childhood vaccine immunogenicity.

The profound impacts of HCMV on host cell immunity could affect vaccine immunogenicity as argued by Falconer et al (118). As summarised in Table 1-2, the few studies conducted by the time of this thesis investigated the influence of HCMV on measles, diphtheria, pertussis, tetanus (DPT), Haemophilus influenzae type B (Hib), meningococcal, polio, Hepatitis B and Bacille Calmette-Guérin (BCG) vaccines with conflicting findings (19-23). Importantly there were no studies that had investigated impact of HCMV on the observed immunogenicity of oral rotavirus vaccines. In Zambia, only a single study was identified which investigated impact of HCMV infection on infant antibody responses to oral polio vaccine (OPV) (24).

a) DPT, Hib and BCG vaccination

In The Gambia, no difference in IgG protective antibody responses to tetanus or Hib was observed between HCMV infected and uninfected infants in one study (19). However, in another study among Gambian infants, HCMV infected infants receiving DPT had lower

tetanus toxoid (Ttx) IgG titres compared to uninfected infant despite no apparent difference observed in attainment of protective Ttx IgG antibody levels (21). In this latter study, diphtheria toxoid (Dtx) antigen, pertussis antigens, pertussis toxoid (Ptx), Fimbriae (Fim), filamentous haemagglutinin (FHA) and Pertactin (Per) specific IgG responses were also not impacted by HCMV infection (21). Similarly among South African infants, no differences were observed in mean concentrations or seroprotective levels of IgG responses to Ttx, but also to Dtx, Ptx, FHA, Hib polyribosylribitol phosphate (PRP, capsular polysaccharide) between congenitally infected infants compared to congenitally uninfected controls, postnatally infected or postnatally uninfected (23). Interestingly, investigations of BCG associated and overall T-cell stimulation have revealed significantly lowered IL-4, GM-CSF, IL-12 and IL-10 cytokine responses in HCMV infected infants (21). HCMV infected infants also had reduced Ttx induced GM-CSF post DPT vaccination (21). In contrast, among infants in Botswana, while HCMV infection was also not associated with infant Tetanus IgG antibody titres, it had no impact on infant IFN-$\gamma$ and IL-2 cellular response to Tetanus or BCG vaccination (22).

b) Hepatitis B vaccination

In one study assessing HCMV influence on Hepatitis B vaccination in African infants neither congenital nor postnatal HCMV infection had any influence on infant seroprotective IgG levels against Hepatitis B surface antigen (HBsAg) post Hepatitis B vaccination (23).

c) Measles and rubella vaccination

HCMV has been associated with significantly lowered anti-measles CD4 IFN-$\gamma$ response in infants receiving measles vaccination and also lowered VEGF cytokine responses to measles antigen one month post vaccination (21). Surprisingly the HCMV specific IFN-$\gamma$ response is also reported to positively correlate with anti-measles haemagglutination inhibition (HAI) titres in some studies (19). This suggests a beneficial effect of HCMV on measles vaccine induced humoral responses but negative effect on cellular responses. Interestingly, HCMV is observed to counteract the negative influence of Epstein-Barr virus (EBV) on vaccine immunogenicity. Infants infected with EBV alone are observed to have nearly two-fold lower median HAI titres compared to EBV and HCMV uninfected infants, however, EBV-HCMV coinfection was linked to restored HAI titres to levels comparable to those observed in EBV and HCMV uninfected infants (20). In contrast, other studies have shown that HCMV infection prior to vaccination was not associated with anti-measles HAI IgG titres one month post vaccination and did not affect ability of infant to mount protective levels of antibodies against measles (21).

d) Meningococcal vaccination

No differences in meningococcal IgG antibody titres or IgG and IgM antibody titres at the time of vaccination or around the timing of vaccine response measurements two months post vaccination respectively have been observed between HCMV infected and uninfected children (20).

e) Oral polio vaccine

A single study which was done in Zambia investigated influence of HCMV on infant immune responses to oral polio vaccine (OPV) (24). No significant differences in polio antibody titres were observed between HCMV infected and uninfected infants. Of note however, HIV positive infants, with HCMV viremia had lower polio antibody responses compared to those without HCMV viremia while HIV unexposed-HCMV infected infants had increased antibody responses to OPV (24).

**Table 1-2. Influence of HCMV on childhood vaccine responses**

| Author, Country, year (Ref) | Child age[1] | Vaccine type | Summary key findings |
|---|---|---|---|
| Miles et al, The Gambia, 2008 (19) | 9, 13 and 18 months | Measles, DPT, Hib | • No significant difference in anti-measles CD8 T cells secreting IFN-γ and IL-2 by HCMV status.<br>• Significantly lowered median anti-measles CD4 IFN-γ in HCMV infected infants<br>• HCMV IFN-γ responses positively correlated to anti-measles HAI titres.<br>• No difference in Tetanus and Hib IgG antibody titres |
| Holder et al, The Gambia, 2010 (20) | 9 and 11 months | Measles and Meningococcal | • No difference in meningococcal IgG and IgM by HCMV infection before or after vaccination<br>• EBV mono infection lowered anti-measles HAI titre but co-infection with HCMV associated with higher HAI titres comparable to EBV-HCMV uninfected. |
| Cox et al, The Gambia, 2020 (21) , | 9 and 10 months | Measles and DPT | • No significant difference in measles HAI titres by HCMV status<br>• HCMV infection significantly lowered Tetanus IgG titres in males<br>• No difference in Diphtheria or Pertussis antigen specific IgG titres or ability to mount protective antibody levels by HCMV status.<br>• HCMV infection lowered BCG specific and overall T cell IL-4, GM-CSF, 1L-12, and IL-10, Eotaxin, TNF, TNF/IL-10 ratio cytokine responses and varied by sex. |

| | | | |
|---|---|---|---|
| | | | • HCMV infection lowered measles-induced VEGF in males, but increased measles-induced GM-CSF in females.<br>• HCMV infection lowered Tetanus-induced GM-CSF in males but enhanced IL-1β, PDGFBB and TNF in females |
| Smith et al, Botswana, 2020 (22) | 6 and 18 months | DPT, BCG | • No association of HCMV infection with Tetanus IgG titres<br>• No association of HCMV infection with IFN-γ and IL-2 cellular response to Tetanus or BCG vaccination |
| Pathirana et al, South Africa, 2021 (23) | 7 months | DPT, Hib, HepB | • No difference in mean concentrations or seroprotective IgG levels to Diphtheria, Tetanus, Pertussis, Hepatitis B or Hib by HCMV status |
| Sanz-Ramos et al, Zambia, 2013 (24) | 18 months | Oral polio vaccine | • Overall, no significant association between HCMV DNAemia or IgG seropositivity and polio neutralising antibody titres.<br>• Lower polio neutralising antibody titres in HIV infected infants with HCMV viremia but did not reach significance.<br>• Evidence of increased polio neutralising antibody titres in HCMV infected HIV unexposed infants |

Abbreviations: BCG (Bacille Calmette-Guérin), DPT (Diphtheria, Pertussis, Tetanus containing vaccine), EBV (Epstein-Barr Virus), GM-CSF, (Granulocyte macrophage colony-stimulating factor), HAI (haemagglutination inhibition), HCMV (Human cytomegalovirus), Hib (Haemophilus influenzae type B), HIV (Human Immunodeficiency Virus), IFN-γ (Interferon gamma), IgG (immunoglobulin G), IL-1β (Interleukin 1 beta), (IL-2 (Interleukin 2), IL-4 (Interleukin 4), IL-10 (Interleukin 10), IL-12 (Interleukin 12), PDGFBB (platelet-derived growth factor-BB),TNF (Tumour Necrosis Factor), VEGF (Vascular endothelial growth factor) [1]Child age at the time of vaccine immune response assessment

Concluding remarks

HCMV is prevalent among infants residing in Africa and infection can modulate infant T cell immune profiles and is linked to altered intestinal environments . The ambiguity of results from studies conducted to date and the absence of data for ORV signal the need for additional research. To fully understand the observed rotavirus vaccine immunogenicity in African settings, all possible predictors such as HCMV infection around the time of oral vaccination must be studied. Such investigations may provide important insights for existing vaccines or implementation of future rotavirus vaccines.

## 1.4 Human Coronavirus

Coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged during the conduct of the study. This thesis explored antibody responses to common cold HCoV and SARS-CoV-2 within the study cohort. Therefore, common cold HCoV and SARS-CoV-2 are summarily introduced in this chapter.

### Human Coronaviruses and the COVID-19 pandemic

Coronaviruses are a group of enveloped viruses of the order *Nidovirales* and *Coronaviridae* family that infect birds and mammals, including humans (119). These coronaviruses can rapidly mutate and recombine leading to novel types that can spread from animals to humans (120). The virion is comprised of an outer lipid membrane with associated structural membrane, envelope, and prominent protruding spike (S) proteins that encompasses a core of nucleocapsid protein complexed with a single-stranded ribonucleic acid (RNA) genome (121). The human coronaviruses (HCoV) NL63 and 229E in the Alphacoronavirus genus and OC43 and HKU1 of the Betacoronavirus genus have been known to commonly infect humans and primarily associated with the common cold and mild to moderate respiratory or gastrointestinal illness (119, 122). More pathogenic HCoV namely severe acute respiratory syndrome coronavirus (SARS-CoV), middle eastern respiratory syndrome coronavirus (MERS-CoV) and the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have emerged between 2002 to date and associated with severe and highly fatal respiratory disease (123).

Since the initial outbreak in China in late December 2019, the COVID-19 pandemic caused the death of 7 million people globally as of June 2024 (124). Zambia recorded its first confirmed cases of SARS-CoV-2 infection in March 2020 (125). Interestingly, early on and over the pandemic children made up the least proportion of COVID-19 infections and had reduced disease severity and mortality compared to adults (126) and in comparison to other global regions, Africa was among regions with the least recorded number of COVID-19 deaths (124). While SARS-CoV-2 infection was widespread, reasons behind the lower mortality were debatable (127) and sparked research into understanding HCoV and SARS-CoV-2 immunity. However, despite the occurrence of the common cold HCoV and SARS-CoV-2 in Southern Africa, seroprevalence estimates were limited (128) creating a knowledge gap in understanding the coronavirus epidemiology in the region.

The host immune response to HCoV infection is characterised by an initial innate interferon and pro-inflammatory cytokine response to limit viral replication followed by an adaptive response mainly comprising cytotoxic T-cells and antibodies to destroy virally infected cells and inhibit viral attachment respectively (119). The HCoV structural S protein is the major inducer of the host neutralizing antibodies targeted against its subunit 1 (S1) that mediates viral attachment and the subunit 2 (S2) involved in fusion and entry during infection (129, 130). HCoV and SARS-CoV-2 infections induce robust IgM, IgA and IgG, with comparatively more durable IgG responses (131). Most children are reported to seroconvert to HCoV during early childhood before 5 years old (132) but only a few studies have profiled kinetics of antibody responses (133). Moreover, the pre-existence of immunity to common cold HCoV was among hypothesis proposed to explain variations in SARS-COV-2 across populations (134) and between adults and children (135).

The common cold HCoV OC43 and HKU1 have higher overall protein sequence homology with SARS-CoV-2 than the Alphacoronaviruses 229E and NL63. All four viruses have >30% homology with SARS-CoV-2 spike (S) antigen, whereas only the Betacoronaviruses have notable homology within its receptor binding domain (RBD), the principle target of neutralizing antibodies (136). This pattern holds for S antigen T cell epitopes where circulating Betacoronaviruses have slightly increased homology with SARS-CoV-2 Spike antigen compared to Alphacoronaviruses (137). Studies have also reported cross-reactivity between the common cold HCoV and SARS-CoV-2 (131, 138). Therefore, knowledge on the natural immune responses differentiated according to these specific coronaviruses can be informative to SARS-CoV-2 specific or pan-coronavirus control.

This thesis addressed a critical COVID-19 related research gap by assessing the degree of pre-existing immunity to common cold HCoV and investigating cross-reactiveness to SARS-CoV-2 in the Zambian setting. The generated knowledge was envisioned to provide useful local knowledge to control of SARS-CoV-2 infection and other emerging HCoV.

## 1.5 Aims

This thesis aimed to address the issue of the underperformance of oral rotavirus vaccines in high burden low-income settings. The goal of the work detailed here was to contribute knowledge towards strategies that can be used to improve vaccine performance in three ways. The first was by evaluating the immune benefits of a booster Rotarix vaccine dose administered in later infancy for improved vaccine immunogenicity. The second was by characterising the T cell immune profiles associated with rotavirus vaccination to better comprehend the immune effectors induced in infants. The third was by interrogating the influence of HCMV, a lifelong infection, on infant immune profiles and response to oral rotavirus vaccination as a potential biological factor to explain the observed variation in vaccine performance across regions. Findings from the works described in this thesis were envisioned to contribute insights for (i) policymakers on the recommendations for rotavirus vaccine booster doses in high burden settings, (ii) the design of next generation vaccines and (iii) evidence basis on whether HCMV infection conferred a vulnerability for vaccine failure in rotavirus vaccinated infants. Post COVID-19 emergence, the thesis work aimed to explore coronavirus immunity to contribute to local coronavirus research efforts.

This thesis aimed to answer the following research questions in Zambian infants:

1. Could an additional third dose of Rotarix vaccine administered at 9 months infant age boost RV-IgA seroresponses?
2. What were the T cell profiles in rotavirus vaccinated infants and was there an association with vaccine seroconversion?
3. Was HCMV infection associated with diminished rotavirus vaccine immunogenicity?
4. What were the pre-existing antibody responses to common cold HCoV in infants and was there an association with SARS-CoV-2 responses during the COVID-19 pandemic?

<u>Specific objectives</u>

1. To measure RV-IgA antibodies in two versus three dose Rotarix vaccinated infants and assess boosting effect of the third dose.
2. To detect rotavirus specific T cell responses in vaccinated infants and investigate the association with vaccine seroconversion.

3. To determine HCMV serostatus around the time of rotavirus vaccination and investigate relationship with vaccine induced RV-IgA responses.
4. To measure common cold HCoV antibodies in infants before and during COVID-19 pandemic and explore its association with SARS-CoV-2 seropositivity.

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**Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.**

**<u>SECTION A – Student Details</u>**

| | | | |
|---|---|---|---|
| **Student ID Number** | 1402541 | **Title** | Miss |
| **First Name(s)** | Natasha Makabilo | | |
| **Surname/Family Name** | Laban | | |
| **Thesis Title** | Antibody and T-cell responses in rotavirus vaccinated Zambian infants: impact of human cytomegalovirus | | |
| **Primary Supervisor** | Martin Goodier | | |

**If the Research Paper has previously been published please complete Section B, if not please move to Section C.**

**<u>SECTION B – Paper already published</u>**

| | | | |
|---|---|---|---|
| Where was the work published? | MDPI Viruses | | |
| When was the work published? | 23rd February 2022 | | |
| If the work was published prior to registration for your research degree, give a brief rationale for its inclusion | N/A | | |
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## Chapter 2: T-cell responses after rotavirus infection or vaccination in children: a systematic review

This chapter contains a research article published in MDPI Viruses 2022

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## 2.1 Abstract

Cellular immunity against rotavirus in children is incompletely understood. This review describes the current understanding of T-cell immunity to rotavirus in children. A systematic literature search was conducted in Embase, MEDLINE, Web of Science, and Global Health databases using a combination of “t-cell”, “rotavirus” and “child” keywords to extract data from relevant articles published from January 1973 to March 2020. Only seventeen articles were identified. Rotavirus-specific T-cell immunity in children develops and broadens reactivity with increasing age. Whilst occurring in close association with antibody responses, T-cell responses are more transient but can occur in absence of detectable antibody responses. Rotavirus-induced T-cell immunity is largely of the gut homing phenotype and predominantly involves Th1 and cytotoxic subsets that may be influenced by IL-10 Tregs. However, rotavirus-specific T-cell responses in children are generally of low frequencies in peripheral blood and are limited in comparison to other infecting pathogens and in adults. The available research reviewed here characterizes the T-cell immune response in children. There is a need for further research investigating the protective associations of rotavirus-specific T-cell responses against infection or vaccination and the standardization of rotavirus-specific T-cells assays in children.

## 2.2 Introduction

Rotavirus is the leading cause of life-threatening diarrhoea among young children, particularly in those below five years of age (1, 2). Globally, rotavirus has been responsible for approximately 258 million diarrheal episodes and an estimated 128,515 diarrhoea deaths in this population with the largest burden within Sub-Saharan Africa (3). Fortunately, rotavirus vaccines are widely available and have significantly contributed to reductions in rotavirus-associated diarrheal morbidity and mortality globally (4-6). However, despite being discovered nearly half a century ago in 1973 and more than a decade since vaccine introduction, immune mechanisms, and correlates of protection against rotavirus remain poorly understood (7).

In humans, rotavirus is transmitted via a faecal-oral route and is known to predominantly infect and replicate in mature enterocytes of the intestinal epithelium inducing innate and adaptive humoral and cellular immune responses (8). In children, repeated rotavirus infection leads to a lower likelihood of subsequent rotavirus infections and reduced occurrence of moderate to

severe diarrheal disease suggesting the development of immune memory (9). This acquired, non-sterilizing immunity is derived from a combination of gut secretory and humoral antibody and cell-mediated immune effectors with neutralizing antibodies directed against the viral capsid proteins and viral epitope recognition by T-cells thought to play an important role in protection (8). However, immune parameters correlating with protection against rotavirus in humans are yet to be demonstrated (10).

Rotavirus-specific antibodies are well documented and frequently studied in children as immune markers of previous infection or vaccination (11). However, even though they are recognized as being important for protection, it is generally appreciated that these antibody markers are sub-optimal correlates of protection (12, 13). In contrast, there is sparse data on the underlying T-cell immune responses to rotavirus infection or vaccination, particularly in children, and even fewer still have studied the role of this T-cell immunity in protection against rotavirus. The current understanding of rotavirus T-cell mediated immunity has for the most part been achieved through studies in animal models which have shown that T-cells have crucial roles in suppression of rotavirus replication, clearance of infection, and generation of antibody responses associated with protection (10, 14-16).

As rotavirus remains a cause of high morbidity and mortality in children, especially in the developing world (3), it is necessary to fully understand the immune mechanisms underlying protection. Improved knowledge on T-cell-mediated rotavirus immunity can inform vaccine development and is particularly important considering the sub-optimal antibody immune correlates and the consistent observation of markedly lower vaccine immunogenicity and efficacy in children within high rotavirus burden regions (17). We, therefore, conducted a systematic review of literature on T-cell responses to rotavirus in children to consolidate currently available knowledge on the characteristics of T-cell immunity to rotavirus in this population including its association with the antibody responses.

## 2.3 Methods

### Literature Search Strategy

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) checklist (Supplementary Table S2-1) in the preparation of the systematic review manuscript (18). The literature search was conducted in Embase (1947 to March 2020), MEDLINE (1946 to March 2020), Web of Science (1970 to 2020), and Global Health (1910 to

week 9 2020) electronic databases using a combination of “T-cell”, “rotavirus” and “child” keywords to identify relevant articles.

Inclusion Criteria

Studies included in this review were those that were primary research, were conducted among children or used child-derived samples in any region of the world, reported T-cell immune responses to rotavirus, had full English text available and had rotavirus as the focus of the study. There was no restriction to study design, but we restricted selection to articles published after 1973, the year rotavirus was discovered.

Exclusion Criteria

We excluded studies that did not include children or child-derived samples, did not report T-cell responses against rotavirus, or had no English full text available. Non-primary research including review articles and conference abstracts were also excluded.

T-Cell Responses

The T-cell responses considered in the systematic review were T-cell quantity (counts, ratios, frequencies), phenotype (activation, cell surface markers, epitopes) function (cytokine secretion), activity (proliferation), and kinetics (pre- and post-infection or vaccination, durability) for all CD4 and CD8 T-cells subsets.

Study Selection and Data Extraction

EndNote reference manager software was used to remove duplicate articles identified from the search strategy. The resulting unique articles were imported into Rayyan web-tool software for additional duplicate identification and article selection. Three reviewers (NML, CC, MS) independently selected potentially eligible articles by screening the title and abstract of all unique articles for the keywords using the Rayyan web-tool software. Full texts of articles selected by all three reviewers combined were retrieved and assessed for eligibility using the inclusion and exclusion criteria. Articles concordantly selected as eligible by the three reviewers were included in the review and those concordantly rejected were excluded from the review. Discordance in selection was discussed and reassessed together by all three reviewers until a consensus on inclusion or exclusion was made. Data were extracted into an excel sheet to capture information on the author, year of publication, study location, study design, characteristics of the child population, sample size, rotavirus context (rotavirus

infection or vaccination), T-cell responses, and laboratory methods used for measures of T-cell immunity.

Quality Assessment and Data Synthesis

We reviewed published articles of similar nature to our systematic review to identify potential appraisal tools and we adapted a recently published quality assessment tool (19) and quality level thresholds (0% to 39% = low, 40% to 69% = moderate, and 70% to 100% = high) (20) for our critical appraisal (Supplementary Table S2-2). One author (NML) conducted the quality assessment which was reviewed by two other authors (SB and ONC). Due to the wide heterogeneity in laboratory methodology and reported T-cell response across the studies included in the systematic review, formal quantitative meta-analysis was not conducted, and results were presented in a thematic narrative format.

## 2.4 Results

Literature Search Results

Articles retrieved from the literature search comprised 937 from Embase, 465 from MEDLINE, 574 from Web of Science, and 125 from Global Health electronic databases giving a total of 2101 articles identified. After the removal of 906 duplicate articles, a resulting total of 1195 articles were screened for eligibility based on title and abstract and an additional 1162 articles were excluded because they were non-primary research (n = 710), were not about rotavirus in humans (n = 288,) did not include children (n = 96), did not report T-cell responses (n = 72). The remaining 33 articles underwent further screening for eligibility by full text based on set inclusion criteria. After full-text screening, a further 16 articles were excluded because they did not have full text available to the reviewers (n = 2), did not report T-cell responses for children (n = 10), and rotavirus was not the focus (n = 4). This resulted in 17 articles that met the inclusion criteria and were included in the systematic review as summarized in Figure 2-1.

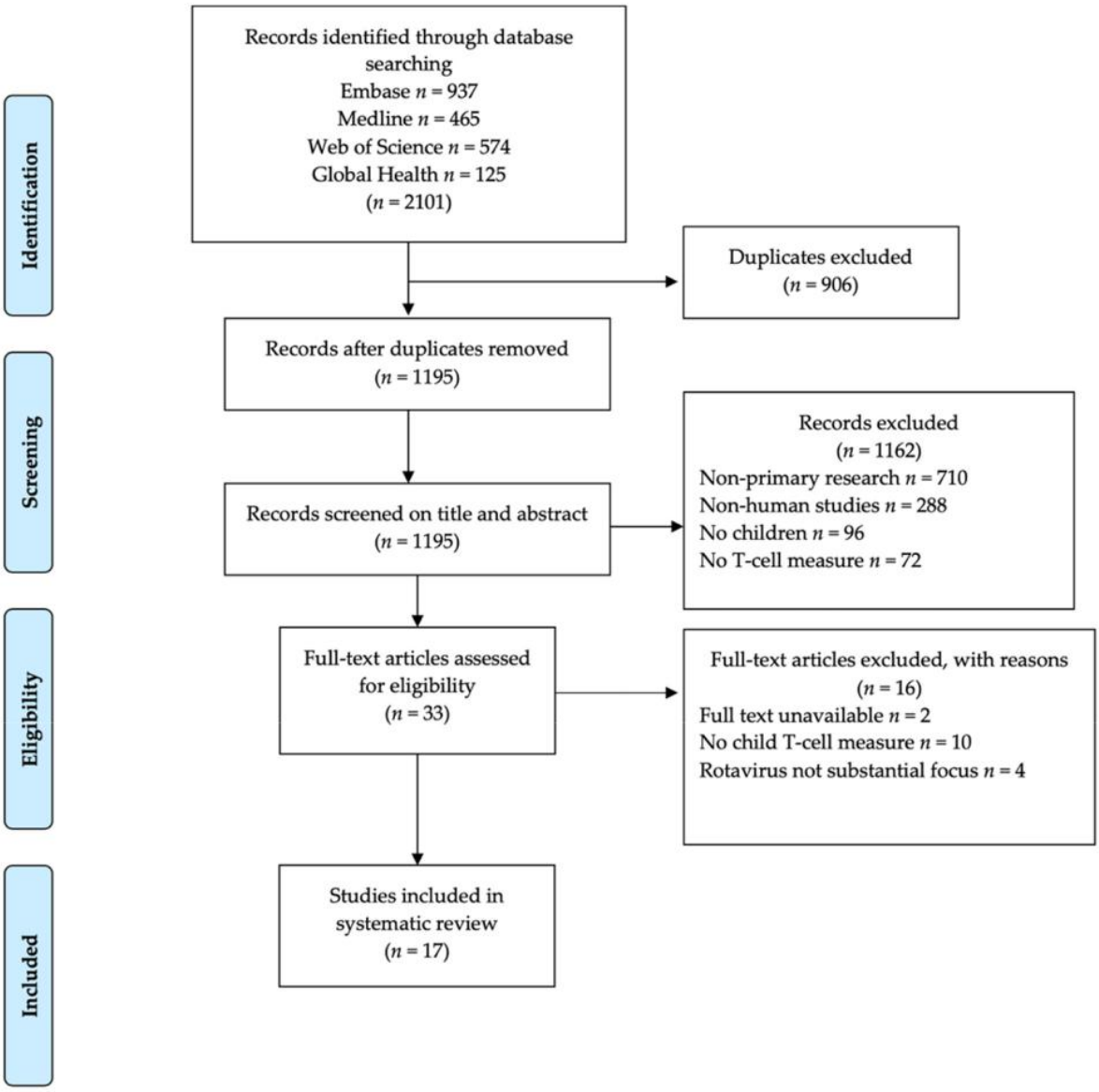


**Figure 2-1. Flow chart of literature search results and article selection process.**

Characteristics of Articles Included in Systematic Review

Among the seventeen studies included in the systematic review, the earliest study identified was published in 1988 and the latest in 2018. Most of the studies were conducted among children in the Americas (9/17) followed equally by Europe (3/17) and Asia (3/17) while the least number of studies (2/17) was conducted among African children. Ten studies reported T-cell immunity in the context of rotavirus infection, two studies reported T-cell responses to rotavirus vaccination, and five studies reported rotavirus-specific T-cell response in healthy children. Laboratory methods used to measure T-cell responses varied across studies and included flow cytometry, lymphoproliferation, microscopy, indirect fluorescence microscopy, gene microarray, and enzyme-linked immunospot (ELISpot) assays. Different types of T-cell

outcomes in response to mitogen, human rotavirus, and non-human rotavirus antigens were reported across studies. More detailed characteristics of the studies included in the systematic review are as outlined in Table 1.

**Table 2-1. Characteristics of studies included in the systematic review.**

| Author Year [Ref] | Country | Design | Child Population, n | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| Dong et al. 2015 **(21)** | China | Observational | RV-AGE, n = 102;<br>Healthy, n = 30 | 3 mos to 3 yr.<br>2 mos to 3 yr. | Rotavirus infection | PMA Ionomycin | Flow Cytometry | • Treg ($CD4^+CD25^+$)<br>• Th17 ($CD4^+$ $IL\text{-}17^+$) |
| Elaraby et al. 1992 **(22)** | Egypt | Observational | RV-AGE, n = 6.<br>Healthy, n = 50 | NR.<br>New-born, 1 to <12 mos, 12 to 24 mos, 24 to 60 mos | Rotavirus infection | Rotavirus antigen, PHA | Light microscopy, Indirect Fluorescent Microscopy | • Stimulation Index, positive > 1.5<br>• helper/suppressor (CD4:CD8) ratio<br>• CD3, CD4, CD8 subsets |
| Iwasa et al. 2008 **(23)** | Japan | Observational | RV-AGE, n = 1 | 6 mos | Rotavirus infection | Nil | Flow Cytometry | • $CD4^{+/}CD8^+$ IFN-$\gamma^+$<br>• $CD4^+/CD8^+$<br>• $CD4^{+/}CD8^+$ ratio |
| Jaimes et al. 2002 **(24)** | Colombia | Observational | RV-AGE, n = 12 | 6 mos to 7 yr. | Rotavirus infection | RRV, SEB, CD28, CD49d | Flow Cytometry | • CD4/CD8 $CD69^+$ IFN-$\gamma^+$,<br>• CD4/CD8 $CD69^+$ $IL\text{-}13^+$ |
| Makela et al. 2006 **(25)** | Finland | Observational | Healthy (T1D at risk), | ≤15 years | N/A | HRV Wa, BRV NCD, CBV, PPD, TT, PHA, PCB | Proliferation assay, PCR | • Stimulation Index, positive ≥ 3 |

| Author Year [Ref] | Country | Design | Child Population, n | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| | | | n = 183 | | | | | • IFN-$\gamma^+$, IL-$4^+$, IL-$10^+$ and TGF-$\beta^+$ PBMC gene expression |
| Makela et al. 2004 **(26)** | Finland | Observational | Healthy children (T1D at risk), n = 20 | 3 mos to 5 yr. | N/A | HRV Wa, BRV NCD, PPD, TT, PHA | Proliferation assay | • Stimulation Index, positive ≥ 3 |
| Mesa et al. 2010 **(27)** | Colombia | Observational | RV-AGE, n = 17. Non-RV-AGE, n = 36 | Median 14 mos (range 4 to 22 mos) | Rotavirus infection | HRV Wa, Simian RRV, SEB, CD28, CD49d | Flow Cytometry | • $CD4^+$ and $CD8^+$<br>• $CD4^+/CD8^+$ IL-$2^+$, IL-$10^+$, IL-$13^+$, IL-$17^+$, IFN-$\gamma^+$<br>• $CD4^+CD25^+$,$CD4^+CD25^+CD127^{low}$, $CD4^+CD25^+CD127^{low}$ TGF-$\beta^+$ |
| Offit et al. 1992 **(28)** | USA | Observational | Healthy, n = 48 | New-born to 18 yrs. old | N/A | HRV Wa, HRV HCR3a strains, | Proliferation assay | • Stimulation Index, positive ≥ 3:1 |

| Author Year [Ref] | Country | Design | Child Population, n | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Simian RRV, concanavalin A | | |
| Offit et al. 1993 **(29)** | USA | Observational | RV-AGE, n= 8 | <2 yrs. old. | Rotavirus infection | HRV HCR3a, HRV W179 | Proliferation assay | • Stimulation Index, positive ≥ 3:1 |
| Parra et al. 2014 **(30)** | Colombia | Observational | Healthy, n = 5 | 2 to 8 yrs. old | N/A | Simian RRV, Influenza vaccine, TT, SEB, CD28, CD49d | Flow Cytometry, Proliferation assay | • $CD4^+/CD8^+$ $IL\text{-}2^+$ $IFN\text{-}\gamma^+$, $TNF\text{-}\alpha^+$<br>• IFN-γ, TNF-α, GM-CSF, RANTES MCP-1 and IL-10, IL-4, IL-6, IL-17A, IL-9, and IL-2 secreting PBMC<br>• $CD4^+$ and $CD8^+$ proliferation |
| Parra et al. 2014 **(31)** | Colombia | Randomised Controlled Trial | Vaccine, n = 35 | 2 to 4 mos | Rotavirus vaccination | RRV, NSP2, VP3-4, VP6-7, SEB, CD28, CD49d | Flow Cytometry | • VP6-7 $tetramer^+$ $CD62L^-$ $CD45RA^{+/-}$ and $CD62L^+$ $CD45RA^-$ $CD4^+$, |

| Author Year [Ref] | Country | Design | Child Population, n | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| | | | Placebo, n = 24 | | | | | • Gut homing ($\alpha4\beta7^+$ and $CCR9^+$) VP6-7 $tetramer^+$ $CD4^+$ |
| Rojas et al. 2003 **(32)** | Colombia | Observational | RV-AGE, n= 15. Non-RV-AGE, n = 13 | 3 mos to 7 yrs. | Rotavirus infection | RRV, SEB, CD28, CD49d | ELISpot | • IFN-$\gamma^+$, IL-$4^+$ secreting PBMC, • IFN-$\gamma^+$, IL-$4^+$ secreting $CD4^+$ and CD8 |
| Rott et al. 1997 **(33)** | USA | Observational | RV-AGE, n = 1 | NR | Rotavirus infection | RRV, concanavalin A | Flow Cytometry, Proliferation assay | • $\beta7^+$ and $\beta7^-$ PBMC • Stimulation Index |

| Author Year [Ref] | Country | Design | Child Population, n | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| Wang et al. 2007 **(34)** | USA | Observational | RV-AGE, n = 10; Healthy, n= 8 | <3 yr. | Rotavirus infection | Nil | Flow Cytometry PCR | • $CD4^+/\alpha\beta CD4^+$, and $CD8^+/\alpha\beta CD8^+$,<br>• $CD4^+/CD8^+$ $CD69^+$, and $CD4^+/CD8$ $CD83^+$<br>• CD1C, CD2, CD3D, CD28, CD96, CD2, αβ TCR, Lck and Lck substrate, LAT, SLP-76, IL-16, CD27, IL-17R, IL-27Rα, IL-7R, RP1, LIGHT, and MAL gene expression |
| Weinberg et al. 2018 **(35)** | Botswana, Tanzania, Zambia, Zimbabwe | Randomised Controlled Trial | Vaccine, n = 42; Placebo n = 47 | 2 to ≤15 wks. | Rotavirus vaccination | Nil | Flow Cytometry | • $CD4^+$<br>• $CD4^+$ $IL\text{-}10^+$<br>• $CD4^+FOXP3^+CD25^+$ $CD8^+FOXP3^+$ $CD25^+$ |
| Wood et al. 1988 **(36)** | England | Observational | RV-AGE, n = 2 | New-born and 11 mos | Rotavirus infection | Simian RRV SA11strain, PHA | Proliferation assay | • Stimulation Index, positive > 2, T-cell frequency |

| Author Year [Ref] | Country | Design | Child Population, n | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| Yasukawa et al. 1990 **(37)** | Japan | Observational | Healthy child, n = 1 | New-born | N/A | HRV Wa strain, BRV NCD strain | Proliferation assay | • Scintillation count/minute |

Abbreviations: α4β7 = alpha 4 beta 7. β7 = beta 7. BRV = bovine rotavirus. CBV = Coxsackie B4 virus. CCR9 = C-C motif chemokine receptor 9. CD1C = cluster of differentiation 1C. CD2 = cluster of differentiation 2. CD3 = cluster of differentiation 3. CD3D = cluster of differentiation 3D. CD4 = cluster of differentiation 4. αβCD4 = alpha beta cluster of differentiation 4. CD8 = cluster of differentiation 8. αβCD8 = alpha beta cluster of differentiation 8. CD25 = cluster of differentiation 25. CD27 = cluster of differentiation 27. CD28 = cluster of differentiation 28. CD45RA = cluster of differentiation 45RA. CD49d = cluster of differentiation 49d. CD62L = cluster of differentiation 62L. CD69 = cluster of differentiation 69. CD83 = cluster of differentiation 83. CD96 = cluster of differentiation 96. CD127 = cluster of differentiation 127. CD28 and CD49d were used as co-stimulators. ELISpot = enzyme linked Immunospot. FOXP3 = Forkhead box protein P3. GM-CSF = granulocyte-macrophage colony-stimulating factor. HCR3a = human cytopathic rotavirus 3a. HRV = human rotavirus. IFN-γ = interferon gamma. IL2 = interleukin 2. IL4 = interleukin 4. IL6 = interleukin 6. IL7R = interleukin 7 receptor. IL9 = interleukin 9. IL10 = interleukin 10. IL13 = interleukin 13. IL16 = interleukin 16. IL17 = interleukin 17. IL17R = interleukin 17 receptor. IL27R α = interleukin 27 receptor alpha subunit. LAT = linker for activation of T cells. Lck = lymphocyte-specific protein tyrosine kinase. LIGHT = tumor necrosis factor superfamily member 14. MAL = myelin and lymphocyte protein. MCP1 = monocyte chemoattractant protein 1. Mos = months. N/A = not applicable. NCD = Nebraska Calf Diarrhoea. NR = not reported. NSP2 = non-structural protein 2. PBMC = peripheral blood mononuclear cells. PCB = purified Coxsackie B4 virus. PCR = polymerase chain reaction. PHA = phytohemagglutinin. NR = not reported. PMA = phorbol myristate acetate. PPD = tuberculin purified protein derivative. RANTES = regulated on activation, normal T-cell expressed and secreted. RP1 (synonym MAPRE2) = microtubule associated protein RP/EB family member 2. RRV = rhesus rotavirus. RV-AGE = rotavirus acute gastroenteritis. SEB = staphylococcal enterotoxin B. SLP-76 = Src homology 2 domain-containing leukocyte protein of 76 kilodalton. TCR = T-cell receptor. αβTCR = alpha beta T-cell receptor. T1D = type 1 diabetes. TGF-β = transforming growth factor beta. Th17 = T helper 17. TNF-α = tumor necrosis factor alpha. Treg = regulatory T-cell. TT = Tetanus Toxoid. USA = United States of America. Yrs. = years. VP6-7 = viral protein 6-7

Quality Assessment of Individual Studies

Of the included studies, 15/17 (88.2%) were observational studies while only 2/17 (11.8%) made use of experimental designs in the form of randomized controlled trials (Table 2-1). Using our adapted appraisal tool and threshold definitions of study quality, most articles were of moderate quality 11/17 (65%). The remaining 6/17 (35%) articles were appraised as high-quality articles of which the majority 5/6 (83%) were published in more recent years (Table 2-1). Most studies included in the review provided adequate information on research gaps around immunity to rotavirus, including research questions and rationales for the study of T-cell-specific responses to rotavirus. However, there was generally poor methodological reporting for most studies with minimal to no detailed information provided on the exact study design employed, calculations, and assumptions for stated samples sizes or specification of inclusion and exclusion criteria for children or child-derived samples included in the studies. In most studies, there was also a poor presentation of participant or sample flow from recruitment to laboratory testing results as well as little to no information on children's background characteristics (Supplementary Table S2-2).

T-Cell Proliferation against Rotavirus Develops and Broadens Reactivity with Increasing Age

Children can mount detectable T-cell proliferation to different strains of rotavirus after in-vitro stimulation which is associated with age. As shown in Table 2-2, six studies reported induction of T-cell proliferation against human and non-human rotavirus strains and its relationship with the child's age. Children with acute rotavirus diarrhoea had more positive and significantly higher T-cell proliferation to rotavirus antigen compared to healthy children. Among healthy children, T-cell proliferation was absent in new-borns, minimally present in children aged <6 months but became more commonly detected in older age groups of children (22, 25, 26, 28, 37). In contrast to this, however, one study also reported evidence of detectable T-cell proliferation in new-born children (28). In healthy children, although T-cell proliferation to a human rotavirus strain was observed to be stronger than that against a bovine rotavirus strain, a positive correlation of T-cell reactivity was observed between the strains (26). By the age of 2 years old and beyond, most children had developed T-cell reactivity against two strains of human rotavirus and against rhesus rotavirus strains (28). However, T-cell proliferation against two different human rotavirus strains has also been observed among children aged <2 years old with acute and convalescent rotavirus diarrhoea caused by different infecting rotavirus strains (29)

**Table 2-2. Relationship between rotavirus T-cell proliferation and child age.**

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Elaraby et al. 1992 (22) | RV-AGE: (n = 6), age NR<br>Healthy: birth (n = 14), 1 to <12 mos (n = 14), 12 to <24 mos (n = 10), and 24 to <60 mos (n = 12). | Lymphoproliferation against rotavirus antigen (strain NR) | • Lymphoproliferation in all 6/6 (100%) children with RV-AGE versus 18/50 (36%) in healthy children.<br>• No lymphoproliferation in newborns but increasing lymphoproliferation in older age groups from 2/14 (14%) in 1 to <12 mos., to 5/10 (50%) in 12 to <24 mos. and up to 11/12 (92%) in 24 to <60 mos. age groups.<br>• Mean (SD) lymphoproliferation lowest at birth 1.11 (0.16) and in the 1 to <12 mos. age group 1.08 (0.22), increased to 1.5 (0.72) in the 12 to <24 mos. age group and highest in the 24 to <60 mos. age group at 3.58 (1.66) | • Rotavirus is an effective T-cell inducer.<br>• T-cell immunity to rotavirus increases with age. |

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Makela et al. 2004 (26) | Healthy: (n = 20), 3 months to 60 months age: sampled 3 mos to 6 mos (n = 23), 9 mos to 12 mos (n = 26), 15 mos to 24 mos (n = 65), 27 mos to 36 mos (n = 38), 39 mos to 48 mos (n = 31), 51 mos to 60 mos (n = 11) | Lymphoproliferation against bovine NCD (P serotype 6, G serotype 6) and human purified and lysate Wa (P serotype 1, G serotype 1A) rotavirus strain | • Lymphoproliferation against both human and bovine rotavirus antigens are more common with increasing age (NS, Fisher's exact test)<br>• Positive correlation between lymphoproliferation against bovine and human lysate rotavirus ($p < 0.0001$, $r_s = 0.52$, Spearman correlation test) and between bovine and purified human rotavirus $p < 0.0001$, $r_s = 0.56$, Spearman correlation test) | • T-cell immunity to rotavirus increases with age and is cross-reactive |
| Makela et al. 2006 (25) | Healthy: (n = 183), age range 3.5 years to 11.3 years | lymphoproliferation against human Wa (P serotype 1, G serotype 1A) and bovine NCD (P serotype 6, G serotype 6) rotavirus strains | • Lymphoproliferation positively correlated with age for human ($r = 0.32$, $p < 0.0001$) and bovine ($r = 0.20$, $p = 0.001$) rotavirus | • T-cell immunity to rotavirus increases with age and is cross-reactive |
| Offit et al. 1992 (28) | Healthy: age groups new-borns (n = 11), 16 days to <6 | lymphoproliferation against human Wa (serotype 1) and | • Few 1/11 (9%) children aged <6 mos. had lymphoproliferation against | • T-cell immunity to rotavirus increases |

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | mos (n = 11), 6 mos to <2 yrs. (n = 8), 2 yrs. to 5 yrs. (n = 8), 5 yrs. to 18 yrs. (n = 10). | HCR3a (serotype 3) and simian rhesus rotavirus strain 2 (serotype 3) antigens | human rotavirus but unexpectedly 4/11 (36%) newborns showed lymphoproliferation against both human and simian rotavirus antigens.<br>• In contrast, 6/8 (75%) and 4/8 (50%) children aged between 6 mos. to 2 yrs. and 10/13 (77%) and 6/16 (38%) aged between 6 mos. to 5 yrs. had lymphoproliferation against human rotavirus and simian rotavirus antigens respectively.<br>• In children aged >5 yrs. old, ~80% had lymphoproliferation against both human and simian rotavirus antigens. | with age and is cross-reactive.<br>• T-cell immunity to rotavirus may occur at birth due to maternal transfer or in-utero rotavirus exposure |
| Offit et al. 1993 (29) | RV-AGE: n = 8, <2 yrs. caused by serotype 1 (P type 1, G type 1, n = 2), serotype 3 (P type 1, G type 3, n = 3) and serotype 4 (P type 1, G type | Lymphoproliferation against human WI79 (P-type 1, G type 1) and HCR3a (P-type non-human, G type 3) rotavirus strain antigens | • During the acute stage, a few 1/8 (13%) children had lymphoproliferation against WI79 rotavirus antigen. | • T-cell immunity is present during acute and convalescent rotavirus infection. |

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | 4, n = 3) rotavirus strains and followed up in convalescence and late convalescence | | • In contrast during convalescence most 6/8 (75%) $p < 0.05$ and 5/6 (83%) $p < 0.05$ children had lymphoproliferation against WI79 and HCR3a rotavirus strains respectively; 4/6 (67%) had lymphoproliferation to both strains, 1/6 (17%) to only HCR3a, 2 to only WI79, while no reactivity to either strain was observed in 1/6 (17%) children studied. At late convalescence, all 4/4 (100%) children studied had lymphoproliferation.<br>• No proliferative response specific for the G type of the infecting rotavirus strain in either convalescent or late convalescent children was observed. | • T-cell immunity to rotavirus is not G-type specific and may recognize T-cell epitopes shared by different rotavirus strains |

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Yasukawa et a. 1990 (37) | Healthy full-term new-born | Lymphoproliferation against human Wa (serotype 1) strain rotavirus antigen | • Lymphoproliferation against human rotavirus antigen absent in the newborn | • T-cell immunity to rotavirus occurs in an antigen-specific manner |

Abbreviations: HCR3a = human cytopathic rotavirus 3a. mos. = months. NCD = Nebraska Calf Diarrhoea. NR = not reported. NS = not statistically significant. p = probability value. RV-AGE = rotavirus acute gastroenteritis. r = Pearson's correlation coefficient. $r_s$ = Spearman's rank correlation coefficient. SD = standard deviation. Yrs. = years

Rotavirus T-Cell Proliferation and Frequency Coincides with Antibody Responses but Is More Transient

Six studies reported T-cell immunity in association with rotavirus antibody responses as shown in Table 2-3. T-cell responses were observed more frequently in rotavirus antibody seropositive than seronegative children and among secondary than primary infections indicating that both memory T-cell and antibody responses are induced by rotavirus exposure and built from repeated exposure (25, 26, 31). The strength and magnitude of T-cell responses occurred in very close association with the antibody response. Makela et al. showed that generally, lower antibody titres to rotavirus were accompanied by minimal or absent T-cell responses while increased antibody responses were associated with stronger T-cell responses. However, strong T-cell immunity was also observed in the absence of increasing antibody responses in a single child in this study and although firm conclusions cannot be made based on this lone observation, it highlights the need to detect both antibody and cellular responses in assessing rotavirus immunity (26). Compared to antibodies that persisted long after infection, T-cell responses were more transient, detectable two to eight weeks and three to five months post-infection but declining as early as 5 months to nearly undetectable levels within 12 months post rotavirus exposure (26, 29). However, both T-cell and antibody responses were minimal during acute rotavirus infection but more frequent during convalescence (29). Unlike antibodies present at birth, T-cell immunity was generally absent in early infancy (<6 months) developing much later in infancy and may therefore be a better indicator of active infant immunity than antibodies and distinguish from passive maternal immunity in the very young infants (28). Both T-cell and antibody responses can be mounted against different infecting rotavirus strains indicating an inability to clearly distinguish rotavirus P and G serotypes (29). Rotavirus-specific CD4 T-cells are positively associated with antibody responses, while regulatory T-cells may either have a positive or negative association with the antibody response to rotavirus (35). One study among T-cell deficient children further emphasized intimate associations between T-cell immunity and antibody response in the context of clearance of rotavirus infection. Wood et al. described chronic rotavirus infection in two children with congenital T-cell deficiency (36). In a child with cartilage hair hypoplasia associated T-cell deficiency and acute rotavirus diarrhoea, no serum antibody immune response to rotavirus was detected. Likewise, no significant proliferative response to rotavirus was observed ~1 year after the onset of diarrhoea and diarrhoea persisted over an 18-month period characterized by poor weight gain and failure for the child to thrive despite treatment. In the same study, a second child with CHARGE congenital abnormalities and DiGeorge syndrome associated T-cell deficiency who was infected with rotavirus, the rotavirus IgG antibody response was undetectable two months after rotavirus infection and despite treatment, this child failed to thrive and died at 5 months old.

**Table 2-3. Rotavirus T-cell proliferation, frequencies, and phenotypes in relation to an antibody response.**

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Makela et al. 2006 (25) | Healthy: rotavirus IgA and/or IgG seropositive (n = 112) or rotavirus seronegative (n = 41) | Lymphoproliferation and IFN-γ producing PBMC against purified and lysate human and bovine rotavirus antigens | • Seropositive children had more frequent lymphoproliferation 50/112 (45%) than seronegative 4/41, 10% children ($p < 0.0001$) and stronger lymphoproliferation against purified ($p = 0.010$), lysate ($p = 0.0031$) human rotavirus and bovine rotavirus ($p < 0.0001$)<br>• Seropositive children had higher IFN-γ producing PBMC compared to seronegative children ($p = 0.084$) | • Prior exposure to rotavirus induces both memory T-cell and B-cell immunity in children. |
| Makela et al. 2004 (26) | Healthy: rotavirus IgA and/or IgG seropositive or seronegative at 3 mos | Lymphoproliferation against purified and lysate human rotavirus | • Minimal or absent proliferation in children with low rotavirus antibody titers. | • T-cell immunity occurs in tight association with rotavirus antibody response. |

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | to 12 mos of age with primary (n = 19) or secondary (n = 5) rotavirus infections | | • Increase in antibody titers accompanied by stronger lymphoproliferation against lysate and purified human rotavirus (p = 0.017 and p = 0.027, respectively, Wilcoxon test) and more positive lymphoproliferation in 9/24 (37.5%) cases. In contrast, one child had lymphoproliferation without a simultaneous increase in rotavirus antibody titers.<br>• Lymphoproliferation more frequent in secondary infections than primary infections (NS)<br>• Rotavirus-specific antibody levels remained elevated throughout follow-up after rotavirus infection but lymphoproliferation declined | • T-cell immunity can occur in absence of detectable increasing antibody response.<br>• More persistent and stronger T-cell immunity develops after repeated rotavirus exposure.<br>• Unlike antibodies, T-cell immunity to rotavirus is transient. |

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | shortly after infection and was detectable less than 12 months after primary infection (mean 5 months) | |
| Offit et al. 1992 (28) | Healthy: age groups new-borns (n = 11), 16 days to <6 mos (n = 11), 6 mos to <2 yrs. (n = 8), 2 yrs. to 5 yrs. (n = 8), and 5 yrs. to 18 yrs. (n = 10) with rotavirus neutralising antibody | Lymphoproliferation against human and simian rotavirus | • More newborns and children < 6 mos. had neutralizing antibodies against at least one rotavirus strain than lymphoproliferation.<br>• In contrast, among older age groups between 6 mos. and 18 yrs., most children had both lymphoproliferation and rotavirus neutralizing antibodies to at least one human or simian rotavirus strain. | • Development of T-cell immunity to rotavirus occurs in conjunction with the development of antibody responses in children.<br>• In young infants aged <6 mos. measurement of T-cell immunity is possibly more reliable in discriminating active from a passively acquired immune response |

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | | • Both T-cell and antibody immunity induced by rotavirus in children can be cross-reactive |
| Offit et al. 1993 (29) | RV-AGE: caused by P-type 1 and different G type strains followed up in convalescence and late convalescence with rotavirus IgA and neutralizing antibodies (n = 8) | lymphoproliferation against human rotavirus | • Neutralizing antibodies were mounted against different P and G serotype infecting rotavirus strains and similarly, lymphoproliferation was also mounted against different infecting G serotypes strains | • Both rotavirus specific neutralizing antibody and T-cell immunity in children may not clearly distinguish P and G infecting serotypes |
| Parra et al. 2014 (31) | Rotavirus IgA seropositive vaccinated (n = 35) | Frequency of CD4 T-cells positive for rotavirus | • Vaccinated seropositive children had a higher frequency of VP6-7 tetramer-positive activated CD4 T- | • Rotavirus-specific antibody responses to vaccination are accompanied by |

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | and seronegative placebo (n = 24 | specific VP6-7 T-cell epitope | cells (40–71%) than placebo seronegative children (0–8%) | rotavirus-specific CD4 T-cells in children. |
| Weinburg et al. 2018 (35) | PHEU and PHIV (n = 42) vaccinated with pentavalent live rotavirus vaccine: IgA and neutralizing IgG | Frequency of several CD4 and CD8 T-cell phenotypes | • Higher CD4 T-cell frequency and counts marginally and significantly associated with higher IgG neutralizing antibodies to 3/5 viral strains tested.<br>• Higher frequencies of $CD4^+FOXP3^+$ $CD25^+$ and $CD8^+$ $FOXP3^+$ $CD25^+$ regulatory T-cells were marginally or significantly ($p < 0.1$) associated with higher rotavirus IgG neutralizing antibodies to 4/5 viral strains in the RV5 vaccinated and significantly associated with higher IgA antibodies. These | • Rotavirus CD4 T-cells are induced in positive association with the antibody response to vaccination.<br>• $FOXP3^+$ $CD25^+$ regulatory CD4 and CD8 T-cells may positively influence antibody responses by the protection of B cells against intense activation and apoptosis while $IL10^+$ regulatory CD4 T-cells may negatively influence this response by |

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | associations remained at least marginally significant after adjustment for CD4 T-cell proportions.<br>• Significant negative correlations with antibody titers were observed for $CD4^+$ $IL10^+$ regulatory T-cells. | downregulation of immune responses via bystander mechanisms. |
| Wood et al., 1988 (36) | CHH (n = 1) and CHARGE associated (n = 1) T-cell deficiency and rotavirus IgG | Lymphoproliferation against mitogens, rotavirus antigen, and proportions of T-cells | • Poor lymphoproliferation and absent rotavirus specific IgG antibody response associated with persistent rotavirus diarrhea. | • Rotavirus-specific T-cell deficiency is associated with impaired antibody response and inability to clear rotavirus infection. |

Abbreviation: CD4 = cluster of differentiation 4. CD8 = cluster of differentiation 8. CD25 = cluster of differentiation 25. CHH = cartilage hair hypoplasia. CHARGE = coloboma, heart defects, atresia choanae growth retardation, genital abnormalities, and ear abnormalities. FOXP3 = Forkhead box protein P3. IFN-γ = Interferon gamma. IgA = Immunoglobulin A. IgG = Immunoglobulin G. IL1–10 = Interleukin 10. mos. = months. NS = not significant. PBMC = peripheral blood mononuclear cells.

PHEU = perinatally HIV exposed but uninfected. PHIV = perinatally HIV infected. p = probability value. RV-AGE = rotavirus acute gastroenteritis. RV5 = pentavalent rotavirus vaccine. VP6-7 = viral protein 6-7. Yrs. = years.

CD4 and CD8 T-Cells Are of Low Circulating Frequency in Acute Rotavirus

Five studies reported a lower circulating frequency of $CD4^+$ and $CD8^+$ T-cells in response to acute rotavirus infection. In one study, while healthy children had normal proportions of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell subsets, children with acute rotavirus diarrhoea had selectively lowered $CD4^+$ T-cell proportion and a low $CD4^+$:$CD8^+$ T-cell ratio (22). A case study of a single child with rotavirus diarrhoea showed a depressed $CD4^+$ T-cell frequency and $CD4^+$:$CD8^+$ ratio in an acute phase that persisted up to one-month post-infection but normalized by convalescent period (23). In another two studies close to half of the children with rotavirus diarrhoea had absolute lymphopenia compared to children with or without previous rotavirus exposure but with non-rotavirus diarrhoea and the majority of children with acute (<7 days after the onset of illness) rotavirus diarrhoea had total whole blood lymphocyte counts less than the lower limit of the normal count range in healthy children (27, 34). Additionally, among children with previous rotavirus exposure and those with rotavirus diarrhoea, few had detectable cytokine-producing rotavirus-specific CD4 or CD8 T-cells (27). Likewise, flow cytometry and gene expression T-cell analysis of children with rotavirus diarrhoea revealed significantly lower mean frequencies of $CD4^+$ and $\alpha\beta^+CD4^+$ T-cells, $CD8^+$ and $\alpha\beta^+CD8^+$ T-cells and T-cell associated gene expression in children with rotavirus diarrhoea in the acute phase than in healthy controls. In the convalescent phase, however, the frequencies of these T-cell populations significantly increased to similar levels observed in healthy children. Exceptionally, one child with rotavirus diarrhoea was observed to have a minimal reduction in $CD4^+$ and $CD8^+$ T-cell frequencies in the acute stage but had a severe reduction in CD4 and CD8 T-cell subsets at convalescence (34). Among vaccinated children, rotavirus antigen-experienced CD4 T-cells were detected in low frequencies two weeks post-vaccination (31). Summary findings of these studies are outlined in Table 2-4.

**Table 2-4. Proliferative, Helper, and cytotoxic T-cell frequency to rotavirus in children compared to adults and other stimulants.**

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Elaraby et al., 1992 (22) | Healthy: (n = 50); RV-AGE: (n = 6) | CD3 (OKT3 pan), CD4 (OKT4 helper), CD8 (OKT8 frequency, CD4:CD8 T-cell ratio | • Depressed CD4 T-cell frequency (33.4%) and a lower CD4:CD8 ratio (1.36) in children with rotavirus diarrhea compared to normal CD4 (range 47.1% to 55.7%) and CD8 (23.8% to 25%) T-cell frequency and helper: suppressor ratio (1.9 to 2.23) in healthy children. | • Lowered CD4 T-cells during acute infection may be a result of CD4 T-cell migration out of circulation to effector sites |
| Iwasa et al., 2008 (23) | RV-AGE: (n = 1) | CD4 and CD8 T-cell frequency, CD4:CD8 T-cell ratio | • Depressed CD4 T-cells frequency (15.7%) and lowered CD4:CD8 ratio (0.41) but normal CD8 T-cell frequency (38.76%) in acute phase. | • Lowered CD4 T-cell during acute infection may be a result of CD4 T-cell migration out of |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | • Depressed CD4 T-cells frequency (14.55%) and lowered CD4:CD8 ratio (0.42) sustained in early convalescence but normalized in late convalescence. | circulation to effector sites.<br>• CD4 T-cells may be more critical effectors than CD8 T-cells in mucosal tissue sites |
| Mesa et al., 2010 (27) | Non-RV-AGE seronegative (n = 15) or seropositive (n = 21) and RV-AGE (n = 17) children. Healthy (n = 21) and RV-AGE adults (n = 5) | Lymphopenia and Th1, Th2, Th17 CD4 and cytotoxic CD8 T-cells | • Absolute lymphopenia in 5/12 (41.6%) children with RV-AGE compared to only 1/25 (4%) in children with non-RV-AGE<br>• Low (<0.06) or undetectable frequencies of IFN-$\gamma^+$, IL-$13^+$, IL-$2^+$, IL-$10^+$ and IL-$17^+$ CD4 T-cells in most children with non- and RV-AGE. The IFN-$\gamma^+$ CD4 and CD8 T-cells were observed in a few 2/12 children with | • Low circulating frequency of Th1, Th2, Th17, and cytotoxic T-cells in acute rotavirus that may result from effector T-cell functions at mucosal sites of infection.<br>• Diminished rotavirus Th1 and cytotoxic responses in children compared to adults |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | previous rotavirus exposure or rotavirus diarrhoea.<br>• In contrast, higher frequencies (≤0.65%) of rotavirus-specific $CD4^+$ IFN-$\gamma^+$ and $CD4^+$ IL-$2^+$ T cells were detected in the majority 14/21 (66.7%) and 6/10 (60%) of healthy adults, respectively. Similarly, $CD8^+$ IFN-$\gamma^+$ and $CD8^+$ IL-$2^+$ T cells were observed in 8/20 (40%) and 1/9 healthy adults, respectively. | |
| Parra et al., 2014 (31) | Seropositive vaccinated: (n = 35) and seronegative placebo: (n = 24) | Rotavirus (VP6-7 tetramer) antigen experienced CD4 T-cells | • Low frequency (0.001–0.1%) rotavirus antigen experienced CD4 T cells in children two weeks post two-dose vaccination | • CD4 T-cells are expanded after rotavirus vaccination but low circulating frequency |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | | |
| Wang et al., 2007 (34) | RV-AGE: (n = 10); Healthy (n = 8) | Lymphopenia, frequencies of CD4, $\alpha\beta^+$ CD4, CD8 and $\alpha\beta^+$ CD8 T-cells | • Lymphopenia in majority 5/7 (71%) of children with RV-AGE and repressed T-cell proliferation, differentiation, activation, survival, and homeostasis mRNA gene expression<br>• Lower mean frequency of CD4 (20%, range 10.4% to 26.8%) and $\alpha\beta^+$ CD4 (17% range 9% to 22.6%) T-cells in RV-AGE than in healthy children (50.9% range 38.6% to 60.5%) and (46.8% range. 36.7% to 53.7%) respectively ($p < 0.01$). CD4 T-cell frequencies significantly increased ($p < 0.01$) to that of | • Altered T-cell homeostasis and low circulating frequency of CD4 and CD8 T-cells in acute rotavirus that may result from effector T-cell functions at mucosal sites of infection |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | healthy children at convalescence.<br>• Similarly, lower mean frequency of CD8 (2.8%, range 1.6% to 3.8%) and $\alpha\beta^+$ CD8 (2.9%, range 1.7% to 3.7%) T-cells in RV-AGE than in the healthy children (10.9%, range 7.4% to 13.5%) and (8.6%, range 6.1% to 10.5%) respectively ($p < 0.05$). Both CD8 and $\alpha\beta^+$ CD8 T-cell frequencies significantly increased ($p < 0.01$) to that of healthy children at convalescence. | |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Jaimes et al., 2002 | RV-AGE children (n = 12), rotavirus exposed asymptomatic and symptomatic adults (n = 19), healthy adults (n = 7) | Th1 and Th2 CD4 and cytotoxic CD8 T-cell frequencies | • Lower mean rotavirus specific CD8 IFN-γ T-cell frequency 0.02% (SEM 0.007% range −0.01 to 0.08%) in RV-AGE children than exposed adult 0.49% (SEM 0.17% range 0.2 to 1.13%); recently infected symptomatic adults 0.28% (SEM 0.11% range, 0.03 to 0.91%); and asymptomatic adults mean, 0.15% (SEM 0.06% range, 0.03 to 0.37%) ($p < 0.05$)<br>• Lower mean rotavirus-specific CD4 IFN-γ T-cell frequency 0.02% (SEM 0.007% range −0.01 to 0.07%) in infected children than in exposed adults 0.1% (SEM 0.02% range, 0.02 | • Lower circulating frequency of Th1 and cytotoxic T-cells in infected children than adults<br>• Mixed Th1 and Th2 responses in children contrasted to predominantly Th1 in adults. |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | to 0.19%); symptomatically infected adults mean 0.18% (SEM 0.10% range 0.02 to 0.94%) and asymptomatic rotavirus infected adults mean 0.05%; SEM 0.01%; range, 0.01 to 0.09%) ($p < 0.01$).<br>• CD4 IL-13 T-cell frequency mean 0.02%; SEM, 0.009%; range, 0 to 0.06% detected in children but not adults but no predominance in CD4 IFN-γ or IL-13 T-cells in children. | |
| Makela et al., 2004 (26) | Healthy (T1D at risk) children: (n = 20); Healthy rotavirus exposed adults (n = 16) | Lymphoproliferation | • Adults had stronger T-cell proliferation to bovine rotavirus (NCD) ($p = 0.0001–0.0067$), human rotavirus lysate ($p = 0.0008–0.011$) and purified human rotavirus ($p = 0.0044–$ | • Children have weaker T-cell responses to rotavirus compared to adults. |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | 0.083) than any age group of children.<br>• Similar T-cell proliferation to PPD in children and adults (p = 0.53–0.91) | • Rotavirus is a poor inducer of T-cells in comparison to mycobacterial tuberculin |
| Makela et al., 2006 | Healthy children (T1D at risk, n = 183) | Lymphoproliferation | • Children had a higher median T-cell proliferative response to TT and PPD than to purified rotavirus, human rotavirus lysate, or bovine rotavirus (NCD) | • Rotavirus is a poor inducer of T-cells in comparison to mycobacterial tuberculin and tetanus toxoid |
| Parra et al., 2014 (30) | Healthy children (n = 5) and healthy adults (n = 25) | Cytokine secreting PBMC. Th1 CD4 and cytotoxic CD8 T-cells. CD4 and CD8 proliferation. | • IFN-γ, TNF-α, GM-CSF, RANTES, MCP-1 and IL-10 secreting PBMC in adults but not children. | • Diminished Th1 responses in children than adults.<br>• Rotavirus is a poor inducer of T-cells in |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | • Lower frequencies of IFN-γ, TNF-α, and IL-2 CD4 T-cells against rotavirus than against TT (p = 0.0313) or Influenza (p = 0.0313) in both children and adults.<br>• Monofunctional (single IFN-γ or TNF-α secreting) rotavirus specific CD4 T-cells predominant in both adults and children | comparison to tetanus toxoid and Influenza.<br>• CD4 T-cell response to rotavirus involves predominantly Th1 subset |
| Rojas et al., 2003 (32) | RV-AGE children (n = 9); Healthy adults (n = 7) | Frequencies of Th1 and Th2 CD4 and cytotoxic CD8 T-cells | • Both IFN-γ CD4 (p = 0.046) and IFN-γ CD8 (p = 0.028) T-cells against rotavirus detected in adults but only IFN-γ CD8 p = 0.018) and not CD4 T-cells (p | • IFN-γ cytotoxic CD8 T-cells may be the main effector in acute rotavirus infected children. |

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| | | | = 0.17). detected in children with diarrhoea.<br>• Low but insignificant frequency of IL-4 CD4 T-cells against rotavirus detected in both adults and children (p = 0.15). | • Th2 CD4 T-cells may have a less significant role against rotavirus |

Abbreviations: CD3 = cluster of differentiation 3. CD4 = cluster of differentiation 4. αβCD4 = alpha beta cluster of differentiation 4. CD8 = cluster of differentiation 8. αβCD8 = alpha beta cluster of differentiation 8. GM-CSF = granulocyte-macrophage colony-stimulating factor. IFN-γ = interferon gamma. IL-2 = interleukin 2. IL-10 = interleukin 10. IL=13 = interleukin 13. IL-17 = interleukin 17. mRNA = messenger ribonucleic acid. MCP1 = monocyte chemoattractant protein 1. NCD = Nebraska Calf Diarrhoea. OKT3 = anti-CD3 monoclonal antibody. OKT4 = anti-CD4 monoclonal antibody. OKT8 = anti-CD8 monoclonal antibody. PBMC = peripheral blood mononuclear cells. p = probability value. PPD = tuberculin purified protein derivative. RANTES = regulated on activation, normal T-cell expressed and secreted. RV-AGE = rotavirus acute gastroenteritis. SEB = staphylococcal enterotoxin B. SEM = standard error of measurement. T1D = type 1 diabetes. Th1 = T-helper type 1. Th2 = T-helper type 2. Th17 = T-helper type 17. TT = tetanus toxoid. TNF-α = tumor necrosis factor alpha. VP6-7 = viral protein 6-7

Proliferative, Helper and Cytotoxic T-Cells Profiles to Rotavirus Differ in Children Compared to Adults and Other Stimulants

Diminished responses and different profiles of proliferative, helper, and cytotoxic T-cell responses are elicited against rotavirus in children compared to adults or other stimulants as shown in Table 2-4. In a study by Jaimes et al., rotavirus-specific $CD4^+$ IFN-$\gamma^+$ Th1, $CD4^+$ IL-$13^+$ Th2, and $CD8^+$IFN-$\gamma^+$ cytotoxic T-cells, were investigated in children with rotavirus diarrhoea in comparison to recently infected, exposed, and unexposed healthy adults. When compared, rotavirus-exposed adults had significantly higher mean proportions of rotavirus-specific Th1 and cytotoxic responses than children whose responses were like those observed in healthy adults. However, while the Th1 and cytotoxic T-cell responses were induced by rotavirus in both adults and children, the Th2 response was additionally observed in children with rotavirus diarrhoea at a similar frequency to the Th1 response but not in adults (24). In contrast, a study by Parra et al. showed a predominance of monofunctional $CD4^+$ IFN-$\gamma^+$ and $CD4^+$ TNF-$\alpha^+$ Th1 response in both adults and children (30). Another study found T-cell proliferative responses to rotavirus were generally weaker in prospectively studied children compared to adults with the adults having significantly stronger T-cell proliferation to both bovine and human rotavirus strains than any age group of children (26). A study looking at frequencies of $CD4^+$ IFN-$\gamma^+$ or IL-$2^+$ Th1, $CD4^+$ IL-$13^+$ Th2, $CD4^+$ IL-$17^+$ Th17 and $CD8^+$ IFN-$\gamma^+$ cytotoxic T-cells in children with rotavirus and non-rotavirus diarrhoea in comparison with healthy and acutely or convalescent rotavirus infected adults found similar observations. Little to no Th1, Th2, or Th17 rotavirus-specific T-cell responses were observed in children with diarrhoea and few responses observed comprised Th1 and cytotoxic responses and were only observed among children with prior exposure to or existing acute rotavirus diarrhoea. In contrast to children, a much larger proportion of adults, both healthy and acutely infected had detectable Th1 and cytotoxic T-cell responses (27). These results are similar to another study that showed secretion of IFN-γ, TNF-α, GM-CSF, RANTES, MCP-1, and IL-10 from rotavirus stimulated cells in adults but not in children (30).

In comparison to other viral and bacterial stimulants, circulating rotavirus-specific T-cell responses are generally diminished. While significantly higher proliferation to rotavirus was observed in adults than children, proliferation in response to mycobacterium purified protein derivative (PPD) in children was as high as that observed in adults (26). Among healthy children, T-cell proliferation to rotavirus was observed to be generally lower in comparison to proliferation against tetanus toxoid (TT), mycobacterium PPD antigens, and Coxsackie B4 virus (CBV) antigen (25, 30). Significantly lower frequencies of IFN-γ, TNF-α and IL-2 producing CD4 T-cells were observed against rotavirus than in response to Influenza virus antigens in children (30)

Rotavirus Activates Proinflammatory, Regulatory and Gut Homing Effector T-Cell Phenotypes

The T-cell immune response to rotavirus in children is characterized by an elevated activated and proinflammatory T-cell profile (Table 2-5). Children with rotavirus diarrhoea show higher proportions of proinflammatory T-helper 17 cells complemented by higher levels of peripheral blood circulating pro-inflammatory IL-6 and IL-17 cytokines at the time of acute infection compared to healthy children (21). Similarly, a case report of a child with rotavirus gastroenteritis reported elevated proportions of IFN-γ producing helper and cytotoxic T-cells in the acute phase of infection although these levels were reduced by convalescence (23). Likewise, another study showed a positive correlation between T-cell proliferative responses to rotavirus and messenger ribonucleic acid (mRNA) expression of proinflammatory IFN-γ and IL-4 cytokines in healthy children (25). Like these findings, a microarray analysis study of immune cell mRNA gene expression by Wang et al. revealed that children with rotavirus diarrhoea had upregulation of genes encoding lymphocyte activation markers, proinflammatory cytokines, chemokines, and immune proteins in the acute stage compared to healthy children. Interestingly, although there was an elevated gene expression of lymphocyte activation markers CD69 and CD83 as well as genes encoding for the differentiation, maturation, activation, and survival of B lymphocytes, there was a reduced expression of genes involved in the proliferation, differentiation, activation, survival, and homeostasis of T lymphocytes in these rotavirus infected children (34).

**Table 2-5. T-cell activation, proinflammatory, regulatory and homing phenotypes in response to rotavirus.**

| Author, Year (Ref) | Child Population | T-Cell Response | Finding | Interpretation |
|---|---|---|---|---|
| Dong et al., 2015 (21) | RV-AGE (n = 102); Healthy (n = 30) | Th17 and Tregs frequency | • Frequencies of $CD4^+$ $IL\text{-}17^+$ Th17 cells and circulating IL-17 and IL-6 proinflammatory cytokines were increased ($p < 0.05$) in RV-AGE than healthy children. ($p < 0.05$)<br>• In contrast, the frequency of $CD4^+$ $CD25^+$ Treg cells and levels of circulating IL-10 and TGF-β regulatory cytokines in children with rotavirus enteritis was significantly decreased when compared with the healthy children ($p < 0.05$). | • Th17 cells play a role in the protective immune response to rotavirus.<br>• $CD4^+CD25^+$ T-cells and regulatory cytokines lowered in rotavirus infection |
| Iwasa et al., 2008 (23) | Infant with acute rotavirus gastroenteritis (n = 1) | Th1 CD4 and cytotoxic CD8 T-cell frequencies | • Elevated IFN-γ $CD4^+$ (14.85%) and $CD8^+$ (77.58%) T-cell frequency during acute stage that decreased one month later to 3.46% and 0.19% respectively | • IFN-γ Th1 CD4 and cytotoxic CD8 T-cells are effectors against acute rotavirus |

| Author, Year (Ref) | Child Population | T-Cell Response | Finding | Interpretation |
|---|---|---|---|---|
| Makela et al., 2006 (25) | Healthy (T1D at risk), n = 183) | IFN-γ, IL-4, IL-10 and TGF-β mRNA expression and T-cell proliferation | • Positive correlation between PBMC IFN-γ, IL-4 and IL-10 mRNA secretion and lymphoproliferation against rotavirus (r = 0.48, p = 0.003, r = 0.46, p = 0.004, and r = 0.36, p = 0.026 respectively). No correlation with TGF-β | • Rotavirus T-cell responses includes Th1 and Th2 effectors.<br>• IL-10 and not TGF-β regulatory T-cells may be important immune regulators of the proinflammatory response |
| Wang et al., 2007 (34) | RV-AGE (n = 10); Healthy (n = 8) | Gene expression of T-cell immune markers | • Elevated gene expression of inflammatory immune markers TNF-α, proIL-1β, IL-1 β, IL-6, IL-8, GRO-β, IL-1R antagonist, IFN- α/β receptor and IFN α/β -stimulated proteins in rotavirus infected children than healthy children<br>• Elevated CD4 T-cell activation CD4/CD69 (from 2.7% to 10.5% [mean, 5.5%]), CD4/CD83 (from 10.5% to 25.8% [mean, 16.6%]), and CD8 T-cell activation CD8/CD69 (from 1.6% to 8.3% (mean, 3.5%), CD8/CD83 (from | • Rotavirus induces a pro-inflammatory immune response.<br>• CD69 and CD83 activated CD4 and CD8 T-cells contribute to antiviral activity and recovery from disease in children |

| Author, Year (Ref) | Child Population | T-Cell Response | Finding | Interpretation |
|---|---|---|---|---|
| | | | 4.4% to 16.1% [mean, 7.8%]) in RV-AGE than in healthy children range 0% to 0.5% (mean, 0.3%) for CD4/CD69, from 0.1% to 4.0% (mean, 1.2%) for CD4/CD83, from 0.1% to 0.7% (mean, 0.3%) for CD8/CD69, and from 0% to 0.4% (mean, 0.2%) for CD8/CD83 respectively. | |
| Mesa et al., 2010 (27) | RV-AGE (n = 53) | $CD4^+CD25^+$, $CD4^+CD25^+CD127^{low}$, $CD4^+CD25^+CD127^{low}$ $TGF\text{-}\beta^+$ and $CD45RA^+$ regulatory T-cells (Tregs) and<br>IFN-γ producing CD4 T-cells | • Rotavirus IFN-γ CD4 T-cells not affected by TGF-β regulation in children but in adults.<br>• No difference in $CD4^+CD25^+$, $CD4^+CD25^+CD127^{low}$ and $CD4^+CD25^+CD127low$ $TGF\text{-}\beta^+$ Tregs in RV-AGE and non-RV-AGE<br>• Most $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^+CD127low^+$ | • TGF-β does not regulate IFN-$\gamma^+$ CD4-T cell to rotavirus in children.<br>• The naïve Treg profiles in children could result in their reduced immunomodulatory effects in response to rotavirus infection |

| Author, Year (Ref) | Child Population | T-Cell Response | Finding | Interpretation |
|---|---|---|---|---|
| | | | TGF-$\beta^+$ Treg cells in children are naïve phenotype (CD45RA) | |
| Parra et al., 2014 (31) | Vaccine (n = 3) | $CD62L^-$ $CD45RA^{+/-}$ and $CD26L^+$ $CD45RA^-$ CD4 T-cells α4β7 and CCR9 | • Most of the rotavirus antigen VP6-7 tetramer$^+$ experienced CD4 T-cells expressed α4β7, or expressed both, α4β7 and | • Majority rotavirus CD4 T-cells are gut homing.<br>• Generation of these T-cell gut homing phenotypes may be important for clearing rotavirus infection and protecting against re-infection |
| Rojas et al., 2003 (32) | RV-AGE (n = 9) | Frequencies of CD4 and CD8 T-cells producing IL-4 and IFN-γ | • Detectable INF-γ CD8 ($p = 0.018$) but not INF-γ CD4 ($p = 0.17$) T-cells. IL-4 CD4 and IL-4 CD8 also not detected ($p = 0.15$). | • INF-$\gamma^+$ cytotoxic CD8 T-cells may be more important for initial clearance of infection than the CD4 T-cell subset |

| Author, Year (Ref) | Child Population | T-Cell Response | Finding | Interpretation |
|---|---|---|---|---|
| Rott et al., 1997 (33) | convalescing RV-AGE (n = 1) | T-cell proliferation | • $\alpha4\beta7^{hi}$ blood lymphocytes showed a 2.6-fold greater proliferative response to rotavirus than $\alpha4\beta7^{-}$ cells (SI 4.07 versus 1.54 respectively) | • Majority of rotavirus T-cell have $\alpha4\beta7^{hi}$ phenotype |

Abbreviations: α4β7 = alpha 4 beta 7. CCR9 = C-C motif chemokine receptor 9. CD4 = cluster of differentiation 4. CD8 = cluster of differentiation 8. CD25 = cluster of differentiation 25. CD45RA = cluster of differentiation 45RA. CD62L = cluster of differentiation 62L. CD69 = cluster of differentiation 69. CD83 = cluster of differentiation 83. CD127 = cluster of differentiation 127. GRO-β = growth-regulated oncogene. IFN-α = interferon alpha. IFN- β = interferon beta. IFN-γ = interferon gamma. IL-1β = interleukin 1β. IL-1R = interleukin 1R. pro-IL-1β = precursor interleukin 1β. IL-4 = interleukin 4. IL-6 = interleukin 6. IL-8 = interleukin 8. IL-10 = interleukin 10. IL-17 = interleukin 17. mRNA = messenger ribonucleic acid. p = probability value. PBMC = peripheral blood mononuclear cells. r = Pearson's correlation coefficient . RV-AGE = rotavirus acute gastroenteritis. Th1 = T-helper type 1. Th2 = T-helper type 2. Th17 = T-helper type 17. T1D = type 1 diabetes. TNF-α = tumor necrosis factor alpha. Treg = regulatory T-cell. TGF-β = transforming growth factor beta.

The proinflammatory T-cell response to rotavirus may occur in association with either a lowered or elevated regulatory T-cell response (Table 2-5). Dong et al. found that rotavirus infected children had a significantly lower proportion of regulatory T-cells compared to healthy children. The lower regulatory cell profile corresponded to significantly lower levels of circulating immunosuppressive IL-10 and TGF-β cytokines (21). In contrast, a study by Mesa et al. showed that a TGF-β dependent regulatory mechanism of rotavirus specific CD4 and CD8 IFN-γ T-cell response was absent in children with acute rotavirus gastroenteritis but present in adults, although only four and three adults were studied respectively, and showed that the lowered circulating frequency of rotavirus specific T-cells was not due to regulation by TGF-$\beta^{+}$ regulatory T-cells as both rotavirus-infected and healthy children had similar proportions of these circulating Treg profiles (27). Furthermore, another study found a positive correlation between T-cell proliferative responses and immunosuppressive IL-10 but supporting the previous studies this was not observed for TGF-β (25). One other study also found elevated expression of other inflammation-modulating proteins IL-1R antagonist, IFN α/β receptors and IFN-stimulated proteins in rotavirus infected children (34).

Two studies reported that a substantial proportion of rotavirus-experienced T-cells express gut homing markers. As shown in Table 2-5, one study by Rott et al. among children convalescing after acute rotavirus infection reported higher T-cell proliferative response to rotavirus in the $\alpha4\beta7^{hi}$ lymphocyte population than $\alpha4\beta7^{-}$ lymphocyte population although this was based on cellular data obtained from one child (33). Likewise, another study among rotavirus vaccinated children found that most of the rotavirus antigen experienced $CD4^{+}$ T cells expressed α4β7 gut homing marker with most cells expressing both, α4β7 and CCR9, gut homing markers (31).

## 2.5 Discussion

We provide an overview of the evidence and characteristics of T-cell immune responses to rotavirus in healthy, rotavirus infected, and vaccinated children. Although many research studies have been done, very few of them specifically address T-cell mediated immunity to rotavirus in children. We found only seventeen articles to include in this review.

Summary Findings and Implications

Most studies identified were within the context of rotavirus infection and only two studies assessed T-cell responses in relation to rotavirus vaccination. This is particularly surprising considering the continued development and introduction of new rotavirus vaccines (6, 38) and the fact that immune correlates of protection for rotavirus vaccines remain elusive to date (7). Additionally, the least number of studies were conducted in African children which is of concern as this region bears the highest burden of rotavirus diarrhoea (3) and rotavirus vaccines within this region consistently exhibit diminished performance (17). These findings highlight the gap in research elucidating the role of T-cell mediated immunity to rotavirus to explore their potential as immune correlates of vaccine protection and the need for a better understanding of rotavirus immune mechanisms. Such research would particularly help understand the reduced vaccine immunogenicity in African children.

T-cell immunity does play a role in the immune response to rotavirus in children. Lymphoproliferative assays provided evidence of circulating rotavirus-specific T-cells in children. The lack of proliferation observed in new-borns, minimal proliferation in infants <1-year-old, and increasing proliferation with age are consistent with the exposure pattern to rotavirus in early life. However, the minimal rotavirus-specific T-cell proliferation in children aged below 1 year of age is of concern as rotavirus vaccines are administered within this period and vulnerability to rotavirus is highest in early infancy. While transplacental maternal antibody immunity is most probably important for protection in this age group, it may be necessary for new rotavirus vaccine formulations to incorporate designs allowing for enhanced T-cell activation such as the addition of adjuvants. Interestingly, evidence of rotavirus T-cell proliferation is also seen in some new-borns that could be a result of in-utero or very early exposure to rotavirus antigens and is of significance for neonatal rotavirus vaccines strategies. Rotavirus vaccines administered at birth have been developed and found to be safe and highly efficacious in new-borns. This birth dose vaccination could potentially impart rotavirus-specific memory T-cells thus providing an opportunity for cell-mediated protection very early on in life (39). This early protection would have a considerable impact on further reduction of rotavirus burden in low-income countries where a sizeable proportion of children are infected with rotavirus before receipt of the first vaccine dose that has been associated with poor vaccine seroconversion (11, 40).

Broadening of cross-reactive T-cells with increasing age is consistent with exposure to different rotavirus strains as children age. These results further implied that rotavirus-specific T-cells recognize epitopes shared by different infecting rotavirus serotypes indicating that T-

cell immunity can provide cross-reactive protection. Rotavirus has a large strain diversity based on varying combinations of G- and P-serotypes and genotypes classified by antibody reactivity to VP7 and VP4 viral proteins respectively (8). Rotavirus strains that cause infections in humans and commonly infect children aged <5 years are well known but evolutionary genetic mutation and reassortments eventually give rise to new strains (41). This observed T-cell proliferation irrespective of infecting G-serotype suggests that rotavirus induced T-cell immunity in children is not G-serotype specific which is important for effective vaccine strategies. For instance, Rotarix, a monovalent G1P [8] rotavirus vaccine has shown protection against non-vaccine serotype rotavirus strains, however, vaccine strain breakthrough still occurs and the extent to which this cross-reactive immunity is mediated by T-cells or antibody responses is unclear and needs further investigation (42). Total circulating antibody and homotypic and heterotypic neutralizing antibodies are associated but not entirely correlated with protection, which has suggested that other immune mechanisms like these cross-reactive T-cells are likely at play (7).

The available literature shows that both memory B and T-cell immunity are developed after rotavirus exposure with T-cell responses occurring in tight association with the antibody response. This review revealed more frequently detected T-cell responses in children that were seropositive than those seronegative for rotavirus-specific antibodies as well as in secondary versus primary infections. However, the antibody response is more persistent and due to the more transient nature of the T-cell response, T-cell immunity detected in children most likely reflects previous rather than active exposure. Therefore, in infants, T-cell immunity may be more useful as a measure of child-specific immune memory and in early infancy to discriminate from passively acquired maternal immune memory in response to infection. Additionally, in the context of vaccination, detection within shorter time periods post-vaccination would be required in the assessment of these effector T-cell responses. Nevertheless, the detection of both T-cells and antibody responses is necessary to adequately describe the immune response to rotavirus in children infection.

Evidence of T-cell proliferation in the absence of increasing antibody titres in some children speaks towards the existence of anti-rotavirus protection mediated via a direct T-cell immune effector in children. The direct effector contribution of T-cells has been shown in murine model depletion and adoptive studies where depletion of CD8 T-cells resulted in the delayed rate of resolution of rotavirus infection, CD4 T-cell depletion was associated with chronic viral shedding and complete loss of protection (14), and adoptive transfer of rotavirus primed CD4

and CD8 T-cells resulted in shorter rotavirus shedding (43). In such murine studies, a significant loss of protection against rotavirus has also been observed in T-cell deficient and T-cell receptor (TCR) knockout mice with the delayed resolution of rotavirus infection attributed to the depletion of the CD4+ T-cell subset, while B-cell and TCR deficient mice remained protected (15). In this review, direct effects of T-cell immunity were exemplified by the impaired rotavirus antibody response, chronic viral shedding, and inability to clear infection observed in T-cell immunodeficient children. In the context of vaccination, it is plausible that lowered antibody responses detected in non-seroconverting children based on fold change in antibody response may not entirely imply reduced protection as T-cell immunity may provide direct protective and immune memory functions. The contribution of T-cell immune memory in the measurement of vaccine immunogenicity may have implications for measures of vaccine efficacy.

The positive association between higher rotavirus CD4 T-helper cell response and rotavirus seropositivity or higher neutralizing IgG in children highlights the particular importance of indirect protection offered via the CD4 T-cell helper function in the production of the antibody response. In adoptive transfer murine models, rotavirus primed CD4 T-cells and not CD8 T-cells are associated with increased production and maintenance of secretory IgA that is important in mucosal immunity, and both serum IgA and IgG are currently recognized as valuable surrogate endpoints for protection (12). Therefore, taking this into account, in regions of poor rotavirus vaccine performance, there is a need for elucidating detailed profiles of these CD4 T-cells in relation to the magnitude and neutralizing ability of the antibody responses among vaccinated children. Magnitude and maintenance of antibody response may be reliant on characteristics of the elicited CD4 T-cell response. Such T-cell studies may provide useful insights for the observed lower vaccine immunogenicity and effectiveness trends in these regions.

In children, these characteristics of CD4 and CD8 T-cell responses to rotavirus include predominantly Th1 but also Th17 responses. Activated CD4 and CD8 T-cells secreting proinflammatory cytokines particularly IFN-γ and IL-17 appear important in this immune response. IFN-γ cytokine has direct anti-viral effects and IL-17 is associated with the provision of protection via recruitment of other immune cells with both cytokines shown to be important in the clearance of rotavirus infection (44). On the other hand, regulatory T-cells which may suppress the proinflammatory immune response in efforts to maintain homeostasis also occur in response to rotavirus. The regulatory T-cells can have a negative or positive influence on

the immune response to rotavirus infection or vaccination. This review revealed $IL10^+$ and $FOXP3^+$ regulatory T-cells as distinct subpopulations with opposing effects on rotavirus antibody immunity. In this context, a distinct population of $CD4^+/CD8a^+$ $CCR6^+CXCR6^+$ Treg cells has been identified in the human colon, which responds to faecal bacterial species and produces IL-10 (45). These cells could indeed drive distinct outcomes during rotavirus infection compared to their $FOXP3^+$ Treg counterparts. For live attenuated rotavirus vaccines, assessing these Th1 and Th17 inflammatory and $FOXP3^+$ and $IL\text{-}10^+$ regulatory T-cell profiles in children may provide insights into the observed vaccine immunogenicity.

In addition to these conventionally studied CD4 and CD8 T-cell subsets, recently identified innate-like T-cells such as the gamma delta T-cell (γδT), mucosal-associated invariant T-cells (MAIT), and natural killer T-cells (NKT) are enriched in mucosal tissues and have been reported to provide protective effector activities against human intestinal infections. Through direct cytokine action or indirectly via recruitment of other immune effector cells cytokine responses, these innate-like T-cells have been suggested to provide early antiviral immune protection in the interface between innate immunity and induction of adaptive immunity and have been associated with inhibited viral replication of important human viral pathogens (46, 47). There is an urgent need to also consider the characterization of these atypical T-cell profiles and how they relate to conventional CD4 and CD8 T-cell subsets in relation to observed rotavirus infection or vaccine immunogenicity.

Circulating rotavirus-specific T-cells in children are generally low in frequency during the acute than convalescent phase and much weaker than those generated in adults and against other pathogens. The lowered frequency of rotavirus-specific T-cells in the initial response may be a direct consequence of their migration from circulation to gut mucosal priming sites to carry out effector function. This is supported by literature documenting higher T-cell proliferation within $\alpha4\beta7^{hi}$ subset and a higher proportion of CD4 T-cells responding to rotavirus expressing α4β7 or CCR9 gut homing markers. Current live attenuated oral rotavirus vaccines aim to mimic natural infection immune priming within the gut. The extent to which such vaccines elicit these gut homing effector T-cell phenotypes may relate to the protective effect of vaccination. With new parenterally administered rotavirus vaccines being introduced, their ability to elicit these gut homing phenotypes must also be studied. While murine models have documented the development of mucosal immunity from parenteral vaccination (48), the generation of gut homing rotavirus specific T-cells in children vaccinated with parenteral rotavirus vaccine remains to be determined although an observed reduction in viral shedding in clinical trials conducted thus far has implied generation of local mucosal effectors (49). It will, therefore, be

important to conduct studies assessing the homing phenotypes elicited by rotavirus vaccination which may influence effector abilities in the protection against rotavirus at the gut.

When compared to tuberculin, tetanus toxoid, and influenza-derived antigens for which childhood vaccines are also administered, the T-cell responses induced by rotavirus antigen were observed to be diminished. Reasons for such variations in antigen-specific responses in early life can include immune dysfunction in antigen-specific presentation and differences in antigen-specific T-cell activation, proliferation, and effector versus memory generating functions. A better understanding of these T-cell phenotypes responding to rotavirus in this context has the potential to be exploited for improved immunity (50). Considering the role of T-cell phenotypes in the child's immune response to infection or vaccinations, it should be important when assessing immune responses in children to account for pathogens that have a strong modulatory effect on these T-cell populations. For instance, cytomegalovirus, a ubiquitous pathogen, and potent T-cell modulator have been shown to influence immune and vaccine-induced T-cell profiles in children (51, 52) but data is unavailable on its modulatory effect on anti-rotavirus T-cell immunity in children.

Strengths and Limitations

To the best of our knowledge, this is the first systematic review of the T-cell response to rotavirus in children using a clearly defined search and screening strategy to obtain existing literature. Our review gives an overview of research done prior to and post introduction of rotavirus vaccines and provides evidence supporting the need for more research on T-cell mediated immunity in children not only as it relates to infection but also vaccination. This review provides current knowledge in the literature on different subsets and characteristics of T-cells response to rotavirus encompassing general proliferation, specific phenotypes, functional cytokine secretion, and migratory profiles. The review also covered the relationship of T-cell responses to widely studied antibody responses.

Limitations in this review primarily arose from the nature of the studies identified. A substantial proportion of studies, particularly those conducted earlier, reported lymphoproliferative activity as an indication of T-cell immunity. However, caution must be taken in their interpretation as the detected proliferation potentially includes that of innate and B-cells. Lymphoproliferative-based measures, while giving insights to T-cell immunity, do not provide specific T-cell immune data in comparison to current more advanced techniques such as multicolour flow cytometry. Additionally, aside from four studies, the majority were conducted within the last decade and as such did not utilize more recent immunological methods such as higher cell marker parameter flow cytometry to provide more comprehensive T-cell knowledge.

Another limitation is that the studies identified used a diverse range of immune stimulants to assess the rotavirus T-cell responses which included different rotavirus strains or mitogens and had variations in reporting format for the T-cell outputs. This introduced large methodological heterogeneity that presented a major challenge in the quantitative synthesis of the evidence that was provided. Additionally, there was a lack of sufficient reporting of statistical data in several studies and more so in studies conducted much earlier on, and for some studies, sample sizes were very small making generalization of findings difficult.

## 2.6 Conclusions

T-cells clearly have a role to play in the immune response to rotavirus in children. This review shows that these responses are heterotypic and although present at low circulating levels and less persistent than antibodies, can be detected in children and develop through repeated exposure. Both CD8 and CD4 T-cell subsets are involved in this response and are primarily of a Th1 and gut homing phenotype. However, there is a paucity of T-cells studies, wide methodological differences, and a lack of sufficient quantitative data sets directly associating T-cell immunity to protection from rotavirus infection or in relation to immunogenicity of rotavirus vaccines. Thus, it is imperative that further research be done investigating T-cell responses against rotavirus and the standardization of rotavirus-specific T-cells assays is needed in this population.

Africa bears a disproportionate burden of rotavirus diarrheal disease and has an urgent need for research in this area. Such studies may also establish whether the observed lower vaccine-induced anti-rotavirus antibodies in African children could be attributed to limited or impaired T-cell responses. There is also a need to address innate-like T-cell subsets and the inclusion of more phenotypic markers using more developed immunological assays to provide comprehensive T-cell immunology data. In rotavirus vaccinology, it will be important to assess T-cell immunity relationship to seroconversion rates and clinical protection against rotavirus infection. Such research could form a good basis for further exploration of T-cells as a potential immune correlate of protection and inform the development of next-generation vaccines.

## 2.7 Funding

This work was supported by the Wellcome Trust grant [211356/Z/18/Z] awarded to Natasha Laban and the EDCTP2 programme supported by the European Union Senior Fellowship grant awarded to Roma Chilengi (grant number TMA2016SF-1511-ROVAS-2)

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## Chapter 3: Methods

### 3.1 Ethics approvals

This study was approved by the University of Zambia Biomedical Research Ethics Committee (UNZABREC, reference number 003-02-18) and the London School of Hygiene and Tropical Medicine (LSHTM) Ethics Committee (reference number 16168 and reference number 26277).

### 3.2 Funding

The PhD was funded by the Second European and Developing Countries Clinical Trials Partnership (EDCTP2) programme supported by the European Union (grant number TMA2016SF-1511-ROVAS-2) and the Wellcome Trust [grant number 211356/Z/18/Z].

### 3.3 Study location and population

This research was conducted in Lusaka province of Zambia within the Centre for Infectious Disease Research in Zambia (CIDRZ) Clinical Research Site (CRS) located at George Health Centre (GHC). The GHC is a government run public health facility serving “George Compound” a peri-urban high-density, low-income community. The health facility offers primary health services including general outpatient screening, maternal and child health (MCH) services, human immunodeficiency (HIV) counselling, testing, and anti-retroviral therapy treatment, and prevention of mother to child transmission, male circumcision, maternity delivery labour ward, emergency obstetric care, laboratory, dental and x-ray services to the catchment population. Majority of the George community residents live in unplanned housing structures characterised by poor water sanitation and hygiene (WASH) infrastructure.

The target population was mother-infant pairs presenting to the MCH department at GHC for the infants’ routine 6-week age vaccination visits according to the expanded program on

immunisation (EPI) schedule in Zambia. At the 6-week MCH visit, infants in Zambia are scheduled to receive oral polio vaccine (OPV) against poliomyelitis, their first dose of pentavalent parenteral vaccine against Diphtheria, Pertussis, Hepatitis, Tetanus, *Haemophylus Influenza* type b (DPT-HepB-Hib), pneumococcal conjugate vaccine 10-valent (PCV) vaccine against Pneumonia and to receive the first dose of the oral rotavirus vaccine Rotarix (Table 3-1).

**Table 3-1. Infant vaccine schedule in Zambia**

| **Vaccines** | **Age of Vaccination** |
|---|---|
| BCG | Birth |
| Polio (OPV) | Birth, 6, 10, 14 weeks and 9 months if OPV missed at birth |
| DPT-HepB-Hib | 6, 10 and 14 weeks |
| Pneumococcal Conjugate Vaccine 10-valent (PCV) | 6, 10 and 14 weeks |
| Rotavirus vaccine (Rotarix) | 6 and 10 weeks |
| Measles-Rubella vaccine | 9 and 18 months |

## 3.4 Study design and sample size

This PhD research study made use of samples collected under a rotavirus vaccine trial. The trial was an open label randomised controlled trial (RCT) acronym ROVAS-2. The control arm was the administration of two-doses of Rotarix vaccine at 6 and 10 weeks of infant age in accordance with the standard of care and the intervention arm was administration of a third dose of Rotarix at 9 months infant age, concomitantly with measles-rubella vaccine. A sample size of 212 mother-infant pairs at 80% power was calculated for the RCT and the PhD study made use of this finite population.

## 3.5 Participant enrolment

Participant recruitment involved prospective accrual from the target population of mother-infant pairs at GHC which was cumulative until the total target sample size of 212 mother-infant pairs for the RCT was reached. The actual enrolled infant sample size was 214 as 2 out of the 212 mothers enrolled had twin infants. Identification and recruitment of potential mother-infant pairs under the RCT was initiated in September 2018 within the MCH department at GHC and conducted by the study Research Nurses. The Research Nurses first approached the mothers in the waiting area of the MCH department at GHC and provided general sensitization information about the ROVAS-2 RCT in either English or two local languages (Nyanja and Bemba), according to the mothers' preference. Mothers that were interested to learn more about the RCT and expressed willingness to participate were invited to the CIDRZ CRS, co-located within the GHC premises a few meters walk from the MCH department where they were provided with further information about the study. At this point, mother's that were still willing to participate in the RCT were individually invited into private rooms where they were screened by the study Clinician for eligibility according to the RCT inclusion and exclusion criteria.

The criteria for enrolment included the mothers' willingness to participate voluntarily and provide written informed consent, the child's eligibility for Rotarix vaccination as per national policy, mothers' willingness for herself and the child to undergo defined study procedures and mother's planned residence in the study area for the duration of the study. Exclusionary criteria constituted the infant's contraindication to rotavirus vaccination, previous receipt of rotavirus vaccine, recent immunosuppressive therapy including high-dose systemic corticosteroids, history of receiving blood transfusion or blood products, including immunoglobulins within the previous 6 months, any condition deemed by the study investigator to pose potential harm to the infant or jeopardize the validity of study result and any existing congenital anomalies constituted exclusionary criteria. Mother-infant pairs that were determined to be eligible were then taken through a voluntary written informed consent process in the mother's preferred language and the informed consent process included administration of an informed consent comprehension quiz to the mother. In cases where the mother was illiterate, a GHC staff member impartial to the RCT or a literate individual identified by the mother was present during the consenting process and signed the written informed consent form as witness. The first participant mother-infant pair was enrolled on 13$^{th}$ September 2018 and follow up was concluded in November 2021.

## 3.6 Study procedures

The timelines and samples collected under the RCT are shown in Figure 3-1. At enrolment (visit 1), each mother-infant pair was assigned a unique participant identification (PID) number and a study file. The mother's and infant's sociodemographic, anthropometric, and clinical data were recorded by the Research Nurses and a full physical exam of the infant performed by the Clinician. The HIV status of the mother and thereby infant HIV exposure was determined using records on the government health facility issued antenatal and infant clinic card that indicated whether the infant was exposed or unexposed to HIV. HIV rapid testing was also offered to all mothers at this enrolment visit. Thereafter, the study Clinician or Research Nurse collected a baseline whole blood sample from both the mother (5ml) and infant (3ml), a breastmilk sample (50ml) from the mother and a passive raw stool sample from infant's diaper if available. The infant was then given the first dose of oral Rotarix vaccine, and all other routine vaccines administered at 6 weeks (OPV, DPT-HepB-Hib, and PCV) as per the Zambian infant vaccine schedule (See Table 3-1). All Rotarix vaccines administered in the study were of a single batch sourced from the Zambian Ministry of Health and stored at the CRS pharmacy. Routine EPI vaccines administered to participating infants were sourced from stocks made available at the GHC MCH pharmacy. The mother was then provided with a post vaccination diary card and counselled to observe and document fever, diarrhoea, vomiting, loss of appetite and any other significant symptoms constituting adverse events post immunisation over the following one week. An appointment card with information on the next expected scheduled clinic visit was also given to the mother.

The next visit (visit 2) was approximately 28 days after first dose Rotarix vaccination of the infant. At this visit, physical examination of the infant was performed and reported history of diarrhoeal episodes, any illness, medication, or clinic visit was recorded. Thereafter, anthropometric measures were taken from both mother and the infant, breastmilk sample (50ml) was collected from the mother and a passive raw stool sample from the infant if available. The infant was then administered the second dose of oral Rotarix vaccine and all routine EPI vaccines (OPV, DPT-HepB-Hib, and PCV) scheduled at the 10-week timepoint (See Table 3-1). Mothers were provided with a new post vaccination diary card and a date for the next scheduled visit on the appointment card. The third visit (visit 3) was approximately 28 days after the second Rotarix vaccine dose was given when infants were aged 14 weeks old. Study clinical procedures at this visit were identical to those conducted at visit 2 with the

exception that a whole blood sample (3ml) was also collected from the infant. This whole blood sample was used in the determination of seroconversion to Rotarix vaccination. At this visit, routine EPI vaccines OPV, DPT-HepB-Hib and PCV were administered to the infant (See Table 3-1). Subsequent clinic visits (visit 4 to 8) in the following five months were scheduled monthly. At these follow up visits, anthropometric measurements of both mother and infant and clinical history of infant including diarrhoeal episodes in the preceding month were recorded. Breastmilk samples (50ml) from the mother and a saliva and passive raw stool sample from the infant were also collected.

At the beginning of the RCT study, based on the targeted sample size, a random allocation list of 212 PID numbers grouped into control or experimental arms were generated by the study Statistician. Allocation cards for each PID were made and kept in sealed opaque envelopes identified only by the PID label at the CRS. Randomisation was implemented when infants attained 9 months of age and presented to the clinic (visit 9) during which PID labelled allocation cards specific for each infant were revealed. Half of the infants were randomised to receive a third dose of Rotarix vaccine coinciding with the EPI administration of measles-rubella (MR) vaccine (See Table 3-1). The other half of infants randomised into the control arm only received the MR vaccine at this visit. Infants who had missed birth dose polio vaccination (OPV) were also given the vaccine (OPV) at this timepoint. Prior to vaccination in each arm at visit 9, a whole blood (3ml), saliva and passive raw stool sample if available was collected from the infant. The next visits (visit 10 and 11) were done monthly during which anthropometric measures and clinical history in preceding month was recorded including a breastmilk sample (50ml) from the mother and passive stool from the infant.

When infants reached 12 months of age (visit 12), a whole blood sample (3ml) was collected from the infant in addition to the anthropometric measures, clinical history, breastmilk (50ml), and stool collection as per previous follow up visits. This blood sample was used to assess the immune boosting effect of the third dose of Rotarix. From this point onwards, stool samples were actively collected from infants every fortnight and anthropometric measures taken every three months (visit 15, 18, 21) until infants were 24 months old (visit 24) at which point a whole blood sample (3ml) was also collected from all infants. During this follow up period between visit 18 and 24, infants were also administered the second measles-rubella vaccine (MR). In the following year, similar procedures were undertaken, with the addition of whole blood sample (3ml) collection from infants when aged 30 months old (visit 30), up to when infants attained 36 months of age (visit 36) which was the study close out timepoint and at which a final whole blood sample was collected from both mother (5ml) and infant (3ml).

During the entire study follow up period, mothers were encouraged to bring the infant to the CRS whenever the infant was unwell and such visits were termed “unscheduled or sick visits” and recorded as adverse events. Presenting complaints, final diagnosis and treatment during these sick visits were documented by the study Clinicians in the adverse event logs. All serious adverse events were communicated to the local regulatory authority and the RCT data and safety monitoring committee (DSMB) within the defined timelines.

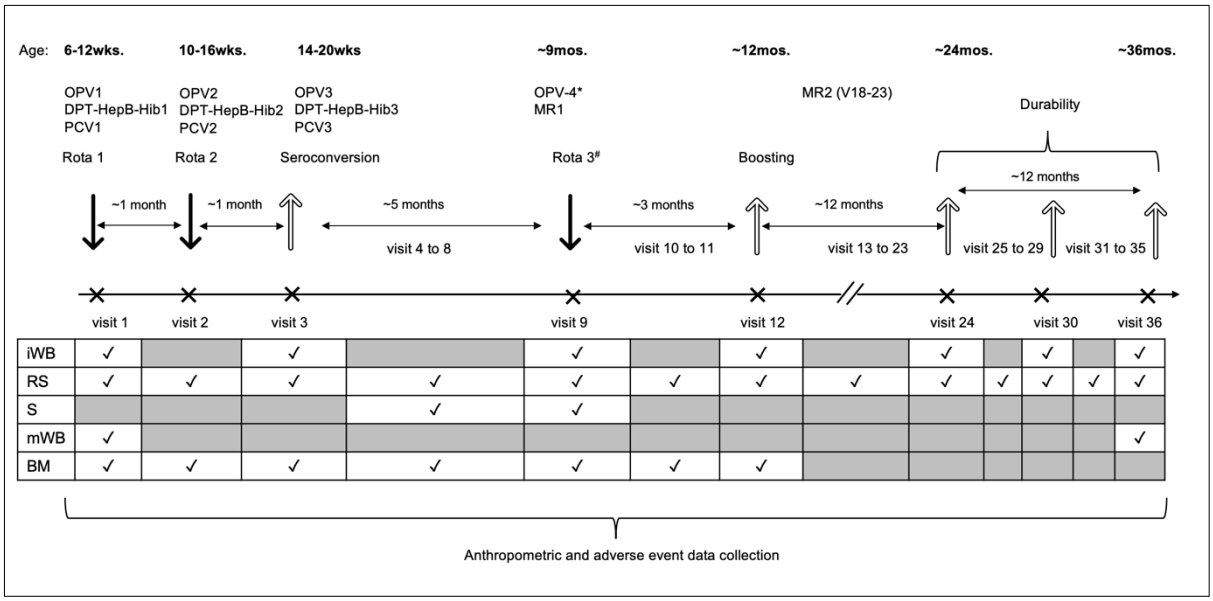


**Figure 3-1. RCT study schema**

Infants were enrolled at ages 6 to 12 weeks (n=214) and sampled for 3ml whole blood (iWB), raw stool (RS) and saliva (S) while enrolled mothers were sampled for 5ml whole blood (mWB) and 50ml breastmilk (BM) at specified timepoints before and after Rotarix and other routine EPI vaccinations. Vaccine seroconversion was assessed one month post receipt of two-doses of Rotarix. Boosting effect was assessed 3 months post-receipt of experiment third dose of Rotarix. Note: *administered only to infants who did not receive a birth dose polio vaccination; #administered to infants randomised to the experimental arm

A subset of the infants enrolled under the RCT had additional blood sampling (1ml) collected prior to receipt of the first, second and third dose (experimental arm) of Rotarix or first measles vaccines (control arm) and within seven days post vaccination respectively to enable assessment of early immune responses to vaccination. Specifically for this subset, peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples collected at all timepoints up to the age of 12 months. This PBMC cohort of infants was the second half of infants that were enrolled in the ROVAS-2 trial as they randomly presented to the study site, that is infant participant number 102 to 214 or n=113 infants. As shown in Figure 3-2, the PhD

research made use of plasma obtained from the whole blood samples of all infants for rotavirus, HCMV and HCoV including SARS-CoV-2 antibody testing by enzyme linked immunosorbent assay (ELISA). PBMC samples were used for T-cell phenotyping.

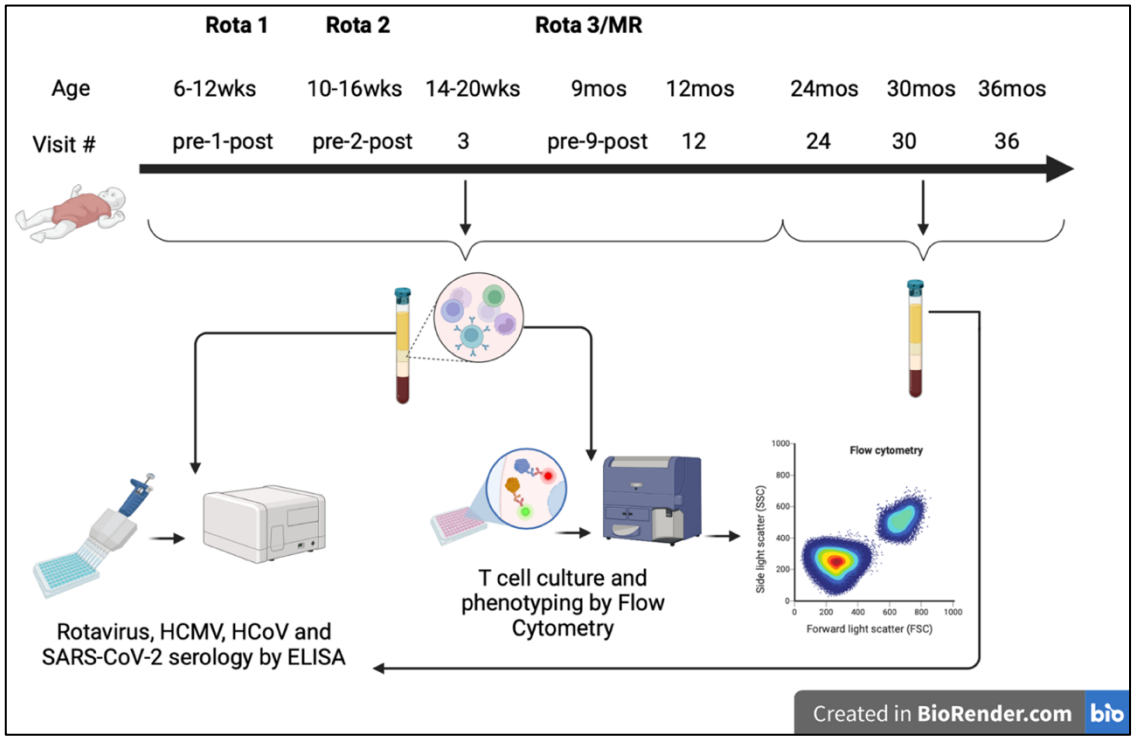


**Figure 3-2. PhD testing schema**

A subset of the infants enrolled under the RCT were additionally sampled for 1ml whole blood (iWB) immediately before and 7 days after first, second and third Rotarix vaccination (experimental arm) or measles-rubella vaccination (control arm). Plasma and peripheral blood mononuclear cells (PBMC) were obtained from infant whole blood samples. Plasma from all infants (n=214) was used to measure rotavirus, human cytomegalovirus (HCMV) and human coronavirus (HCoV) immunoglobulins by enzyme immunoassays. PBMC from the infant subset (n=113) were used to measure innate and adaptive immune cell subsets (including T cells, Natural Killer cells and unconventionall T cells by flow cytometry. Abbreviations: Rota1 (Rotarix dose 1); Rota 2 (Rotarix dose 2); Rota 3 (Rotarix dose 3); (MR (measles- rubella). Created with BioRender.com.

## 3.7 Laboratory assays

### Plasma and PBMC isolation

Whole blood samples were collected from infants via venepuncture into ethylenediaminetetraacetic acid (EDTA) anticoagulant treated vacutainers (Becton Dickinson, and Company, USA). All blood samples were transported from the CRS at GHC to the CIDRZ

research laboratory within 4 hours from the time of blood draw. Plasma and PBMC were isolated from whole blood using density gradient centrifugation. The 3 ml or 1ml whole blood samples were layered onto Histopaque® 1.077 g/ml density media (R8889, Sigma-Aldrich, Germany) at a 1:1 ratio in a 15 ml or 5ml tube respectively. Layered blood samples were then centrifuged for 30 minutes at 1,700 revolutions per minute (rpm), 20° Celsius, maximum acceleration and no brake. Following centrifugation, plasma at the topmost layer of the gradient fractions was reserved as 'plasma' and aliquoted into screw capped microtubes. A minimum of two plasma aliquots were made for all blood samples collected and all plasma was stored at minus 20° Celsius.

PBMC from the gradient separation were then collected from above the Histopaque® layer and transferred to a new 15ml or 5ml tube respectively. Collected PBMC were then resuspended in Roswell Park Memorial Institute (RPMI) – 1640 media (R0883, Sigma Aldrich, Germany) supplemented with 1% volume/volume of 100x Penicillin-Streptomycin-L-Glutamine (PSG) (10378016,ThermoFisherScientific, USA). Resuspended PBMC were mixed by inversion and centrifuged for 10 minutes at 1,800rpm, 20° Celsius, maximum acceleration and medium brake. Following centrifugation, the supernatant media was carefully decanted off in one smooth action without disturbing the PBMC pellet. For the 3ml whole blood sample derived PBMC, the pellet was loosened by flicking the tube, resuspended in 5ml media, and filtered through a 40µm cell strainer (542040, Greiner Bio-One International GmbH, Austria) using additional 5ml media to make a total 10ml resuspension volume. For the 1ml whole blood derived PBMC, the pellet was loosened by flicking the tube and resuspend in 1ml media without filtration. Next, PBMC were counted by mixing 50µL of PBMC resuspension with 550µL of 1 x phosphate buffered saline (PBS) reagent (BSS-1005-B, Sigma Aldrich, Germany ) in a counting chamber before counting using an automated Vi-CELL™ XR cell count, and viability analyser (Beckman Coulter, USA) based on the trypan blue dye exclusion method. Cell counts and viability for each PBMC sample were recorded using the Vi-CELL™ XR machine printouts. After cell counting, remaining PBMC suspension was centrifuged for 7 minutes at 1,700rpm, 20° Celsius, maximum acceleration and medium brake. The supernatant was decanted off and the PBMC pellet loosened by flicking the tube. PBMCs were then resuspended in 500µL and 250µL media for the 3ml and 1ml whole blood derived cells respectively, resulting in a concentration of between 4x10^6/ml and 1.2 x10^7/ml. Resuspended cells were transferred into Nunc™ CryoTube™ vials (Thermo Scientific, USA) placed in a cold Nalgene® Mr. Frosty™ rate controlled freezing container (5100-0001, ThermoFisher Scientific, USA) and an equal volume of cold freezing media containing sterile filtered 20% dimethyl sulfoxide (DMSO) (D2650, Sigma Aldrich, Germany) in fetal calf serum (FCS, 10500064, Gibco, Paisley, UK) was added. The loaded Mr. Frosty™ freezing container was then immediately placed in minus 80° Celsius freezer overnight before transfer to

cyroboxes and placement in temperature monitored, vapour phase, liquid Nitrogen storage tanks. PBMC samples were cryopreserved in the liquid Nitrogen prior to testing.

Rotavirus IgA ELISA

As illustrated in Figure 3-3, plasma rotavirus-specific immunoglobulin A (IgA) antibodies were measured using a quantitative sandwich ELISA protocol and lysate from WC3 rotavirus strain and mock infected African green monkey kidney (MA104) cell line. This was an in-house ELISA assay set up at CIDRZ post training and technology transfer from Christian Medical College (CMC) Vellore, India. The assay used anti-rotavirus IgG as a capture antibody to bind to the rotavirus antigen in the viral lysate. The bound rotavirus antigens were then used to capture the anti-rotavirus antibody present in the test plasma sample. Detection of the anti-rotavirus IgA was achieved with biotinylated secondary anti-human IgA antibody and amplified by colour development from an enzymatic reaction of avidin-biotin horseradish peroxidase complex with substrate. The concentration of the anti-rotavirus IgA in the plasma test sample was then calculated from a standard curve generated using a provided reference pooled plasma that had an assigned quantity of anti-rotavirus IgA. The ELISA assay was performed at the CIDRZ research laboratory. The rotavirus viral and cell lysate was provided by CMC while the reference pool rotavirus IgA standard was provided by Monica McNeal from Cincinnati Children's Hospital Medical Centre (CCHMC). An in-house rotavirus IgA standard was created from pooled plasma obtained from Zambian adult donors and the concentration of the in-house standard was extrapolated from the CCHMC standard. This in-house standard was then calibrated and used for the subsequent ELISA testing.

Initially a 96-well microtitre assay plate (655061,Greiner Bio-One International GmbH, Austria ) was coated with 100µL of rabbit anti-rotavirus IgG capture antibody (ABIN308233, Antibodies-online, USA) that was diluted 1000-fold in sodium carbonate- (S7795, Sigma Aldrich, Germany) bicarbonate (GRM849, HIMEDIA® Laboratories, Germany) coating buffer solution at pH 9.6±0.2. The coated plate was then sealed with plastic film and incubated overnight at 4° Celsius in a refrigerator. Plates were then washed five times with 200µL/well 1X wash buffer. The 1X wash buffer was prepared from a 5X concentrated stock phosphate-buffered saline buffer solution containing sodium chloride (GRM031, HIMEDIA® Laboratories, Germany), potassium phosphate monobasic (GRM249, HIMEDIA® Laboratories, Germany), sodium phosphate dibasic (S0876 Sigma Aldrich, Germany) and 0.25% Tween® 20 (P1379, Sigma Aldrich, Germany) (PBST). Thereafter, a 50µL/well volume of rotavirus cell lysate and cell lysate diluted 2-fold in 1% weight/volume skim milk (GRM1254, HIMEDIA® Laboratories,

Germany) in PBST (1% blotto) was added to the assay plate in alternating columns and incubated for 1hour minutes at 37° Celsius on a rotating platform set to 270-rpm. After lysate incubation, the plate was washed five times with 200µL/well 1X wash buffer. Next, 50µL/well of eight two-fold serial dilutions of rotavirus IgA standard starting at 12.5 Units/mL (U/mL), four two-fold serial dilutions starting at 1:20 to 1:160 of test plasma, high, medium and low positive controls samples and blank (1% blotto) were added to appropriate to the plate according to the test plate layout and the plate was sealed and incubated for 1hour minutes at 37° Celsius on a 270-rpm rotating platform.

Following this sample incubation, the plate was washed five times with 200µL/well of the 1X wash buffer before addition of 50µL/well of biotinylated rabbit anti-human IgA (SAB3701234, Sigma Aldrich, Germany) diluted 1500-fold in 1% blotto. The plate was then sealed and incubated for 30 minutes at 37° Celsius on a 270-rpm platform. After this incubation step, the plate was again washed five times with 200µL/well of 1X wash buffer followed by addition of 50µL/well avidin-biotin-peroxidase complex solution diluted in 1X wash buffer. This complex was prepared using a commercially available VECTASTAIN® kit (PK-4000, Vector Laboratories, USA). The plate was incubated for 30 minutes at 37° Celsius on a 270-rpm rotating platform. Finally, the plate was then washed five times with 200µL/well of 1X wash buffer and once with 200µL/well citric acid phosphate buffer prepared by dissolution of citric acid monohydrate (C1909, Sigma Aldrich, Germany) and sodium phosphate dibasic (S0876, Sigma Aldrich, Germany) in distilled water. This wash step was followed by addition of 50µL/well of o-Phenylenediamine dihydrochloride (OPD) substrate (reference number P5412, Sigma Aldrich, Germany) diluted in the citric acid phosphate buffer to the plate and incubation for 30 minutes at room temperature (between 20 and 25°Celsius) protected from light for color development. The color development was stopped by addition of 100µL/well of 1M sulphuric acid to the plate. The absorbance was read immediately at 492nm wavelength using an Epoch 2 microplate reader (Agilent formerly BioTek, South Africa) and linked computer with Gen5 software.

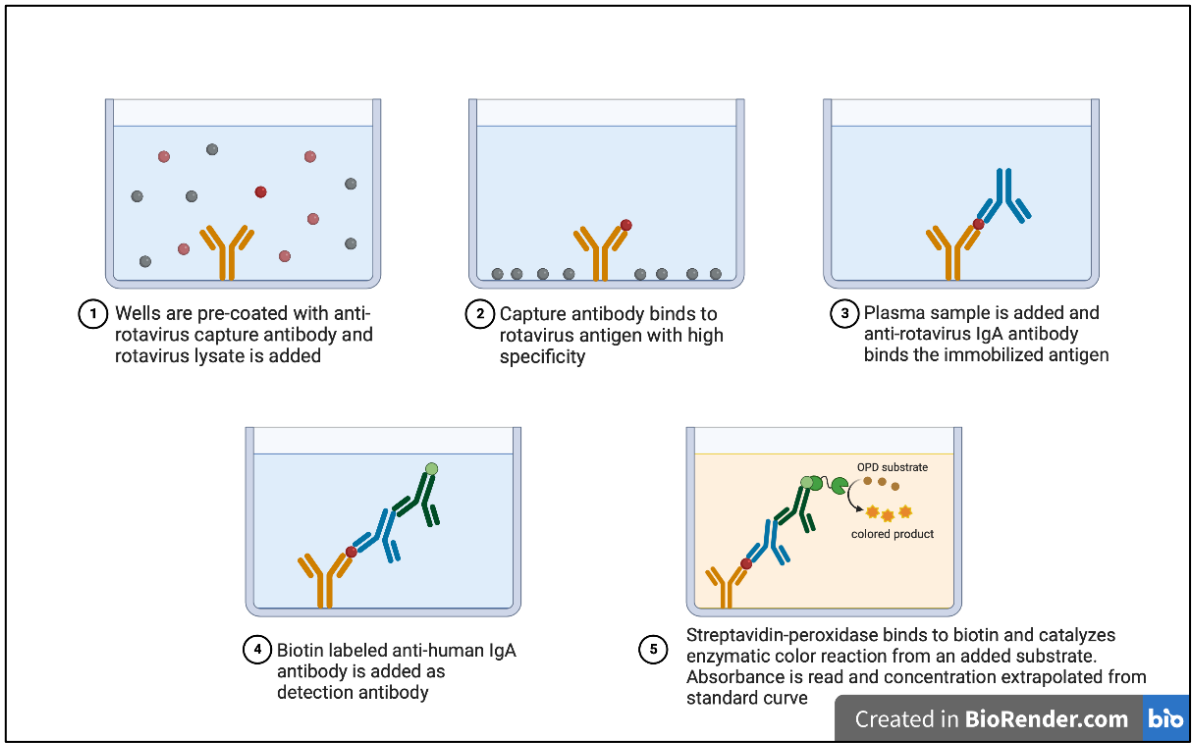


**Figure 3-3. Illustration of in-house rotavirus IgA immunoassay**

Detection of rotavirus immunoglobulin A was done using an ELISA method. Microtitre wells were coated with rotavirus capture antibody (1) which bound rotavirus antigen present in added viral lysate (2). Rotavirus specific antibodies in infant plasma was detected by binding to the captured antigen (3) and quantity measured by absorbance readings resulting from a complex with biotinylated anti-human secondary antibody (4) and streptavidin-peroxidase reaction with substrate (5). Created with BioRender.com.

Calculation of rotavirus antibody titres from the absorbance readings and relative to the standard curve was done using automated software (available from MyAssays.com). Test sample titres were reported in U/mL and accepted if the readings from two consecutive sample dilutions out of the four sample dilutions fell within the linear quantifiable range of the standard curve with a percent coefficient of variation (%CV) that was ≤20% and the mean titre result was reported. Samples whose values fell below the lower limit of quantification (LLOQ) for the assay were reported with titres half of the lowest detectable titre. Samples whose values fell above the quantification range of the standard were re-tested at higher dilutions. Seropositivity was defined as an RV-IgA titre ≥20U/mL while seroconversion was defined as a four-fold or greater change in RV-IgA titre one month after second Rotarix® dose if pre-vaccination titre was < 20 U/mL.

HCMV ELISA

HCMV specific IgM antibodies were measured using commercially available ELISA kits (Demeditec Diagnostics GmbH, Germany and Alpha Diagnostic International, USA) (according to manufacturer's recommendations. Diluted plasma samples were tested in parallel with the assay controls provided in the kit on a microtiter plate pre-coated with HCMV antigen. The assay was developed using horseradish peroxidase (HRP)-conjugated anti-human IgM and 3,3'5,5' Tetramethylbenzidine (TMB) substrate. Absorbances were read at 450nm wavelength in microplate reader. Plasma HCMV-IgM serostatus was reported as seropositive or seronegative using the cut-off control values for each assay and kit.

Coronavirus ELISA

The HCoV and SARS-CoV-2 IgG antibodies were measured using in house ELISA and the following coronavirus recombinant antigens obtained commercially (Sino Biologicals Incorporation, China): SARS-CoV-2 Spike S1-His recombinant protein (40591-V08H), HCoV NL63 Spike S1 subunit, His Tag (40600-V08H), HCoV OC43 Spike S1 protein, His Tag (40607-V08H1), HCoV 229E Spike S1 protein subunit, His Tag (40601-V08H) and HCoV HKU1 Spike S1 protein subunit, His Tag (40021-V08H).

For the SARS-CoV-2 ELISA, a 96-well microtiter plate (655061, Greiner Bio-One) was coated with 50μL of the SARS-CoV-2 recombinant protein at a concentration of 1μg/ml in sodium carbonate-bicarbonate buffer pH 9.6±0.2. and incubated overnight at 4°Celsius. The following day, 200μL/well of 1% blotto was added to the plate wells to block any unbound sites and the plate was sealed with film and incubated at room temperature for 3 hours. The plate was then washed five times with 200μL/well of PBST after which 50μL/well of 100-fold diluted plasma sample, positive, negative and blank (1% blotto) control was added. Eight 2-fold serial dilutions of plasma from a known SARS-CoV-2 infected adult were also included in each assay plate as reference sample. All test plasma samples, controls and reference were assayed in duplicate, and the plate was incubated overnight at 4°Celsius. Following another five washes with 200μL/well of PBST, 50μL/well of peroxidase-conjugated goat anti-human IgG antibody (A0170, Sigma Aldrich, Germany) diluted 1:15000 in PBST was added to each well and plate was incubated for 3 hours at room temperature. Thereafter, plate was washed a further five times with 200μL/well of PBST, once with 200μL/well citric acid phosphate buffer pH 5±0.2 before addition of 50μL/well of OPD substrate (P4664, Sigma Aldrich, Germany) diluted in citric acid phosphate buffer. Colour development for 20 minutes protected from light and stopped by addition of 100μL/well 1M Sulphuric acid. The absorbance was read at 492nm wavelength using the Epoch2 microplate reader (Agilent, formerly BioTek, South Africa). Test

sample absorbance readings were reported as relative absorbance units relative to the reference sample (rAU).
Measurement of HCoV HKU1, OC43, NL63 and 229E IgG was performed in a similar manner to the SARS-CoV-2 ELISA. The exception was that coating recombinant antigen concentration used for each HCoV was 0.5µg/mL and the inclusion of plasma controls with known exposure to the four HCoVs to each assay plate.

PBMC recovery

To recover PBMC from cryopreservation in liquid Nitrogen, PBMC samples were thawed in media (RPMI with 1% PSG) pre-warmed to 37° Celsius. To thaw, 1mL of the pre-warmed media from a 15mL tube was pipetted into the PBMC vial immediately after retrieval from liquid Nitrogen, gently mixed and solution transferred back into the 15ml tube. This was repeated until the PBMC was completely thawed and transferred. Thereafter, the thawed PBMC were centrifuged at 1,800 rpm for 10 minutes at 20°Celcius with maximum acceleration and brake applied. Supernatant was discarded and PBMC pellet resuspended in 10ml of media followed by a second centrifugation at 1,700rpm for 7 minutes at 20°Celcius with maximum acceleration and brake applied. Resulting supernatant was discarded, the pellet resuspended in 3ml of media and PBMC counting done using light microscope and trypan blue exclusion method for viability. to obtain quantity of recovered PBMC. Based on the PBMC/mL that was recovered, a final resuspension in appropriate volume of media was done to obtain the required concentration for cellular assays. PBMC samples for the flow cytometry assay that were obtained from 3mL and 1mL whole blood were resuspended to obtain a minimum 5x10^6 PBMC/ml up to 1x10^7 PBMC/mL and 5x10^5 or 1x10^6 PBMC used for the assay respectively.

In-vitro T cell stimulation and activation induced marker (AIM) assay by flow cytometry

For the in-vitro T cell culture and stimulation, 1x10^6 (or 5x10^5) PBMC in a volume of 100uL/well was added to a 96-well U-bottomed culture plate (Cat 163320, ThermoFischerScientific, USA). This was followed by addition of 50uL/well of 20% heat inactivated human AB serum (H4522, Sigma Aldrich, Germany) diluted in media (RPMI 1% PSG) and 50uL/well of rotavirus VP6 peptide pool (PSACM0039-01, Mimotopes Pty Ltd, Australia), HCMV pp65 peptide pool (donated by Dr. Clive Michelo), PHA mitogen (L4144, Sigma Aldrich, Germany) or 1% PSG supplemented RMPI with a DMSO concentration equivalent to that in the rotavirus VP6 peptide pool as the vehicle control (unstimulated control) to respective wells. The plate was thereafter incubated at 37°Celcius 5% $CO_2$ for 20 hours.

The following day, the plate was centrifuged at 1,800rpm for 5 minutes, supernatant removed then plate was vortexed to release pellet. The pellet was resuspended in 200μL/well FACS buffer (1 x PBS, 5mM EDTA, 1% Sodium Azide) and the centrifugation wash and release by vortex repeated. Next, 10μL/well of 1:50 diluted Fc blocker (130-059-901, Miltenyi Biotec Ltd. UK) was added to the plate and incubated for 5 minutes at room temperature. This was followed by addition of 10μL/well activation induced marker (AIM) antibody panel cocktail and incubation in refridgerator at 4°Celcius for 30 minutes. As shown in Table 3-1, the AIM antibody panel cocktail comprised allophycocyanin-cyanine7 (APC-Cy7) conjugated CD14 (398708, Biolegend USA) and CD19 (302218, Biolegend USA) as monocyte and B-cell dump channels respectively, eFluor780 fixable Viability Dye which was on same channel as APC-Cy7 (65-0865-14, eBiosciences, ThermoFischerScientific, UK), fluorescein-isothiocyanate (FITC) conjugated alpha-beta TCR (αβTCR FITC) (306706, Biolegend, USA), peridinin chlorophyll protein-cyanine5.5 (PerCPCy5.5) conjugated CD4 (344608, Biolegend USA), phycoerythrin (PE) conjugated CD69(310906, Biolegend USA), phycoerythrin-cyanine7 (PE-Cy7) conjugated CD134 (350012, Biolegend USA) and allophycocyanin (APC) conjugated CD137 (309810, Biolegend, USA) anti-human antibodies to enable phenotypic characterisation into activated CD4 and CD8 T cell phenotypes.

**Table 3-2. In-vitro T cell activation induced marker (AIM) antibody panel**

| **Antibody** | **Phenotype** |
|---|---|
| CD14 APC-Cy7 | Monocytes (dump channel) |
| CD19 APC-Cy7 | B-cell (dump channel) |
| Viability Dye-eFluor780 | Viability (same channel as dump) |
| αβTCR FITC | αβ T cell |
| CD4 PerCP-Cy5.5 | CD4 T cell |
| CD69 PE | Activation |
| CD134 PE-Cy7 | Activation |
| CD137 APC | Activation |

Next, 200μL/well FACS buffer was added to the plate, spun at 1,800rpm for 5 minutes after which supernatant was discarded, and pellet was released by vortex. This was followed by addition of 75μL/well CytoFix Perm solution (51-2090KZ BD Biosciences, USA), a solution containing 4.2% formaldehyde and used in this assay for the main purpose of fixing the cells to stabilise them prior to flow cytometry acquisition, and incubation for 15 minutes at room temperature in the dark. Next, 175μL/well Perm wash buffer (554723, Beckton Dickinson, USA) was added to the plate and spun at 2000rpm for 5 minutes, supernatant discarded, and pellet released by vortex once more. Finally, 150μL/well of FACS buffer was added to plate and mixed by pipetting before transfer to 1.2ml microtubes tubes (QS845, Alpha Laboratories, UK) containing 100μL FACS buffer to give a final resuspension volume of 250μL. Resuspended stained PBMCs were acquired on a 6-colour BD FACSVerse™ Flow Cytometer (Reference number 651154 Beckton Dickinson, USA) and the flow cytometry standard (FCS) files imported and gating analysed in FlowJo™ Software (Beckton Dickinson, Belgium). Statistical analysis and graphical presentation were done using GraphPad Prism 9.0 (GraphPad Software, USA).

Determination of rotavirus VP6 specific T cell responses

Data on the αβTCR+ CD4+ (CD4) and αβTCR+ CD4+ AIM+ T cell frequency and count and on the αβTCR+ CD4- (CD8) and αβTCR+ CD4- (CD8) AIM+ T cell frequency and count acquired from the Rotavirus VP6, HCMV pp65 and PHA stimulated samples from baseline, 1 month post second Rotarix dose, at 9 months before the third Rotarix dose and/or MR vaccination and three months post third Rotarix and/or MR at 12 months old was exported from FlowJo™ Software as excel files for statistical analysis.

First, a quality control check based on the αβTCR+ CD4+ (CD4) T-cell count acquired from the DMSO background and antigen stimulated wells was done on the exported data. Samples with less than 500 CD4 T-cells were excluded. Samples included in the subsequent analysis had an average acquired CD4 T cell count of 19748 for the DMSO background, 21513 for rotavirus VP6, 20942 for HCMV pp65 and 12318 for PHA stimulated wells respectively.

Rotavirus VP6, HCMV pp65 and PHA antigen specific CD4+CD134+CD137+ (CD4+AIM+) and CD8+CD69+CD137+ (CD8+AIM+) T-cell responses were determined for each child at each timepoint after subtraction of the corresponding DMSO background frequency and in combination with a calculated stimulation index. The steps taken in the determination of

antigen specific T-cell response for each infant at each timepoint is exemplified for rotavirus as follows:

1. The CD4+ AIM+ T cell frequency in the DMSO background wells was subtracted from the rotavirus VP6 specific CD4+ AIM+ T-cell frequency. The resulting rotavirus VP6 specific CD4+ AIM+ T-cell frequency was the "net rotavirus VP6 specific CD4+ AIM T-cell frequency".
2. Infants were categorized into whether they had a detectable net rotavirus VP6 specific CD4+ AIM+ T-cell frequency (net frequency greater than zero) or if they did not (net frequency equal to or less than zero. Net frequency values were less than zero in cases were the DMSO background frequency was higher than the rotavirus specific frequency)
3. In parallel, a rotavirus VP6 specific stimulation index was calculated as the rotavirus VP6 specific CD4+ AIM+ T-cell frequency divided by the DMSO background CD4+ AIM T-cell frequency.
4. A rotavirus VP6 specific CD4+ AIM+ T cell responder was defined by the below criteria combination:
    a) A detectable net rotavirus VP6 specific CD4+ AIM+ T cell frequency AND
    b) A rotavirus VP6 specific CD4+ AIM+ T cell frequency equal to or greater than one and a half times that of the DMSO background CD4+ AIM+ T-cell frequency (i.e. Stimulation Index $\geq$1.5)
5. A rotavirus VP6 specific CD4+ AIM+ T cell non-responder was defined by the below criteria combination:
    a) No detectable net rotavirus VP6 specific CD4+ AIM+ T-cell frequency OR
    b) A detectable net rotavirus VP6 specific CD4+ AIM+ T-cell frequency AND
    c) Net rotavirus VP6 specific CD4+ AIM+ T-cell frequency was less than one and a half times that of the DMSO background CD4+ AIM+ T-cell frequency (or Stimulation Index <1.5)

These steps were also followed for CD8 AIM+ T-cells and were done for the other antigens HCMV pp65 and PHA.

Ex-vivo NK and T-cell phenotyping by flow cytometry

Ex-vivo NK and T cell phenotyping was conducted in parallel with the in-vitro stimulation assay. A 20uL/well PBMC volume containing 2x10^5 cells were added to a 96-well U-bottomed culture plate (Cat 163320, ThermoFischerScientific, UK) followed by 150uL/well volume of FACS buffer. The plate was spun at 1,800rpm, for 5 minutes at 20°Celcius with

maximum brake and acceleration to pellet PBMC after which supernatant was discarded and PBMC pellet was released by vortex, resuspended in 10uL/well Fc blocker (diluted 1:50 in FACS buffer) and incubated for 5 minutes at room temperature. Next, 10uL/well of ex-vivo NK and T cell (conventional and atypical) antibody panel cocktails were added to appropriate wells of the plate and incubated in refridgerator at 4°Celcius for 30 minutes. As shown in Table 3-2, a total of five ex-vivo antibody cocktail panels were used to phenotype innate NK, conventional and unconventional T cells using anti-human antibodies comprising CD3 FITC (344804, Biolegend USA), CD8 PE (344706, Biolegend USA), CD56 PE-Cy7 (335826, BD Biosciences USA), CD8 APC (344722, Biolegend USA), CD25 PE-Cy7 (302612, Biolegend USA), CCR9 PE-Cy7 (358910, Biolegend USA), γδTCR APC (331212, Biolegend USA), Ki67 PerCP-e eFluor710, same channel as PerCP-Cy5.5 (ThermoFisher eBioscience, USA), CD161 PE-Cy7 (339918, Biolegend USA), β7 PerCP-Cy5.5 (121008, Biolegend USA), Vδ1TCR PE-Vio770 with same channel as PE-Cy7 (130-137-697, Miltenyi Biotec UK), Vα7.2TCR APC (351708, Biolegend USA), CD4 PE (344606, Biolegend USA), CD57 APC (359610, Biolegend USA) and NKG2C PE (FAB138P-100, Biotechne Ltd UK).

The change in frequencies of these ex-vivo immunophenotypes was investigated at seven timepoints post-vaccination in comparison to baseline and between vaccine seroconverters and non-seroconverters. For the comparisons across the multiple timepoints, the observed ex-vivo frequencies were log transformed to allow for repeated measures analysis in GraphPad Prism using a mixed model approach. In this log transformation process, ex-vivo frequencies with a value of zero yielded undefined values in the statistical software and thus, to facilitate the repeated measures analysis, ex-vivo data were recoded as outlined in the steps below:

1. Ex-vivo T cells frequencies for a particular visit (e.g. baseline) and subset type (e.g. CD4+CD25+) were sorted in order of lowest to highest value.
2. The lowest ex-vivo T cell frequency value above zero (e.g. 0.01) was identified and half of that value (0.005) was calculated.
3. The ex-vivo T cell frequency that was originally zero was replaced with this newly calculated frequency value (0.005) and then the log transformed value obtained and used in the repeated measures analysis.
4. Graphing was done using original ex-vivo T cell frequency.

**Table 3-3. Ex- vivo NK and T cell antibody staining panel.**

| Panel | Antibody | Phenotype |
|---|---|---|
| 1 | CD3 FITC | Pan T cell |
| | CD4 PE | CD4 T cell |
| | CD8 APC | CD8 T cell |
| | CD25 PE-Cy7 | Activation/Regulatory |
| | Ki67 PerCP-Cy5.5 | Proliferation |
| | Viability dye eFluor780 | Viability |
| 2 | CD3 FITC | Pan T cell |
| | CD4 PE | CD4 T cell |
| | CD8 APC | CD8 T cell |
| | CCR9 PE-Cy7 | Intestinal homing |
| | β7 PerCP-Cy5.5 | Intestinal homing |
| | Viability dye eFluor780 | Viability |
| 3 | CD3 FITC | Pan T cell |
| | CD56 PE-Cy7 | Pan NK cell |
| | CD57 APC | Differentiation |
| | NKG2C PE | Activating receptor |
| | Ki67 PerCP-Cy5.5 | Proliferation |
| | Viability dye eFluor780 | Viability |
| 4 | CD3 FITC | Pan T cell |
| | CD8 PE | CD8 T cell |
| | Vα7.2TCR APC | Mucosal associated invariant cells |

| | | |
|---|---|---|
| | CD161 PECy7 | Mucosal associated invariant cells |
| | Ki67 PerCP-Cy5.5 | Proliferation |
| | Viability dye eFluor780 | Viability |
| 5 | CD3 FITC | Pan T cell |
| | CD8 PE | CD8 T cell |
| | γδTCR APC | γδ T cell |
| | Vδ1TCR PE-Vio770 | Vδ1 subset |
| | Ki67 PerCP-Cy5.5 | Proliferation |
| | Viability dye eFluor780 | Viability |

This incubation was followed by addition of 200uL/well of FACS buffer and the plate was spun at 1,800rpm, for 5 minutes, supernatant discarded, and pellet released by vortex. A 50uL/well volume of Fix Perm solution was then added, and plate incubated for 30 minutes at room temperature in the dark. Next, 175μL/well Perm wash buffer was added to the plate and spun at 2000rpm for 5 minutes, supernatant discarded, and pellet released by vortex again. In panel 2 only, 150μL/well FACS buffer was added to the respective wells and resuspended PBMC transferred to 1.2ml microtubes tubes (QS845, Alpha Laboratories, UK) containing 100μL FACS buffer to give a final resuspension volume of 250μL. For all other ex-vivo panel wells (panels 1, 3, 4 and 4), a10μL/well volume of Ki67 cocktail (diluted in Perm wash buffer and neat Fc blocker) was added and incubation done in the dark for 30 minutes. After incubation, 200μL/well Perm wash buffer was added to the plate and spun at 2000rpm for 5 minutes, supernatant discarded, and pellet released by vortex once more. Finally, 150μL/well of FACS buffer was added to plate wells before transfer to 1.2ml microtubes tubes containing 100μL FACS buffer to give a final resuspension volume of 250μL. Resuspended stained PBMCs were acquired on a 6-colour BD FACS Verse flow cytometer (Reference number 651154 Beckton Dickinson, USA) and data analysed using FlowJo™ Software (Beckton Dickinson, Belgium) and GraphPad Prism 9.0 (GraphPad Software, USA).

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# RESEARCH PAPER COVER SHEET

**Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.**

**<u>SECTION A – Student Details</u>**

| | | | |
|---|---|---|---|
| **Student ID Number** | 1402541 | **Title** | Miss |
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| **Surname/Family Name** | Laban | | |
| **Thesis Title** | Antibody and T-cell immune responses in rotavirus vaccinated Zambian infants: impact of human cytomegalovirus infection | | |
| **Primary Supervisor** | Martin Goodier | | |

**If the Research Paper has previously been published please complete Section B, if not please move to Section C.**

**<u>SECTION B – Paper already published</u>**

| | | | |
|---|---|---|---|
| Where was the work published? | MDPI Vaccines | | |
| When was the work published? | 3rd February 2023 | | |
| If the work was published prior to registration for your research degree, give a brief rationale for its inclusion | N/A | | |
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## Chapter 4: Evaluation of ROTARIX® booster dose vaccination at 9 months for safety and enhanced anti-rotavirus immunity in Zambian children: a randomised controlled trial

This chapter contains a research article published in MDPI Vaccines 2023

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## 4.1 Abstract

Oral rotavirus vaccines show diminished immunogenicity in low-resource settings where rotavirus burden is highest. This study assessed the safety and immune boosting effect of a third dose of oral ROTARIX® (GlaxoSmithKline) vaccine administered at 9 months of age. A total of 214 infants aged 6 to 12 weeks were randomised to receive two doses of ROTARIX® as per standard schedule with other routine vaccinations or an additional third dose of ROTARIX® administered at 9 months old concomitantly with measles/rubella vaccination. Plasma collected pre-vaccination, 1 month after first- and second-dose vaccination, at 9 months old before receipt of third ROTARIX® dose and/or measles/rubella vaccination, and at 12 months old were assayed for rotavirus-specific IgA (RV-IgA). Geometric mean RV-IgA at 12 months of age and the incidence of clinical adverse events 1 month following administration of the third dose of ROTARIX® among infants in the intervention arm were compared between infants in the two arms. We found no significant difference in RV-IgA titres at 12 months between the two arms. Our findings showed that rotavirus vaccines are immunogenic in Zambian infants but with modest vaccine seroconversion rates in low-income settings. Importantly, however, a third dose of oral ROTARIX® vaccine was shown to be safe when administered concomitantly with measles/rubella vaccine at 9 months of age in Zambia. This speaks to opportunities for enhancing rotavirus vaccine im-munity within feasible schedules in the national immunization program.

## 4.2 Introduction

Diarrhoeal disease is ranked third among the global leading causes of morbidity and mortality in young children, responsible for approximately 1.53 million deaths and contributing to over 80 million disability-adjusted life years, most of which occur within Sub-Saharan African children aged below 5 years (1). Among several infectious aetiologies of diarrhoeal disease, rotavirus is the most common cause of moderate to severe and less severe diarrhoea (2, 3) and the leading cause of diarrhoeal disease-associated mortality that has been attributed to 128,515 deaths in a single year in this population (4).

The orally administered and widely introduced rotavirus vaccines ROTARIX® (GlaxoSmithKline) and RotaTeq® (Merck) have proved important early life interventions in mitigating the diarrhoeal disease burden in this population, with substantial reductions in rotavirus-associated and all-cause diarrhoea morbidity and mortality observed since their introduction (5). However, vaccine immunogenicity and efficacy when administered in early infancy is consistently lower and variable in highly burdened and high mortality settings with several reasons postulated (6, 7). Improved vaccine performance is an important way in which rotavirus infections that occur even among vaccinated infants can be further prevented. For these oral rotavirus vaccines to provide maximal benefit in these settings, it is important to assess potential methods to enhance the immunogenicity of existing vaccines as their routine use continues.

Removal of the age restrictions for child vaccination and alternate schedules with booster doses of existing oral rotavirus vaccines have the potential to improve vaccine performance with benefits outweighing potential associated intussusception risks (8, 9). Modelling data predicts enhanced anti-rotavirus immunity from booster dose administration at 9 or 12 months of age and prevention of up to 19,600 additional rotavirus-associated deaths in the second year of life annually (10). Administration of monovalent ROTARIX® and pentavalent RotaTeq® concomitantly with measles vaccine at 9 months has been demonstrated to induce significantly increased anti-rotavirus antibody titres without interference with measles seroresponses in Bangladeshi and Malian infants, respectively (11, 12).

Zambia introduced the ROTARIX® vaccine in 2013 and recorded a seroconversion rate of 60.2% (13). Although a significant decline in rotavirus-attributable childhood diarrhoea has been recorded especially in infants (14), it remains necessary to further reduce residual infection and disease burden. Newer oral rotavirus vaccines have been evaluated in our setting with similar low rates of vaccine seroconversion observed (15). To date, no study has been conducted on safety and potential immunogenicity benefits of a booster ROTARIX® vaccine dose administered at 9 months of age in Zambian infants.

We aimed to assess a booster dose of ROTARIX® vaccine administered at 9 months of age as an alternative to the current two-dose schedule to enhance anti-rotavirus immunity in Zambian infants.

## 4.3 Methods

The study was a single-centre, open-label, randomised, controlled trial assessing the safety and immunogenicity of a booster dose of the monovalent ROTARIX® vaccine at 9-month infant age. We anticipated a 15% or greater increase in log10 RV-IgA response after the booster ROTARIX® dose. Using a two-sample t-test and assuming equal SD at 5% level of significance, we therefore required a total of 196 infants (98 per arm) to detect an increase to 3.13 log10 RV-IgA due to the booster dose of ROTARIX® at an 80% power. We made an upward sample size adjustment of 9% to account for potential loss to follow-up to reach the total of 214 infants to be recruited in this study. The estimation was performed using Stata 14 MP "power" command (StataCorp™, College Station, TX, USA).

### Study Participant Selection and Enrolment

The study enrolled 214 infants aged 6 to 12 weeks from 13th September 2018 to 15th November 2018 at George Health Centre (GHC), a government-run peri-urban health facility serving a high-density, low-income population in Lusaka, Zambia. Mothers presenting with their infants for routine immunization visits were approached by study staff and sensitized about the study. Interested mothers were provided further study information at the clinical research site located within the GHC premises. Mothers that were willing to participate were individually taken through an informed consent process and simple comprehension assessment test in private rooms. Eligibility criteria included that the infant was aged 6 weeks to 12 weeks old; the mother participated voluntarily, provided written informed consent (with a witness in the case of illiterate participant) and agreed to all study procedures; and the mother was resident in the study area and willing to come for scheduled visits for the duration of the study. Infants were not eligible if they had a contraindication to rotavirus vaccination; previously received rotavirus vaccination; had a recent history of immunosuppressive therapy; had a recent history of blood or blood product transfusion; existing congenital anomalies; or any condition deemed by the study investigator to pose potential harm to the participants or jeopardize the validity of study results.

Study Procedures and Randomization

Enrolled mother-infant pairs were followed up until the infant was 36 months old. At baseline, eligible infants were randomised at a ratio of 1:1 using masked allocation into either the intervention arm to receive a booster dose of ROTARIX® concomitantly with measles/rubella (MR) vaccination or into the control arm to receive only MR vaccination at 9 months old. All children in the study received routinely administered first and second ROTARIX® vaccine doses (given from 6 weeks and ideally 4 weeks apart before the age of 2 years). ROTARIX® is an orally administered live, attenuated G1P [8] monovalent vaccine in routine use in Zambia. The batch number of ROTARIX® used in the study was AROLC044AA. Infants in both arms also received polio, Bacillus Calmette–Guérin (BCG), pentavalent diphtheria /pertussis/ tetanus/ Hepatitis B /Haemophilus influenza-type (DPT-HepB-Hib) and pneumococcal conjugate vaccines (PCV) according to the routine Zambian expanded immunization schedule.

Baseline sociodemographic and clinical data were collected from the participating mother/infant pairs. From all enrolled infants, whole blood samples (3–4 mL) were collected before receipt of the first ROTARIX® dose, 1 month after two-dose ROTARIX® vaccination, before receipt of MR vaccine (control arm) or MR and booster dose ROTARIX® at 9 months of age and when 12 months old. In a subset of infants, additional blood sampling was performed within 1 month after the first ROTARIX® dose. From baseline to the time the infant was 36 months old, anthropometric growth measurements were taken and data on incidence of clinical illness were recorded.

Immunogenicity Assessment

Plasma from whole blood samples was tested for anti-rotavirus immunoglobulin A (RV-IgA) titres using an adaptation of a published and validated sandwich enzyme-linked immunosorbent assay (ELISA) based on the use of WC3 rotavirus antigen and mock infected African green monkey kidney (MA104) cell lysate (16). All plasma testing for RV-IgA was performed at the Centre for Infectious Disease Research in Zambia Enteric Disease and Vaccine Research Laboratory in Lusaka, Zambia. In-house-generated pooled plasma from rotavirus-vaccinated adults was validated for use as the standard in the ELISA assay using pooled serum with known assigned RV-IgA units per millilitre (U/mL). The primary immunogenicity endpoint was the geometric mean titre of anti-rotavirus IgA at 12 months of

age. The study also investigated RV-IgA seropositivity and vaccine seroconversion using published definitions. Seropositivity was defined as an RV-IgA titre ≥ 20 U/mL. Seroconversion was defined as a four-fold or greater change in RV-IgA titre 1 month after dose two if pre-vaccination titre was < 20 U/mL (13).

Safety Assessment

All enrolled infants received ROTARIX® vaccination together with other routine vaccines as per the Zambian immunization schedule. Prior to vaccination, all participants were screened for any medical condition. Following vaccination, all infants were reviewed by the study staff to identify any immediate adverse events (AE). Participant mothers or guardians were provided with and trained in completing a post-vaccination diary card to record presence or absence of solicited AE including fever, diarrhoea, vomiting, loss of appetite and irritability over the next 5 days following immunization, which was returned to the study clinic at the next study visit. Mothers were also encouraged to bring the infant to the study clinic whenever the child was unwell, at which point standard of care was given and the presenting AE was recorded using structured case report forms. For the AE, information collected included but was not limited to the presenting symptoms, evolution of the presentation of symptoms, examination findings, investigations and drugs given (dosage, route, and duration). In the case of serious adverse events (SAE), every effort was made to make physical contact and access the medical records in the admitting health facility. For both AE and SAE, the infants were followed up until resolution whilst offering the necessary standard medical care. Once resolved, the study participant documents were updated accordingly and where required, the local authorities were updated accordingly as per regulatory guidelines. All SAE were also reviewed at regular intervals by the study Data Safety and Monitoring Board (DSMB) comprised of clinicians from the study, those independent of the study and reported to the relevant national regulatory authorities. During routine scheduled study visits to the clinic, mothers were also specifically asked about diarrhoea occurrence and any other illnesses that the infant may have had in the period preceding the visit. All stool samples passively collected from children presenting with diarrhoeal disease during clinic visits were tested for rotavirus. Genotyping was performed on all rotavirus-positive stool samples to determine infecting strains. We documented and described the incidence of clinical AE and SAE within a month following administration of the third dose ROTARIX® + MR and MR alone in infants in the intervention and control arm respectively as the primary measure on safety.

Statistical Analysis

For the immunogenicity analysis, the characteristics of participating infants at 9-month follow-up were tabulated for each arm. Analysis was based on the intention-to-treat population. In the primary analysis, we used two-sample t-test to test the difference in RV-IgA titre at 12-month infant age between the two arms. Linear regression model on log-transformed RV-IgA titre at 12-month infant age was used to estimate the geometric mean ratio and 95% confidence interval (CI), adjusted for potential confounders. p-values were considered significant at 5%. For the safety analysis, AE, and SAE incidence within 1 month after receipt of booster ROTARIX® dose and MR vaccine or MR vaccine alone were tabulated for each arm and 95% CI was calculated for the proportion of infants with any AE or SAE in each arm. All analyses were performed in Stata 17 MP (StataCorp, College Station, TX, USA) and R-Software.

## 4.4 Results

Participant Enrolments and Baseline Characteristics

As summarised in Figure 4-1, the study enrolled and randomised 214 infants between 13th September 2018 and 15th November 2018. Pre-vaccination whole blood was obtained from 211/214 (98.6%) enrolled infants at baseline. 170/214 (88/170 in intervention and 82/170 in control arm) infants had a clinic visit 28 days after their second dose of ROTARIX®. A total of 168 out of 214 (78.5%) infants attended and gave a whole blood sample at their 9-month-age study visit of which 88/168 (52.4%) infants were in the intervention (ROTARIX® + MR vaccination) arm and 80/168 (47.6%) infants in the control (MR vaccination) arm. Of these, 159/168 (94.6.2%), of which 85/159 (53.5%) and 74/159 (46.5%) were in the intervention and control arm, respectively, also attended and gave a whole blood sample at their 12-months-of-age study visit. Infants that had 9- and 12-months-of-age whole blood samples collected were included in the final analysis, whereas others were not included due to dropouts caused by mother's relocation from study site, withdrawal of consent, non-study related infant deaths, and losses to follow-up of participating mothers during follow-up period.

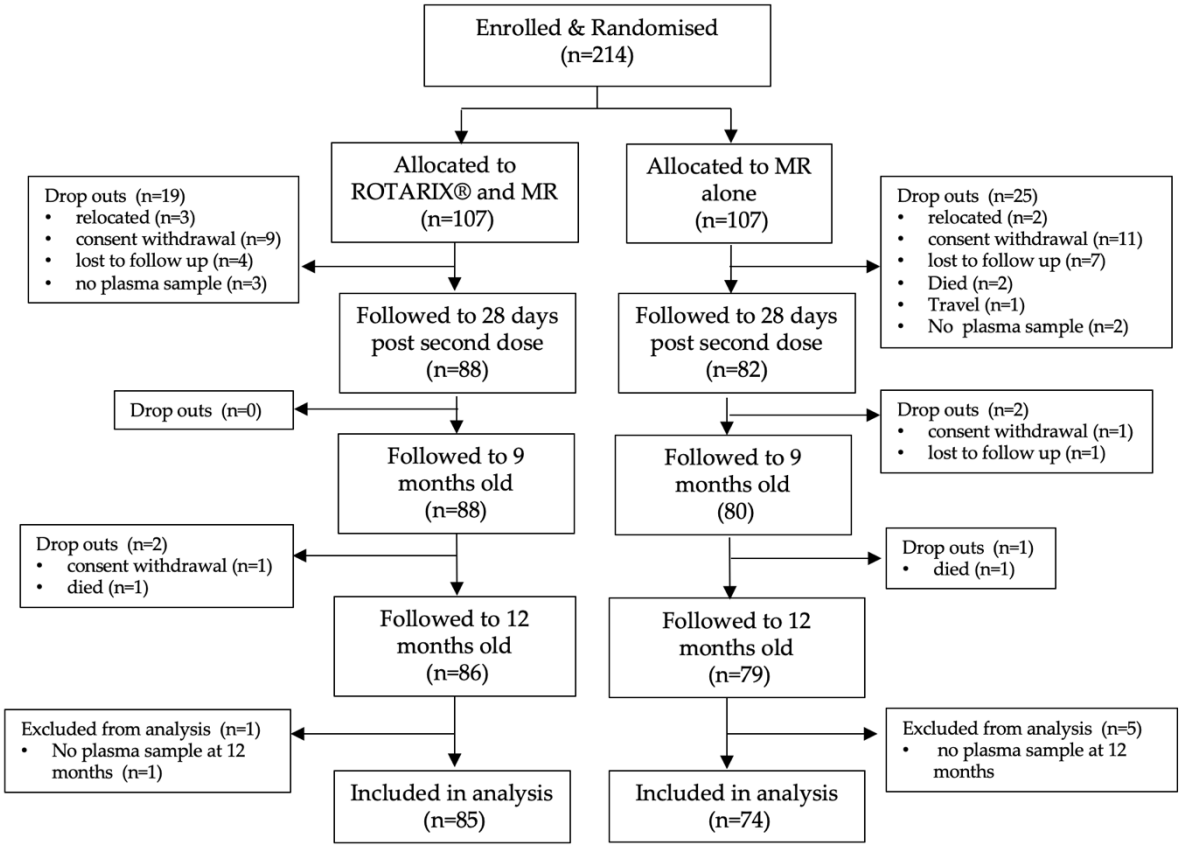


**Figure 4-1. Study participant flow chart**

Abbreviations: MR (measles/rubella vaccine).

As outlined in Table 4-1, infants were from low-income households with poor water sanitation and hygiene (WASH). Most of the infants were from households with shared toilet facilities and using public water sources. Infants at enrolment had a median age of 6 weeks, the majority were HIV unexposed, full-term with normal weight at birth, generally healthy and mostly breastfed. The RV-IgA seropositivity (RV-IgA titre ≥ 20 U/mL) rate was low at baseline at 4.8% overall and 3.5% and 6.3% in the intervention and control arms, respectively. There was no statistically significant difference in these baseline characteristics between the two study arms.

**Table 4-1. Baseline characteristics of mother/infant pairs by trial arm.**

| | Total Population (N = 168 a) | ROTARIX® + MR (n = 88) | MR (n = 80) |
|---|---|---|---|
| | n (%) | n (%) | n (%) |
| Infant Characteristic | | | |
| Age, weeks | | | |
| Median (IQR) | 6 (6–6) | 6 (6–6) | 6 (6–6) |
| Sex | | | |
| Male | 89 (53.0) | 38 (43.2) | 41 (51.3) |
| Female | 79 (47.0) | 50 (56.8) | 39 (48.8) |
| Gestation | | | |
| Pre-term | 11 (6.6) | 5 (5.7) | 6 (7.5) |
| Full-term | 157 (93.5) | 83 (94.3) | 74 (92.5) |
| Mode of Delivery | | | |
| Vaginal | 160 (95.2) | 84 (95.5) | 76 (95.0) |
| Caesarean | 8 (4.8) | 4 (4.6) | 4 (5.0) |
| Feeding | | | |
| Expressed/direct Breastmilk | 158 (94.1) | 83 (94.3) | 75 (93.8) |
| Mixed breast and formula | 10 (6.0) | 5 (5.7) | 5 (6.3) |
| Birth weight, kg (N = 167) | | | |
| <2.5 | 11 (6.6) | 3 (3.5) | 8 (10) |
| ≥2.5 | 156 (93.4) | 84 (96.6) | 72 (90.0) |
| Weight at enrolment, kg | | | |

| | | | |
|---|---|---|---|
| Mean (SD) | 4.6 (0.6) | 4.6 (0.6) | 4.7 (0.7) |
| Length at enrolment, cm | | | |
| Median mean (SD) | 54 (2.6) | 54 (2.7) | 54 (2.6) |
| Malnourished (WLZ<-2) (N = 167) | | | |
| No | 164 (98.2) | 85 (97.7) | 79 (98.8) |
| Yes | 3 (1.8) | 2 (2.3) | 1 (1.3) |
| Stunting (LAZ<-2) | | | |
| No | 138 (82.1) | 70 (79.6) | 68 (85.0) |
| Yes | 30 (17.9) | 18 (20.5) | 12 (15.0) |
| Wasting (WAZ<-2) | | | |
| No | 153 (91.1) | 79 (89.8) | 74 (92.5) |
| Yes | 15 (8.9) | 9 (10.2) | 6 (7.5) |
| HIV exposure | | | |
| Unexposed | 119 (70.8) | 60 (68.2) | 59 (73.8) |
| Exposed | 49 (29.2) | 28 (30.8) | 21 (26.3) |
| RV-IgA seropositive (N = 166) | | | |
| No | 158 (95.2) | 84 (96.6) | 74 (93.7) |
| Yes | 8 (4.8) | 3 (3.5) | 5 (6.3) |
| Maternal characteristics | | | |
| Age, years | | | |
| <20 | 23 (13.7) | 10 (11.4) | 13 (16.3) |
| 20–24 | 53 (31.6) | 29 (33.0) | 24 (30.0) |
| 25–29 | 51 (30.4) | 27 (20.7) | 24 (30.0) |

| | | | |
|---|---|---|---|
| ≥30 | 41 (24.4) | 22 (25.0) | 19 (23.8) |
| Parity | | | |
| Low parity (1–2) | 98 (58.3) | 50 (56.8) | 48 (60.0) |
| Multiparity (3–4) | 54 (32.1) | 27 (30.7) | 27 (33.8) |
| Grand multiparity (5+) | 16 (9.5) | 11 (12.5) | 5 (6.3) |
| Education level | | | |
| No education | 6 (3.6) | 5 (5.7) | 1 (1.3) |
| Some/complete primary | 55 (32.7) | 29 (33.0) | 26 (32.5) |
| Some/complete secondary | 102 (60.7) | 52 (59.1) | 50 (62.5) |
| Attended/completed university | 5 (3.0) | 2 (2.3) | 3 (3.8) |
| Monthly household income, ZMW | | | |
| <500 | 64 (38.3) | 35 (39.8) | 29 (36.7) |
| 500–1000 | 49 (29.3) | 25 (28.4) | 24 (30.4) |
| >1000 | 54 (32.3) | 28 (31.8) | 26 (32.9) |
| Share toilet facilities | | | |
| No | 33 (19.6) | 23 (26.1) | 10 (12.5) |
| Yes | 135 (80.4) | 65 (73.9) | 70 (87.5) |
| Source of water | | | |
| Public tap/pipe | 93 (55.4) | 45 (51.1) | 48 (60.0) |
| Piped into house/yard | 33 (37.5) | 33 (37.5) | 26 (32.5) |
| Yard/public borehole | 8 (4.8) | 3 (3.4) | 5 (6.3) |
| Protected/unprotected well | 8 (4.8) | 7 (8.0) | 1 (1.3) |

[a] Infants that attended the 9-month visit. Abbreviations: cm (centimetre); HAZ (height-for-age Z-score); HIV (human immunodeficiency virus); IQR (interquartile range); kg (kilogram); MR (measles/rubella vaccine); RV-IgA (rotavirus-

specific immunoglobulin A); WAZ (weight-for-age Z-score); WLZ (weight-for-length Z-score); ZMW (Zambian Kwacha).

Seroconversion Rates and Anti-Rotavirus IgA Titres in Two-Dose and Booster Dose ROTARIX® Vaccinated Infants

As shown in Figure 4-2, pre-vaccination mean RV-IgA antibody titres were low in the infants but increased after each ROTARIX® vaccine dose. Statistically significant increases in mean RV-IgA titres were observed between baseline and 1 month after the first dose of ROTARIX® in both the control arm ($p = 0.046$) and intervention arm (0.012). However, this increase was less apparent between the first and second doses for both control ($p= 0.447$) and intervention arms ($p = 0.068$). Interestingly, after two-dose vaccination, significant increases in RV-IgA titres in the control ($p = 0.001$) and intervention arms ($p < 0.001$) were observed by 9 months of age. Similarly, a significant increase ($p < 0.001$) in RV-IgA titres was seen in both arms by 12 months of age.

In general, mean RV-IgA antibody titres were similar in the intervention and control arms at baseline ($p = 0.06$), 1 month after the first dose ($p = 0.944$) and 1 month after the second dose ($p = 0.644$). Similarly, mean RV-IgA titres in the two arms at 9 months old were not significantly different ($p = 0.207$), but the mean RV-IgA titres at 9 months old showed a higher trend among infants in the intervention arm. At 12 months old, the difference in mean RV-IgA titres between the control and intervention arms did not reach statistical significance ($p = 0.688$).

Vaccine seroconversion approximately 1 month after two-dose ROTARIX® was low in this study population with 47/169 (27.8%) infants seroconverting, of which 25/47 (53.2%) were from the intervention arm and 22/47 (46.8%) from the control arm.

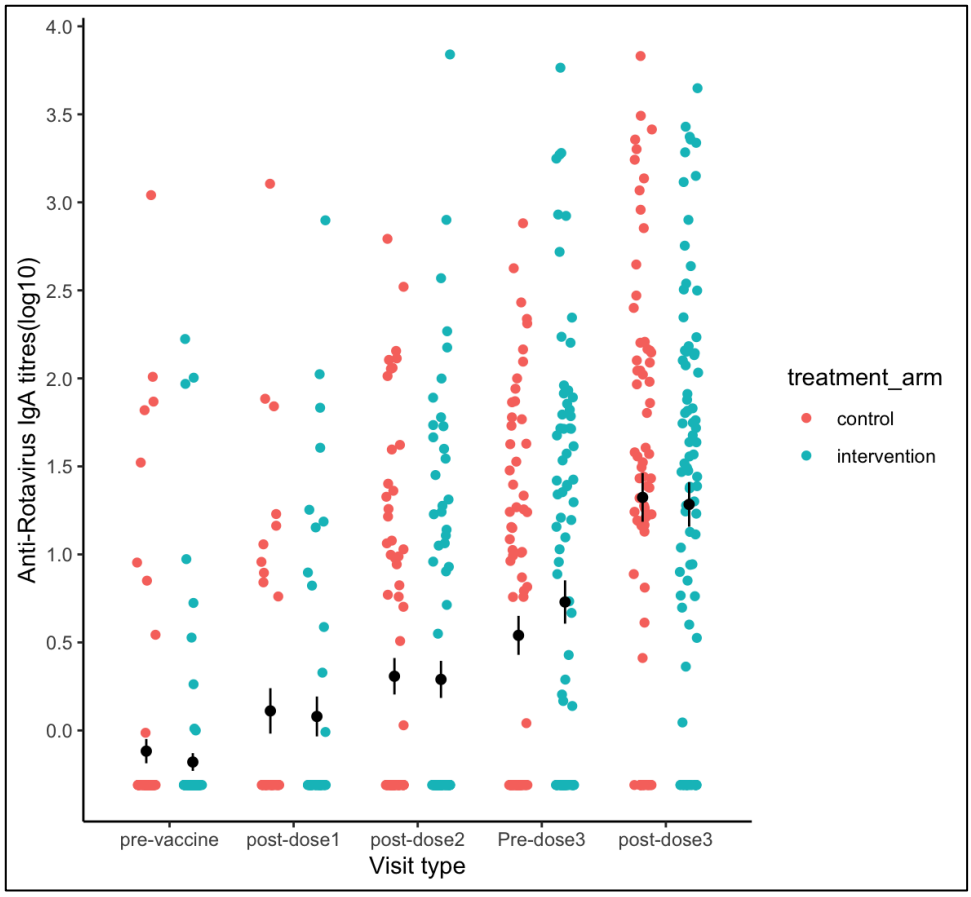


**Figure 4-2. Rotavirus specific immunoglobulin A (RV-IgA) responses.**

Trends in rotavirus-specific immunoglobulin A (RV-IgA) titres pre and post rotavirus vaccination compared between the control (red circle) and intervention (blue circle) arms. Each circle represents an infant's log10 RV-IgA titre. Black circle represents mean and standard error of log RV-IgA titre.

## Effect of Booster Dose ROTARIX® at 9 Months on Anti-Rotavirus IgA Geometric Mean Titres at 12 Months of Age

We observed no statistically significant differences in RV-IgA GMT ratios at 12 months of age between infants that received the third ROTARIX® vaccine dose and those that did not (Table 4-2).

**Table 4-2. Rotavirus IgA geometric titre mean ratio at 12 months by study arm.**

| Arm | N (% of total) | GMT (95% CI) | Two-sample t-test, p-value | GMT Ratio (95% CI) | p-Value | Adjusted GMT Ratio * (95% CI) | p-Value |
|---|---|---|---|---|---|---|---|
| MR | 74 (46.5) | 3.98 (3.50–4.51) | | 1 | 0.689 | 1 | 0.223 |
| ROTARIX + MR | 85 (53.5) | 3.85 (3.41–4.35) | 0.688 | 0.84 (0.35–2.00) | | 0.61 (0.27–1.35) | |

* Adjusted for malnutrition, sex, water source, income, pre-dose three RV-IgA titres using linear regression on log-transformed titres. Abbreviations: MR (measles/rubella vaccine); GMT (geometric mean titre).

Safety: Incidence of Adverse Events and Serious Adverse Events by Trial Arm

Primary safety assessment was conducted on infants who successfully attended the 9-months-of-age study visit and remained in follow-up 1 month thereafter. In these infants, respiratory tract illness (RTI) was the most common AE, followed by diarrhoeal disease with comparable incidence between the intervention and control arms (Table 4-3). Other AEs observed included conjunctivitis, dermatitis, candidiasis, febrile illness, emesis, and otitis with comparable incidences between the two arms (Table 3). Out of 76 stool samples that were passively collected from infants presenting with diarrhoea during unscheduled visits, 4 (5.3%) tested positive for rotavirus. Genotyping of 3 out of the 4 stool samples that had sufficient volumes revealed two G3 and one G4 genotype. Of the G3 genotype infections, one was in an infant in the intervention arm and the other was an infant in the control arm. The G4 genotype was observed in an infant from the control arm.

**Table 4-3. Incidence of adverse events within 1 month after third dose ROTARIX® (+MR) compared to MR vaccination.**

| Arm | Diarrhoea (n), Incidence * (95% CI) | RTI (n), Incidence (95% CI) | Conjunctivitis (n), Incidence (95% CI) | Dermatitis (n), Incidence (95% CI) | Candidiasis (n), Incidence (95% CI) | Febrile Illness (n), Incidence (95% CI) | Emesis (n), Incidence (95% CI) | Otitis (n), Incidence (95% CI) |
|---|---|---|---|---|---|---|---|---|
| MR | 8<br>3.33 (1.7–6.7) | 12<br>5.0 (2.8–8.8) | 1<br>0.4 (0.05–3.0) | 0 | 1<br>0.4 (0.06–3.0) | 1<br>0.4(0.05–3.0) | 3<br>0.8 (0.2–3.3) | 1<br>0.4 (0.06–3.0) |
| ROTARIX + MR | 4<br>2.4 (0.6–4.0) | 8<br>3.0 (1.5–6.1) | 2<br>0.8 (0.2–3.0) | 3<br>1.1(0.2–1.8) | 1<br>0.4 (0.1–2.7) | 1<br>0.4 (0.05–2.7) | 1<br>0.4 (0.05–2.7) | 0 |
| Rate ratio (95% CI), p-value | 1.75<br>(0.14–1.51),<br>0.186 | 1.23<br>(0.25–1.48),<br>0.268 | 1.82 (0.17–20.05),<br>0.620 | - | 0.91 (0.06–14.53),<br>0.946 | 0.91 (0.06–14.5)<br>0.946 | 0.46 (0.04–5.01), 0.509 | - |

* Incidence per 1000 infant days. Abbreviations: CI (confidence interval); MR (measles/rubella vaccine); RTI (respiratory tract illness).

Throughout the three-year study follow-up period, a total of 30 SAEs were recorded. Among these SAEs, 7/30 (23%) had acute gastroenteritis among the presenting symptoms. The study recorded four deaths among these SAE, of which three were in the control arm and one was in the intervention arm. Only two SAEs, one within each arm, occurred within 1 month after the intervention at 9 months. The SAE recorded in the control arm was acute gastroenteritis with severe dehydration in severe anaemia and failure to thrive. The SAE recorded in the intervention arm was acute gastroenteritis with severe dehydration. None of these SAEs recorded were related to the study (Table 4-4).

**Table 4-4. Occurrence of serious adverse events in intervention (ROTARIX® +MR) compared to control (MR) arm.**

| Arm | At Least One SAE,<br>Incidence * (95% CI) | At Least One Related SAE,<br>Incidence (95% CI) | Deaths |
|---|---|---|---|
| MR | 1<br>0.4 (0.06–3.0) | 0 | 3 |
| ROTARIX + MR | 1<br>0.4 (0.06–2.8) | 0 | 1 |
| Rate ratio,<br>p-value | 0.94 (0.06–15.0),<br>0.9633 | | |

## 4.5 Discussion

In this clinical trial, we assessed the safety and immune boosting effects of a third dose of ROTARIX® vaccine administered at 9 months of age. This is the first clinical trial assessing administration of this oral rotavirus vaccine in Zambia outside of the recommended age range and our data show that a third dose of ROTARIX® given at 9 months of age in Zambian infants is well tolerated. Our results are consistent with studies conducted elsewhere, where no difference in AE and/or SAE frequency was observed between intervention and control arms (11, 12).

We found no difference in geometric mean titres and ratios of anti-rotavirus IgA at 12 months of infant age in the intervention arm from a booster third dose of ROTARIX® vaccine given at 9 months compared to the control arm. This contrasts with findings from a study conducted in Mali where a three-fold or greater rise in RV-IgA and greater seropositivity rate 28 days after vaccination was seen among infants who received the booster dose of pentavalent ROTATEQ at 9 to 11 months of age (in addition to doses given at 6, 10 and 14 weeks of age) compared to those who did not. (12). Another study in Bangladesh also observed an increase in RV-IgA seropositivity and geometric mean titres in infants given a booster dose of ROTARIX® at 9 to 10 months when immunogenicity outcome was assessed 2 months later. This was in comparison to infants that received measles/rubella vaccine alone in which no apparent changes were observed (11). Both these studies made use of the same WC3 based ELISA methods as used in our current study.

A notable difference of these two studies with our study was that immunogenicity assessment was performed earlier at 1 month and 2 months after rotavirus booster vaccination, whilst our study measured the immunogenicity effect 3 months later. The peaking of RV-IgA tends to occur within 1 month after vaccination, and it is possible that the 3-month period in our study saw a waning of vaccine induced immune responses in the intervention arm such that by our outcome sampling timepoint RV-IgA levels became comparable to the control arm. We chose to assess boosting at 12 months of age as we believed the timepoint was close enough to detect a boosting effect and gave a window between blood sampling timepoints that reduced the frequency of blood draws.

Additionally, of note is the influence that natural rotavirus immunity may have on observed booster dose immunogenicity. The Malian study observed rise in RV-IgA seroresponses among infants who did not receive the additional ROTATEQ dose, suggesting natural rotavirus exposure may have contributed to a rise in titres (12). We observed similar increase in RV-IgA among infants who did not receive the third dose within the 3 months after intervention. This may indicate that infants in our study had exposure to wild-type infection and the exposure during the three-month period after intervention in our study may have factored into results observed between arms. In Mali, about half of the infants had RV-IgA titres below <20 U/mL (seronegative) prior to receiving the booster dose (12). In Bangladesh, pre-boost RV-IgA seropositivity was ~52.7%; however, an improvement in boosting effect was observed among infants that were seronegative pre-boost. In our study, higher levels of RV-IgA titres relative to post-two-dose vaccination were apparent in infants at 9 months of age with slightly higher levels in the intervention arm though difference did not reach significance. These higher pre-boost titres in the intervention arm could perhaps have influenced responses observed in diminishing immunogenicity of the booster dose. Nevertheless, differences in population ages, time post-boost and vaccines assessed (monovalent versus pentavalent) could also play roles in these contrasting findings.

This study had the opportunity to investigate pre-vaccination seropositivity and vaccine seroconversion as secondary immune measures. We found minimal baseline rotavirus seropositivity and low post-ROTARIX®-vaccination seroconversion rate comparable to estimates reported in a study performed within a similar population in the same setting (15). These findings show that while ROTARIX® vaccine is immunogenic among infants in our setting, the phenomenon of modest immunogenicity persists. Although our study was not designed to assess the protective effect of vaccination, rotavirus infections were present, and incidence of diarrhoea was among the commonly reported illnesses among vaccinated infants. Detected rotavirus infections were G3 and G4 non-vaccine strains. Whilst ROTARIX® is a monovalent vaccine containing G1P [8] strain protection against non-vaccine infecting strains has been shown (17). Nevertheless, detection of non-vaccine strains of rotavirus infections among ROTARIX® vaccinated infants may reduce the effectiveness of these vaccines within our settings and speaks towards the need for vaccines covering multiple strains. Such findings in this study emphasize need for continued surveillance of circulating rotavirus strains including other viral, bacterial, and parasitic enteric pathogens that may become important in the post-vaccine era.

Among the strengths of the study was the that it was a randomised control design and was conducted in a population in which rotavirus vaccines would be of most benefit. The local implementation of an ELISA method that is widely employed in other rotavirus vaccine trials elsewhere was another strength that enabled comparison of findings to other similar studies. Generally, there are limited studies assessing booster rotavirus vaccine doses at later ages in Africa and this study was the first to be performed in Zambia. Another strength was the ability in our study to demonstrate rotavirus immunity status of the children from pre-vaccination. Our study design enabled determination of pre-vaccination immune status and seroconversion rates after routine two-dose vaccination and accounting for this in our interpretations which was not done in the two studies conducted in Bangladesh and Mali (11, 12). This study design also allowed determination of seroresponses of the vaccine in different localities and sub-population but within the same setting of Zambia by comparison to that performed previously when vaccination was introduced (13).

Notable study limitations included the high losses to follow-up encountered early during the trial which may have reduced the power to detect the boosting effect of the third dose. We measured RV-IgA as an immunogenicity outcome, and, while being the most widely utilised measure for rotavirus vaccine immunogenicity, it is a sub-optimal correlate (18). Measurement of other rotavirus-specific humoral and cellular immune responses to vaccination is necessary to further inform immunogenicity and potentially correlates of protection. We did not assess the potential impact of the third rotavirus vaccine dose on immunogenicity of the measles/rubella vaccine in our setting; however, studies conducted elsewhere have observed no influence of booster oral rotavirus vaccine given at this age on measles vaccine responses and attainment of sero-protection (11, 12).

## 4.6 Conclusion

Despite showing evidence that ROTARIX® vaccine is well tolerated at 9 months of age, our study findings do not support improved immunogenicity by 12 months of age from a booster dose vaccination at this age in our study setting. However further research is needed to generate stronger clinical evidence for policymakers. Evaluation of alternative vaccine

formulations for improved immunogenicity may be important in our setting to increased effectiveness and further reduce the burden of rotavirus.

## Author Contributions

Conceptualization R.C., S.B. and M.S.; methodology, R.C., S.B. and M.S.; formal analysis, S.B. and M.C.; investigation, N.M.L., A.C., M.C.-C., N.S., C.C., R.V., K.N., C.M. and I.M.; writing—original draft preparation, N.M.L.; writing—review and editing, R.C., S.B., M.S., M.C., A.C., M.C.-C., N.S., C.C., R.V., K.N., C.M., I.M. and M.R.G.; visualization, S.B. and M.C.; funding acquisition R.C.; All authors have read and agreed to the published version of the manuscript.

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## Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the University of Zambia Biomedical Research Ethics Committee (UNZABREC Ref: 003-02-18). The study also received approval from the Zambia Medicines Regulatory Authority (ZAMRA Ref: CT 078) and the National Health Research Authority (NHRA) prior to study initiation. The study was registered in the Pan African Clinical Trial Registry (PACTR) (Ref: PACTR201804003096919).

## Informed Consent Statement

Written informed consent was obtained from all subjects in-volved in the study.

## Data Availability Statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to institutional data policy re-strictions.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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# RESEARCH PAPER COVER SHEET

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| **Student ID Number** | 1402541 | **Title** | Miss |
| **First Name(s)** | Natasha Makabilo | | |
| **Surname/Family Name** | Laban | | |
| **Thesis Title** | Antibody and T-cell immune responses in rotavirus vaccinated Zambian infants: impact of human cytomegalovirus infection. | | |
| **Primary Supervisor** | Martin Goodier | | |

**If the Research Paper has previously been published please complete Section B, if not please move to Section C.**

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# Chapter 5: Human Cytomegalovirus Seropositivity and Its Influence on Oral Rotavirus Vaccine Immunogenicity: A Specific Concern for HIV-Exposed-Uninfected Infants

This chapter contains a research article published in Clinical and Experimental Immunology 2024


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## 5.1 Abstract

Oral rotavirus vaccines demonstrate diminished immunogenicity in low-income settings where human cytomegalovirus infection is acquired early in childhood and modulates immunity. We hypothesized that human cytomegalovirus infection around the time of vaccination may influence immunogenicity. We measured plasma human cytomegalovirus specific immunoglobulin M antibodies in rotavirus vaccinated infants from 6 weeks to 12 months old and compared rotavirus immunoglobulin A antibody titres between human cytomegalovirus seropositive and seronegative infants. There was no evidence of an association between human cytomegalovirus serostatus at 9 months and rotavirus specific antibody titres at 12 months (geometric mean ratio 1.01, 95%CI: 0.70,1.45; p=0.976) or fold-increase in RV-IgA titre between 9 and 12 months (risk ratio 0.999, 95%CI: 0.66,1.52; p=0.995) overall. However, HIV-exposed-uninfected infants who were seropositive for human cytomegalovirus at 9 months old had a 63% reduction in rotavirus antibody geometric mean titres at 12 months compared to HIV-exposed-uninfected infants who were seronegative for human cytomegalovirus (geometric mean ratio 0.37, 95%CI: 0.17, 0.77; p=0.008). While the broader implications of human cytomegalovirus infections on oral rotavirus vaccine response might be limited in the general infant population, the potential impact in the HIV-exposed-uninfected infants cannot be overlooked. This study highlights the complexity of immunological responses and the need for targeted interventions to ensure oral rotavirus vaccine efficacy, especially in vulnerable subpopulations.

## 5.2 Introduction

Rotavirus, a leading cause of diarrhoeal disease in children (1), remains a public health concern particularly in low- and middle- income countries (LMICs). The use of oral rotavirus vaccines (ORV) (2) has decreased the degree of diarrhoeal disease caused by rotavirus in children residing in LMICs especially in Africa (3, 4). The impact has been to bring down hospitalizations for rotavirus diarrhoea in those children aged 5 years and below (4). However, these vaccines demonstrate diminished seroconversion rates in LMICs, a phenomenon not yet fully understood (5). Zambia has seen a decrease in rotavirus diarrhoea since ORV introduction (6, 7), but low seroresponse rates persist, estimated between 27% to 60% (8, 9).

Researchers have pinpointed numerous factors that could play a role (5), but the impact of persistent viral infections when receiving the vaccinee is yet to be examined.

Human cytomegalovirus (HCMV), a beta-herpesvirus (10), is common and can be transmitted congenitally and during nursing across different regions including Africa (11). HCMV infection occurs early in childhood in Africa, with over 80% of infants infected by their first birthday (12). In Zambia, about 83% of infants acquire HCMV infection by 18 months of age (13). The high HCMV prevalence, its effects on host immunity and the observed poor ORV immunogenicity in these settings necessitate longitudinal studies to investigate temporal associations with childhood vaccine responses as argued by others (14).

Studies regarding HCMV's influence on immunogenicity of childhood vaccines in Africa are scarce and show inconsistent findings. For some vaccines such as measles, HCMV has been found to have no effect (15), beneficial effects (16, 17) but also associated with reduced immune responses (17). The effect of HCMV on other vaccinations like meningococcal (16), Hepatitis B (HepB) (15, 18), diphtheria-pertussis-tetanus (DPT) (15, 17-19), and Bacille Calmette-Guérin (BCG) (15, 19) has been conflicting, with studies showing varying associations with vaccine induced cellular and humoral responses. In Zambia, no significant associations between HCMV and oral polio vaccine antibody responses have been observed (20).

The current ambiguity in the direction of HCMV's influence on infant vaccine responses and the absence of data for ORV signal the need for additional research. This study explores HCMV-IgM seroconversion in the first year of life in Zambia and its effect on rotavirus specific antibody responses among rotavirus vaccinated infants. It addresses the complex relationship between HCMV and vaccine immunogenicity in the context of low-income settings, infant health, and current vaccination strategies, shedding light on an understudied yet vital area of pediatric infectious disease management.

## 5.3 Methods

### Ethics and consent

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) (reference number 003-02-18) and the London School of Hygiene and Tropical Medicine (LSHTM) Research Ethics Committee (reference number 16168). Informed consent was obtained from all study participants.

### Study design and participants

We conducted a longitudinal study nested within an open label, two-arm parallel group, randomised controlled trial (RCT). The RCT compared a two-dose (control arm) and three-dose (intervention arm) Rotarix™ vaccination schedule among Zambian infants. The details of the study design have been published elsewhere (21). Briefly, 214 infants aged 6 to 12 weeks were enrolled in the parent RCT and followed up until they were 3 years of age between 2018 and 2021. During the first year of follow up, all infants were given two doses of an ORV (Rotarix, GlaxoSmithKline) with the first dose administered from 6 weeks old and the second dose administered from 10 weeks old, along with polio, BCG, DPT-HepB-Hib, and pneumococcal conjugate vaccines as part of the regular Zambia national immunization schedule. When the infants reached 9 months of age, they were randomly assigned to either a control arm (receiving only a measles-rubella (MR) vaccination) or an intervention arm (receiving MR vaccine and a third dose of Rotarix).

Plasma samples were collected at specific intervals: at enrolment (baseline, aged 6-12 weeks) before the first Rotarix dose, one month after the second Rotarix dose (aged 14-20 weeks, when vaccine seroconversion was determined), at 9 months (before receipt of the third Rotarix dose and/or MR vaccine), and at 12 months (when the immune-boosting effect of third Rotarix dose was assessed) (Figure 5-1). These plasma samples were tested for rotavirus specific immunoglobulin A (RV-IgA) antibodies. RV-IgA seropositivity was defined as an RV-IgA titre ≥ 20 Units/millilitre. Vaccine seroconversion was defined as a four-fold or greater change in RV-IgA antibody titre one month after dose two of Rotarix if pre-vaccination titre was less than 20 U/mL (21).

For this nested study, infants with available RV-IgA results and sufficient plasma sample to test for HCMV-IgM antibodies at baseline and at least one of the three subsequent time points

up to 12 months of age were included. The design thus facilitated the examination of the association between HCMV infection and ORV immunogenicity by focusing on multiple factors and time points, enabling a comprehensive analysis of the relationship.

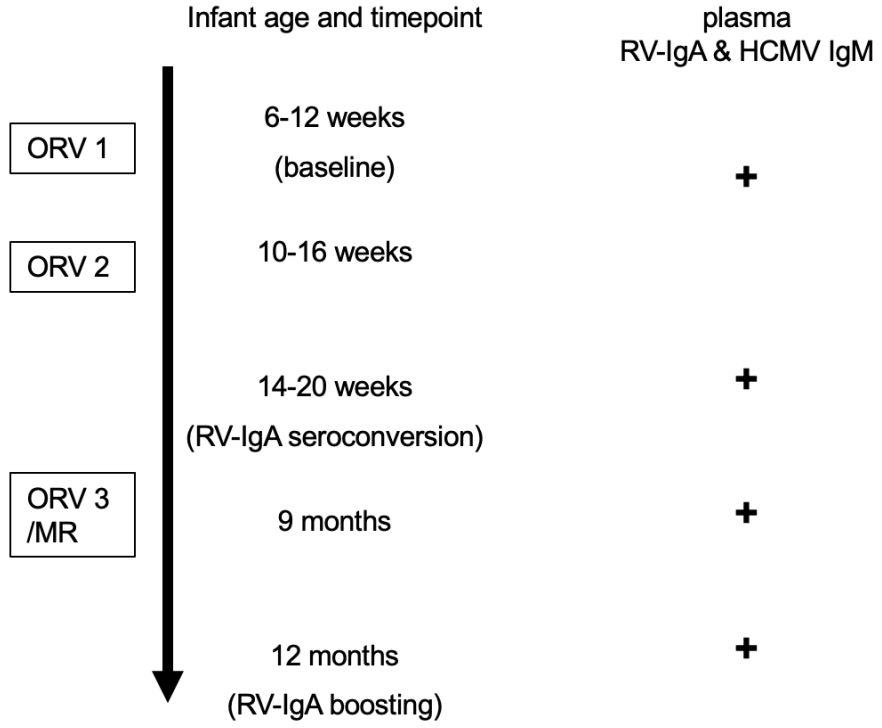


**Figure 5-1. Study design**

An illustration of the parent RCT study design and plasma collection timepoints

Laboratory procedures

Determination of HCMV serostatus: HCMV-IgM antibodies were measured using enzyme-linked immunosorbent assay (ELISA) kits from Demeditec Diagnostics GmbH (Germany) and Alpha Diagnostic International (USA). The procedure followed the manufacturer's guidelines. Infant plasma samples were diluted and added to a 96-well microtiter plate that had been pre-coated with a purified HCMV antigen. To detect any HCMV-IgM in these samples, they were treated with horse-radish peroxidase (HRP)-conjugated anti-human IgM. Subsequently, the tetramethylbenzidine (TMB) substrate was added, initiating an enzyme-substrate hydrolysis reaction, which resulted in colour development. The colour's absorbance was immediately measured at a 450nm wavelength using an Epoch 2 microplate reader by Agilent (South Africa). Proper quality control of each experiment was ensured using the calibrators and

controls provided in the kit. To check consistency in results, selected HCMV seropositive samples were tested with both kits. The test outcomes for HCMV-IgM serostatus in the plasma samples were categorised as either positive or negative based on specific cut-off control values for each experiment.

Quantification of rotavirus specific immunoglobulin A: The measurement of rotavirus-specific immunoglobulin A (RV-IgA) was carried out using a sandwich ELISA method, as outlined in the parent rotavirus vaccine trial (21). In the procedure, infant plasma samples were placed on a 96-well microtitre plate, which was coated with alternating columns of rotavirus infected and uninfected cell lysate. To detect the RV-IgA, the samples underwent a subsequent treatment with biotinylated anti-human IgA and an avidin-biotin-peroxidase complex. The addition of the o-Phenylenediamine dihydrochloride substrate initiated a colour change, whose intensity was measured at 492nm wavelength with a microplate reader. The concentration of RV-IgA was determined based on these readings, compared against a standard curve created from a known rotavirus IgA plasma standard.

Statistical analysis

Background characteristics were summarised with mean and standard deviation (SD) or median and interquartile range (IQR) for continuous variables. Categorical variables were summarised using frequency and proportion. Pearson's chi-square or Fisher's exact test was used to compare the distribution of categorical background characteristics by HCMV-IgM serostatus at 9 months and four-fold change in RV-IgA titres between 9 and 12 months. For RV-IgA titres at 12 months, we used student t test on log-transformed values. For anthropometric indices, we calculated z-scores using the 2006 World Health Organization child growth standards. The exposure of interest was HCMV-IgM serostatus at 9 month and the primary outcome was RV-IgA titres at 12 months. Secondary outcome was proportion with four-fold-change in RV-IgA titres between 9 and 12 months. The primary analysis was conducted among infants that had HCMV serostatus result at the 9 month time point and RV-IgA titre result at both the 9 month and 12 month timepoints.

We used linear regression model of log-transformed RV-IgA titres to estimate the effect of HCMV-IgM serostatus at 9 months or cumulative HCMV-IgM seroconversion by 9 months on RV-IgA GMT at 12 months, adjusting for potential confounders. P-values less than 0.05 were

considered statistically significant. The RV-IgA titre below the range of the standard curve were imputed as “1” prior to log-transformation. We used generalized linear model, adjusted for potential confounders, to estimate the effect of HCMV-IgM serostatus at 9 months or cumulative HCMV-IgM seroconversion by 9 months on the proportion with a four-fold or greater change in RV-IgA titres between 9 and 12 months. In a subgroup analysis, we used likelihood ratio test of interaction to investigate whether the effect of HCMV-IgM serostatus at 9 months on RV-IgA titre at 12 months varied by two-dose versus three-dose vaccination or by infant human immunodeficiency virus (HIV) exposure. In exploratory analyses, we examined the proportion of infants testing seropositive for HCMV-IgM for each time point and the relationship between HCMV-IgM point seropositivity or cumulative HCMV-IgM seroconversion and vaccine seroconversion after two dose vaccination. All analyses were performed in Stata 17 (StataCorp, College Station, TX, USA) and GraphPad Prism v9 (GraphPad Software, LLC).

## 5.4 Results

The parent Rotarix RCT enrolled and quantified RV-IgA titres for 214 infants of which 177 had sufficient plasma available at baseline and for at least one other timepoint; these were also tested for HCMV-IgM. Of these a total of 155/177 (88%) infants met the criteria for inclusion in our primary analysis and included HIV-exposed-infected, HIV-exposed-uninfected and HIV unexposed infants (Figure 5-2).

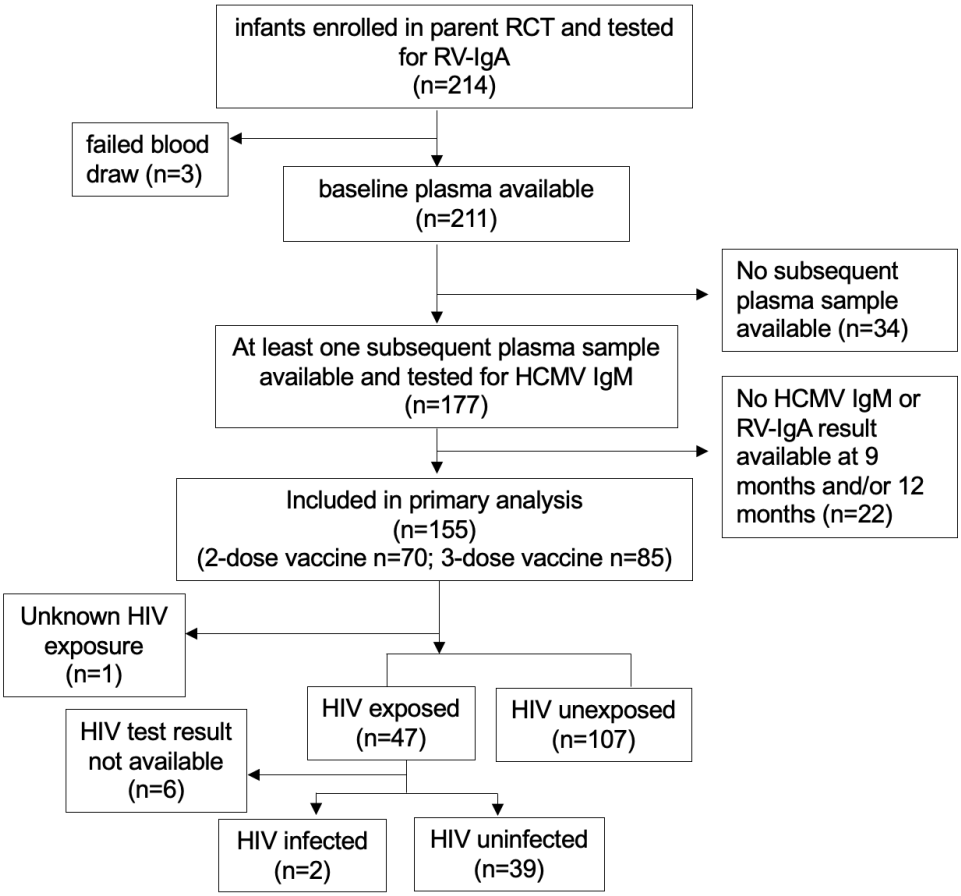


**Figure 5-2. Flow diagram of infant samples included in the study.**

A schema of the participant flow and criteria used in the selection of plasma samples and infant subgroups included in analysis.

## Infant characteristics

As shown in Table 5-1, among the infants included in the primary analysis (n=155), median age at baseline was 6 weeks, majority were vaginally delivered (95%) at full term (94%) , with normal birthweight (90%), and were predominantly breastfed exclusively (94%). Most infants came from homes with less than ideal water and sanitation, often sharing toilets with other households (79%) and getting water from public sources (65%) and approximately one third of infants had HIV-positive mothers. There were 60 HCMV-IgM seropositive and 95 HCMV-IgM seronegative infants at 9 months of age. There was no statistically significant relationship between baseline characteristics and HCMV-IgM serostatus at 9 months of age (Table 1).

**Table 5-1. Baseline characteristics by HCMV-IgM serostatus at 9 months.**

| | **HCMV-IgM serostatus at 9 months old** | | | |
|---|---|---|---|---|
| | | Seronegative | Seropositive | |
| **Characteristic** | N (% of total) | n (% of total) | n (% of total) | p- value |
| Age | | | | |
| Median(IQR) | 6 (6.6) | 6 (6.6) | 6 (6.6) | 0.927 |
| Sex | | | | |
| Female | 73 (47.1) | 41 (43.2) | 32 (53.3) | 0.249 |
| Male | 82 (52.9) | 54 (56.8) | 28 (46.7) | |
| Gestation | | | | |
| Full-term | 146 (94.2) | 89 (93.7) | 57 (95.0) | 1.000 |
| Pre-term | 9 (5.8) | 6 (6.3) | 3 (5.0) | |
| Mode of Delivery | | | | |
| Caesarean | 8 (5.2) | 4 (4.2) | 4 (6.7) | 0.712 |
| Vaginal | 147 (94.8) | 91 (95.8) | 56 (93.3) | |
| Feeding | | | | |
| Breastmilk | 145 (93.5) | 91 (95.8) | 54 (90.0) | 0.187 |
| Breastmilk + formula | 10 (6.5) | 4 (4.2) | 6 (10.0) | |
| Birth weight, kg (n=154) | | | | |
| <2.5 | 16 (10.4) | 10 (10.5) | 6 (10.2) | 1.000 |
| ≥2.5 | 138 (89.6) | 85 (89.5) | 53 (89.8) | |
| Stunting (LAZ <-2) | | | | |
| No | 129 (83.2) | 75 (79.0) | 54 (90.0) | 0.081 |

| | | | | |
|---|---|---|---|---|
| Yes | 26 (16.8) | 20 (21) | 6 (10.0) | |
| Malnourished (WLZ <-2) | | | | |
| No | 152 (98.1) | 92 (96.8) | 60 (100.0) | 0.284 |
| Yes | 3 (1.9) | 3 (3.2) | 0 (0.0) | |
| Maternal HIV (n=154) | | | | |
| negative | 107 (69.5) | 65 (68.4) | 42 (71.2) | 0.857 |
| positive | 47 (30.5) | 30 (31.6) | 17 (28.8) | |
| Toilet facility sharing across households | | | | |
| not shared | 32 (20.6) | 22 (23.2.7) | 10 (16.7) | 0.416 |
| shared | 123 (79.4) | 73 (76.8) | 50 (83.3) | |
| Water source | | | | |
| piped into household | 55 (35.5) | 35 (36.8) | 20 (33.3) | 0.731 |
| wells/public taps and boreholes | 100 (64.5) | 60 (63.2) | 40 (66.7) | |
| Number of children in household | | | | |
| 1-3 | 122 (78.7) | 76 (80.0) | 46 (76.7) | 0.872 |
| 4-6 | 29 (18.7) | 17 (17.9) | 12 (20.0) | |
| 7-9 | 4 (2.6) | 2 (2.1) | 2 (3.3) | |
| **Total** | **155 (100)** | **95 (61.3)** | **60 (38.7)** | |

We additionally assessed for associations between infant baseline characteristics and the primary outcomes of RV-IgA at 12 months old and secondary outcome of four-fold increase in RV-IgA titre between 9 and 12 months old. We found that infants residing in households that

did not share toilet facility had higher RV-IgA GMT at 12 months compared to infants from households with shared toilet facility (p=0.027) but for all other baseline characteristics, no statistically significant relationship was observed (Supplementary Table S5-1). There was no statistically significant relationship observed between infant baseline characteristics and four-fold or greater increase in RV-IgA titre between 9 and 12 months (Supplementary Table S5-2).

HCMV-IgM serostatus by age

To assess HCMV-IgM seropositivity by age, we included infants that had an HCMV-IgM result at all the four age timepoints 6-12 weeks, 14-20 weeks, 9 months and 12 months (n=148) out of the 177 that had a baseline and at least one follow-up sample collected. The proportion of infants that were HCMV-IgM seropositive at each age timepoint increased from 9.5% (14/148) at ages 6-12 weeks, to 27.0% (40/148) at 14-20 weeks, 37.2% (55/148) at 9 months and 59.5% (88/148) at 12 months (Figure 5-3A). We also assessed cumulative HCMV-IgM seroconversion with infants defined as HCMV-IgM seroconverters when they became HCMV-IgM seropositive after having HCMV-IgM seronegative results for all preceding timepoints. By 12 months old, the cumulative HCMV-IgM seroconversion was 79.1% (117/148) and 20.9% (31/148) infants were HCMV-IgM seronegative throughout (Figure 5-3B)

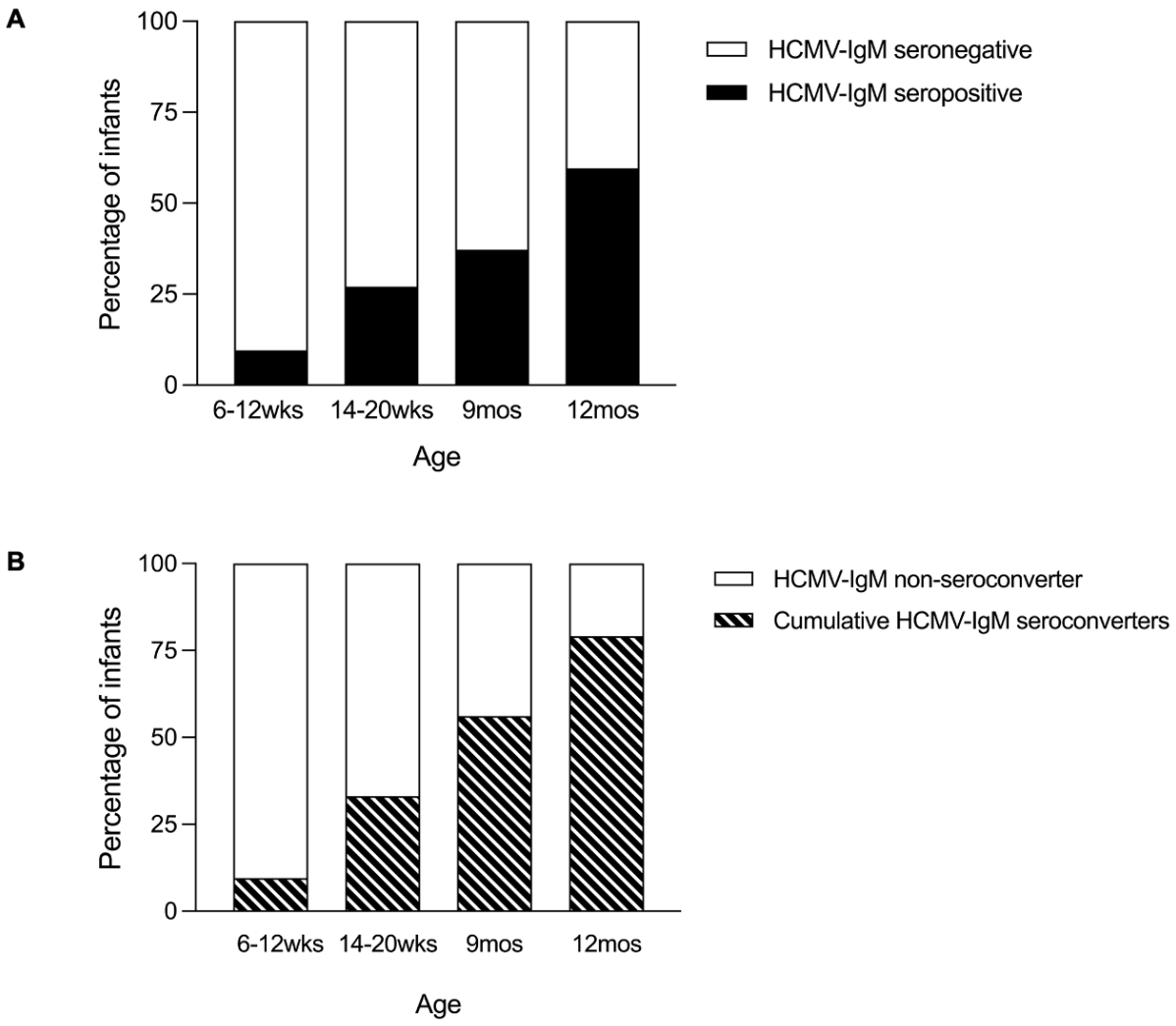


**Figure 5-3. Infant HCMV-IgM seropositivity by age**

The percentage of HCMV-IgM seropositive and seronegative infants (panel A) and cumulative HCMV-IgM seroconverters and non-seroconverters (panel B) at each age timepoint is shown as bars (n=148).

Effect of HCMV-IgM serostatus on rotavirus antibody response

In the overall study population (n=155), the RV-IgA GMT were 1.3 units/mL (95%CI: 1.1, 1.6) at 6-12 weeks (n=154), 3.2 units/mL (95%CI: 2.3, 4.4) at 14-20 weeks (n=149), 6.8 units/mL (95%CI: 4.7, 9.8) at 9 months (n=155) and 24.8 units/mL (95%CI:16.6, 36.9) at 12 months (n=155). There were 7/154 (4.6%), 27/149 (18%), 49/155 (31.6%) and 84/155 (54.2%) infants that were RV-IgA seropositive at 6-12 weeks, 14-20 weeks, 9months and 12 months respectively. A total of 148 infants had RV-IgA results at both 6-12 week and 14-20 weeks and among these, 40/148 (27.0%) were vaccine seroconverters and 108/148 (73%) were vaccine non-seroconverters.

At 12 months, the RV-IgA geometric mean titres (GMT) were 23.2 units/mL (95%CI: 12.32, 43.5) among infants seropositive for HCMV-IgM at 9 months and 25.8 units/mL (95%CI: 15.3, 43.7) among those that were HCMV-IgM seronegative. As shown in Table 5-2, irrespective of the number of vaccine doses and after adjusting for the potential confounding effect of sex, breastfeeding, stunting, wasting, and toilet facility, there was no statistically significant difference in RV-IgA GMT at 12 months between HCMV-IgM seropositive and HCMV-IgM seronegative infants at 9 months (geometric mean ratio (GMR) 1.01, 95%CI: 0.70,1.45; p=0.976). A four-fold or greater increase in RV-IgA titre between 9 months and 12 months of age was observed in 61/155 infants (39.4%) and 23/60 (37.3%) were from HCMV-IgM seropositive and 38/95 (40.0%) were from HCMV-IgM seronegative infants. Irrespective of the number of vaccine doses and after adjusting for the potential confounding effect of sex, breastfeeding, stunting, wasting, and toilet facility, there was no evidence of an association between HCMV-IgM serostatus at 9 months and the four-fold or greater increase in RV-IgA titre (risk ratio (RR) 0.99, 95% CI: 0.66,1.52; p=0.995) (Table 2). Similarly, we found no statistically significant relationship between cumulative HCMV-IgM seroconversion status by 9 months and RV-IgA GMT at 12 months (GMR 1.24, 95%CI: 0.86, 1.78; p=0.239) or a four-fold or greater increase in RV-IgA titre between 9 months and 12 months (RR 0.88, 95% CI: 0.59,1.32; p=0.539) (Table 5-2).

**Table 5-2. Effect of HCMV-IgM serostatus on RV-IgA titres and four-fold increase.**

| HCMV at 9 months | Number infants N (% of Total) | RV-IgA GMT at 12 months (95%CI) | RV-IgA *GMR at 12 months (95% CI) | p-value | Mounted ≥ four-fold rise in RV-IgA titre between 9 and 12 months. n (%) | * RR (95% CI) | p-value |
|---|---|---|---|---|---|---|---|
| HCMV-IgM point serostatus | | | | | | | |
| HCMV IgM - | 95 (61.3) | 25.8 (15.3,43.7) | 1 | | 38 (40.0) | 1 | |
| HCMV IgM + | 60 (38.7) | 23.2 (12.3,43.5) | 1.01 (0.70,1.45) | 0.976 | 23 (38.3) | 0.99 (0.66,1.52) | 0.995 |
| HCMV-IgM cumulative seroconversion | | | | | | | |
| HCMV-IgM ns | 67 (43.2) | 20.67 (11.7,36.6) | 1 | | 28 (41.8) | 1 | |
| HCMV-IgM s | 88 (56.8) | 28.4 (16.2,49.8) | 1.24 (0.86,1.78) | 0.239 | 33 (37.5) | 0.88 (0.59,1.32) | 0.539 |
| Total | 155 (100) | 24.76 (16.6,36.9) | - | | 61(39.4)t | | - |

Subgroup analysis by infant HIV exposure and vaccine dose schedule

Of the 47/154 infants maternally exposed to HIV, 41/47 (87.2%) had an HIV result available. Of these, 39/41 (95.1%) were uninfected (HIV-exposed-uninfected, HEU) and 2/41 (4.9%) were infected (HIV-exposed-infected). We excluded the HIV-exposed-infected (n=2) from

subsequent analysis. As shown in Figure 5-4A, analysis of HEU and HIV-unexposed infants (HU, n=146), demonstrated an effect of point HCMV-IgM serostatus at 9 months on RV-IgA titres at 12 months according to infant HIV exposure status (likelihood ratio test of interaction p=0.002). In contrast, there was no evidence of an interaction between infant HIV status and the effect of cumulative HCMV-IgM seroconversion by 9 months on RV-IgA titres at 12 months (likelihood ratio test of interaction p=0.138) in this grouping (Figure 5-4B).

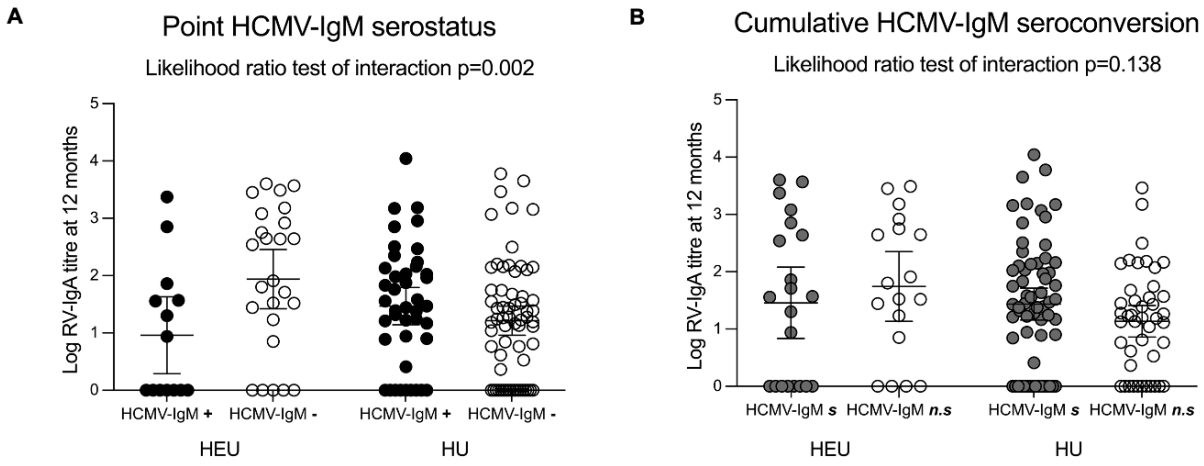


**Figure 5-4. Mean RV-IgA titres at 12 months infant age by point and cumulative HCMV-IgM serostatus at 9 months old stratified by infant HIV exposure**

Each circle represents the log-transformed RV-IgA titre for a single infant (n=146) among HIV-exposed-uninfected (HEU, n=39) and HIV-unexposed (HU, n=107) infants. Black and white circles indicate HCMV-IgM seropositive and HCMV-IgM seronegative infants at 9 months, respectively (panel A). Grey and white circle indicate cumulative HCMV-IgM seroconverting (s) and non-seroconverting (n.s) infants at 9 months respectively (panel B). Solid horizontal bar and error bars indicates the mean value with 95% confidence intervals. As shown in Figure 5-4, we found no evidence that the effect of HCMV-IgM seropositivity at the 9 months timepoint (Figure 5-4A) or cumulative HCMV-IgM seroconversion by 9 months (Figure 5-4B) on RV-IgA titre at 12 months of age varied by the vaccine dose schedule for the entire cohort (two versus three doses of Rotarix, n=155) (likelihood ratio test of interaction p=0.318 and p=0.737 respectively).

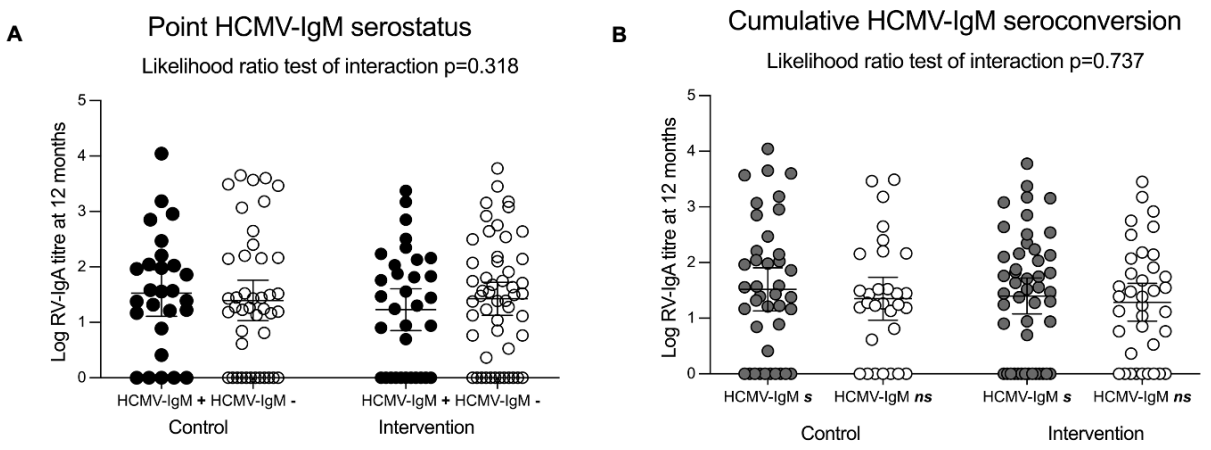


**Figure 5-5. Mean rotavirus antibody titres at 12 months infant age by point and cumulative HCMV-IgM serostatus at 9 months old stratified by vaccine dose schedule**

Each circle represents the log-transformed RV-IgA titre for a single infant (n=155) among those randomised to the intervention arm (n=85) and control (n=70) infants. Black and white circle indicates HCMV-IgM seropositive (+) and seronegative (-) infants at 9 months respectively (panel A). Grey and White circle indicates cumulative HCMV-IgM seroconverting (s) and non-seroconverting (ns) infants at 9 months respectively (panel B). Solid horizontal bar and error bars indicates the mean value with 95% confidence intervals.

Among the HU infants (n=107) there was no statistically significant difference in RV-IgA GMT at 12 months between infants that were HCMV-IgM seropositive (GMT 29.4, 95%CI: 13.9, 61.9) and HCMV-IgM seronegative (GMT 16.4, 95%CI: 9.1, 29.4) at 9 months (GMR 1.35, 95%CI: 0.88, 2.06; p=0.166) (Table 3). In the HEU group, there was evidence that the RV-IgA GMT at 12 months was decreased by 63% in infants that were HCMV-IgM seropositive compared to those that were HCMV-IgM seronegative at 9 months (GMR 0.37, 95%CI: 0.17,0.77; p=0.008) (Table 5-3).

**Table 5-3. Effect of HCMV-IgM serostatus on RV-IgA titres by infant HIV status**

| **Subgroups[a])** | **Number of infants N (% of total)** | **RV-IgA GMT at 12 months (95%CI)** | **RV-IgA *GMR (95% CI)** | **p-value** |
|---|---|---|---|---|
| HIV-unexposed | 107 (73.3) | 20.6 (13.0,32.5) | 1.35 (0.88,2.06) | 0.166 |

| | | | | |
|---|---|---|---|---|
| HCMV-IgM- | 65 (60.8) | 16.4 (9.1, 29.4) | | |
| HCMV-IgM+ | 42 (39.2) | 29.4 (13.9, 61.9) | | |
| HIV-exposed-uninfected | 39 (26.7) | 38.8 (14.8, 102.0) | | **0.008** |
| HCMV-IgM- | 25 (64.1) | 87.2 (26.5, 286.4) | | |
| HCMV-IgM+ | 14 (35.9) | 9.1 (2.0, 42.8) | 0.37 (0.17,0.77) | |
| Total | 146 | 24.4 (16.0, 37.1) | | |

As shown in Figure 5-6, there was no statistically significant difference in the frequency of vaccine seroconversion one month after two dose vaccination either by HCMV-IgM serostatus at 6-12 weeks (n=148, p=0.528) and 14-20 weeks timepoints (n=148, p=0.407) or by cumulative HCMV-IgM seroconversion at 14-20 week timepoint (n=147, p=0.166).

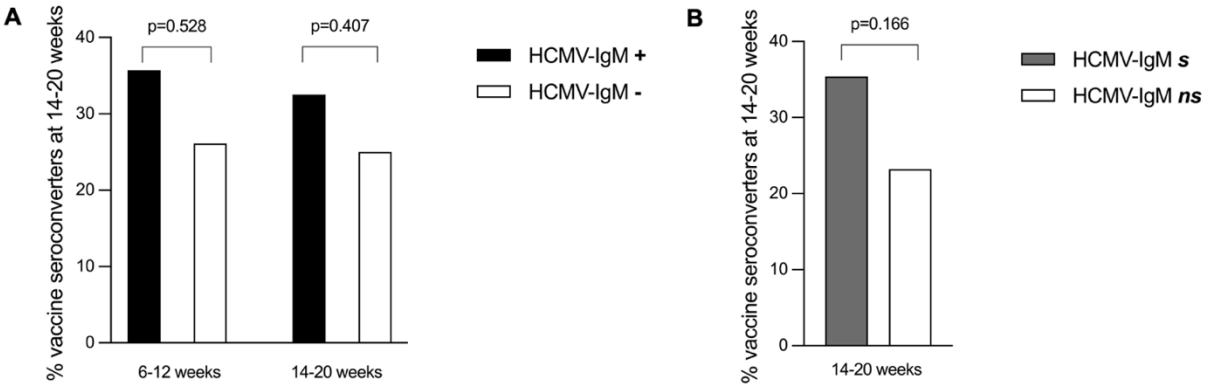


**Figure 5-6. Vaccine seroconversion by HCMV-IgM serostatus**

Each bar represents the percent vaccine seroconverters at 14-20 weeks one month after two dose vaccination among infants that were HCMV-IgM seropositive (n=14) and HCMV IgM seronegative (n=134) before vaccination at 6-12 weeks old (n=148); HCMV IgM seropositive (n=40) and HCMV IgM seronegative (n=108) at 14-20 weeks old (n=148); and cumulative HCMV-IgM seroconverting (n=48) and non-seroconverting (n=99) infants at 14-20 weeks old (n=147). Black and white bars indicate HCMV-IgM seropositive (+) and seronegative (-) infants respectively (panel A). Grey and White bars indicate cumulative HCMV-IgM seroconverting (s) and non-seroconverting (ns) infants respectively (panel B).

## 5.5 Discussion

Our study aimed to investigate the influence of HCMV infection around the time of oral rotavirus vaccination on the vaccine immunogenicity in a low-income setting where early childhood HCMV infection is prevalent and may modulate immune responses. We measured HCMV-IgM in vaccinated infants when they were 9 months of age, which coincided with the time of a third dose of ORV. We specifically examined any association between the presence of HCMV-IgM (indicative of recent HCMV infection or reactivation) and the antibody response to rotavirus vaccine (measured as RV-IgA titres). Overall, there was no evidence of association, at 5% level of significance, between the presence of HCMV-IgM at 9 months of age and RV-IgA titres at 12 months. This suggests that for most infants, HCMV infection does not seem to notably affect the vaccine's immunogenicity. However, among HEU infants who were HCMV-IgM seropositive at 9 months, a 63% reduction in RV-IgA titre at 12 months was observed compared to their HEU- HCMV-IgM seronegative counterparts. This points to a possible specific immune modulation effect of HCMV in HEU infants in our setting.

Our findings are like what has been reported for another orally administered pediatric vaccine, oral polio vaccine (OPV), where a study in Zambia showed no effect of HCMV DNAemia or HCMV serostatus in 18 month old infants on poliovirus antibody titres or proportion of infants with seroprotective levels in the overall study population(20). In contrast to our findings on oral rotavirus vaccine, while significantly reduced poliovirus antibody responses were observed in maternally HIV exposed infants and HIV seropositive infants, the OPV study did not find any difference in poliovirus antibody responses by HCMV DNAemia or HCMV serostatus among the HEU infants(20). Notably, trends of reduced OPV antibody responses among HIV positive infants that had HCMV DNAemia compared to those without HCMV DNAemia were observed(20) although differences in determination of HCMV serostatus with our study (HCMV-IgG versus IgM) may limit comparisons. In our rotavirus vaccine study and the OPV study, while vaccine immunogenicity in the broader population was not impacted by HCMV infection, reduction of vaccine responses was seen within specific subgroups (HEU and HIV positive infants). This nuanced finding underscores the complexity of immunologic responses, especially in populations with various health challenges. Interestingly, in studies elsewhere with reported HIV prevalence of below 5%, HCMV infected infants are observed to have

reduced antibody responses to tetanus toxoid after DPT vaccination and lowered T cell effector responses post measles vaccination compared to HCMV uninfected infants despite no impact on infants' attainment of vaccine specific seroprotective levels, however, the infant HIV status in these studies was not ascertained (15, 17). We could not speculate on the clinical significance of the reduced RV-IgA titre in HEU HCMV-IgM seropositive infants as there is currently no defined seroprotective threshold level of RV-IgA, however, higher RV-IgA titres are associated with reduced risk of rotavirus infection and diarrhoea (22). Thus, factors like HCMV associated with reductions in these RV-IgA responses among HEU infants have the potential to negatively impact overall vaccine efficacy and effectiveness.

HCMV affects the immune system by committing a substantial proportion of T cells towards its immune response (23) and causing accumulation of differentiated immune cells (23-25) and restricted T-cell repertoires (26) characteristic of immune senescence. HCMV can also disrupt antigen presentation to T cells, suppress immune effector function and limit immune cell proliferation (27-29). Expansion of differentiated T cell immunity by HCMV is consistent with immunological profiles observed in HEU infants compared to HIV unexposed infants (30). In the HEU population, HCMV may therefore attenuate vaccine immune responses via this T cell immune perturbation (31). We found evidence of an impact of HCMV on rotavirus specific antibody response in HEU infants thus for rotavirus vaccines, further studies investigating the effect of HCMV infection on T cell immune responses to vaccination in HEU infants are merited to elucidate such possible effects. HCMV also alters intestinal microbiome (32) favoring increased composition of Bacteroidetes(33). Abundance of specific Bacteroidetes genera, Bacteroides and Prevotella, have been significantly correlated with a lack of seroresponses to rotavirus vaccination in infants (34) and are reported to be significantly increased in HEU compared to HIV unexposed infants (35). Human microbiome colonization and immune development are intimately related and influence infant immune responses to vaccines (36). Early life HCMV may therefore be an important determinant of oral vaccine responses in HEU via its effect on the intestinal microbiome composition, but additional studies are needed to confirm this.

A major strength of this study is its focus on a vulnerable population (infants) within a low-income setting. We addressed an important prevailing question as to why oral rotavirus vaccines perform sub-optimally in such regions. Measuring plasma HCMV-IgM prior to vaccination provided a clear temporal relationship between HCMV infection and vaccine immunogenicity. However, some limitations exist. The sample size might not have been sufficiently large to detect more subtle differences across the infant groups. Also, the study

does not delve into potential biological mechanisms underlying the observed association in HEU infants, which might provide clearer insights. We determined HCMV infection based on HCMV-IgM without confirmation of DNAemia by molecular methods, which could have introduced classification bias. HCMV-IgM serology can identify infant HCMV-specific responses in early life, as opposed to passively acquired transplacental maternal HCMV-IgG antibodies, but may show false positives due to cross reactivity with other herpesviruses such as EBV (37). HCMV-IgM can also be transient and cleared rapidly in some individuals, thus contributing to misclassification of HCMV status (38). We also acknowledge that the phenomena of reduced oral rotavirus vaccine immunogenicity may occur in areas with lower HIV seroprevalence and thus limit the generalizability of our findings. Furthermore, genetic, environmental and maternally derived factors known to influence oral rotavirus vaccine immunogenicity (5) were not included in this analysis but which may be involved in the immune responses observed.

Our findings shed light on a potentially critical area of vaccine research, especially in Zambia where HIV prevalence is high. More robustly designed studies may be needed to verify the observed association between HCMV infection and reduced oral rotavirus vaccine response in HEU infants. It would be pertinent to investigate the underlying immunological mechanisms that may be driving this impaired response. Also, it would be useful to study other vaccines' immunogenicity in relation to HCMV within the HEU infant population to understand if this observation is rotavirus vaccine-specific or a broader immunological phenomenon. If future studies corroborate these findings, it could have profound implications for vaccine policy in regions with high HIV prevalence. There might be a need to revisit vaccine schedules, dosages, or even the development of specific vaccine formulations tailored to the needs of HEU infants. Additionally, understanding such interactions can guide health campaigns and interventions, especially in low-income settings where both HCMV and HIV are prevalent. Public health officials might need to consider additional interventions or strategies to enhance vaccine efficacy in vulnerable subpopulations.

### 5.6 Conclusion

While the broader implications of HCMV infections on oral rotavirus vaccine response might be limited in the general infant population, the potential impact on HEU infants cannot be overlooked. This study highlights the complexity of immunological responses and the need for targeted interventions to ensure vaccine efficacy, especially in vulnerable subpopulations.

## Author Contributions

N.M.L.: conceptualization, investigation, formal analysis, visualization, writing—original draft preparation, writing—review and editing, funding acquisition; S.B.: formal analysis, visualization writing—review and editing; R.C.: funding acquisition, writing—review and editing; M.S.: writing—review and editing; C.C.: writing—review and editing; H.N.: investigation, writing—review and editing; K.M.C.: investigation, writing—review and editing; M.G.: conceptualization, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

## Data availability

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to institutional data policy restrictions.

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## Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## Clinical Trial Registration

The parent trial is registered under the Pan African Clinical Trial Registry as PACTR201804003096919.

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| **Student ID Number** | 1402541 | **Title** | Miss |
| **First Name(s)** | Natasha Makabilo | | |
| **Surname/Family Name** | Laban | | |
| **Thesis Title** | Antibody and T-cell immune responses in rotavirus vaccinated Zambian infants: impact of human cytomegalovirus infection. | | |
| **Primary Supervisor** | Martin Goodier | | |

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| Stage of publication | **Not yet submitted** |

## Chapter 6: Limited VP6-specific T-cell responses among rotavirus vaccinated infants in Zambia.

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## 6.1 Abstract

There is limited data on cellular immune responses to oral rotavirus vaccination in infants. Here, we investigated circulating innate NK and T-cell phenotypes and assessed rotavirus specific T-cell responses after rotavirus vaccination in Zambian infants. Ex-vivo flow cytometric analysis of fluctuations in peripheral NK cell , conventional and gut homing T-cells, NKT, MAIT and gamma delta T-cell immunophenotypes were measured within PBMC collected from infants before and after the first, second and third dose of a rotavirus vaccine. An activation induced marker assay was used to assess CD4 and CD8 T-cell activation following in-vitro stimulation with rotavirus VP6 peptide by flow cytometry. We detected low-frequency rotavirus specific CD4 and CD8 T-cell responses following in-vitro stimulation with rotavirus VP6 peptide in vaccinated infants but observed higher frequency of the VP6 specific CD4+ T-cells among vaccine seroconverters. These findings demonstrate limited peripheral rotavirus VP6-specific T-cells in vaccinated Zambian children but indicates an enrichment of VP6-specific CD4 T-cells in vaccine seroconverters.

## 6.2 Introduction

Oral rotavirus vaccines (ORV) have significantly impacted on reduction of rotavirus associated diarrhoea among children since introduction globally (1). However, lower vaccine effectiveness and seroconversion rates prevail in low-income countries (LIC) where higher rotavirus diarrhoeal illness and mortality occurs compared to higher-income countries (2, 3). This reduced vaccine performance and global estimates of >120,000 annual diarrhoea deaths in children aged below 5 years old attributed to rotavirus (4) call for continued research into understanding rotavirus vaccine immunology.

The immunogenicity of ORV in children is widely assessed by rotavirus specific antibodies associated with reduced risk of rotavirus infection and moderate to severe diarrhoea (5) however virus-specific antibodies only partially explain vaccine induced protection (6) implying other immune parameters are at play. Rotavirus infection also induces innate cellular and virus specific adaptive T-cell immunity in children (7) but these cellular responses are under studied in vaccinated infants (8). Knowledge on cell-mediated immune responses after rotavirus vaccination may give mechanistic insights into observed seroresponses or lack thereof among vaccinated children in LIC with direct implications for vaccine efficacy and design.

T-cell activation induced marker (AIM) assays can provide a broader picture of the antigen specific T-cells response for vaccine studies (9) and may prove informative in rotavirus vaccine T-cell immunology studies. The investigation of unconventional T-cell subsets including TCR gamma delta (TCR$\gamma\delta$) T-cells, Natural Killer T-cells (NKT) and mucosal associated invariant

T-cells (MAIT) that are enriched within intestinal tissues and linked to intestinal and viral immune protection (10, 11) but their relationship with oral rotavirus vaccine immunogenicity has not been fully investigated.

We aimed to detect rotavirus-specific T-cells using the AIM assay and profile NK and T-cell phenotypes in rotavirus vaccinated infants in Zambia.

## 6.3 Methods

Ethical approval was obtained from the University of Zambia Biomedical Research Ethics Committee (reference number 003-02-18) and the London School of Hygiene and Tropical Medicine (LSHTM) Research Ethics Committee (reference number 16168). Informed consent was obtained from all study participants.

### Study population, vaccination schedule and PBMC sampling

The study population was a subset of infants among those enrolled at ages 6 to 12 weeks into a rotavirus vaccine randomised controlled trial (RCT) conducted in Zambia, details of which have been published elsewhere (12). Infants received two doses (at approximately 6 and 10 weeks old) or three doses (at approximately 6 and 10 weeks and at 9 months old) of ORV (Rotarix, GlaxoSmithKline, UK) alongside polio, BCG, DPT-HepB-Hib, pneumococcal conjugate at 6, 10 and 14 weeks old, and measles-rubella (MR) vaccines at 9 months old according to the national immunization schedule. In this study, we included infants from whom peripheral blood mononuclear cells (PBMC) were collected during the RCT and who had data on rotavirus-specific antibody responses at pre- and post-rotavirus vaccination. This PBMC cohort of infants was the second half of infants prospectively enrolled under the RCT as they randomly presented to the study site. PBMC from this infant cohort were collected over a total of eight timepoints: at baseline (before vaccination, T1), seven days after first ORV dose (T2), before the second ORV dose (T3), seven days after the second ORV dose (T4) one month post second ORV dose (T5), at 9 months (T6), seven days after the third ORV dose or MR vaccine (T7) and at 12 months of age (T8). Ex-vivo immunophenotyping of T-cells was done using PBMC collected at all eight timepoints (T1-T8). Rotavirus specific in-vitro stimulation and T-cell AIM assay was performed using PBMC collected at T1, T5, T6 and T8 (Figure 6-1).

| T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 |
|---|---|---|---|---|---|---|---|
| Pre-vaccine | 7days post dose 1 | Pre-dose 2 | 7days post dose 2 | 1 month post-dose 2 | Pre-dose 3 | 7days post dose 3 | 3 months post dose 3 |
| Ex-vivo phenotyping | Ex-vivo phenotyping | Ex-vivo phenotyping | Ex-vivo phenotyping | Ex-vivo phenotyping | Ex-vivo phenotyping | Ex-vivo phenotyping | Ex-vivo phenotyping |
| In-vitro stimulation | | | | In-vitro stimulation | In-vitro stimulation | | In-vitro stimulation |

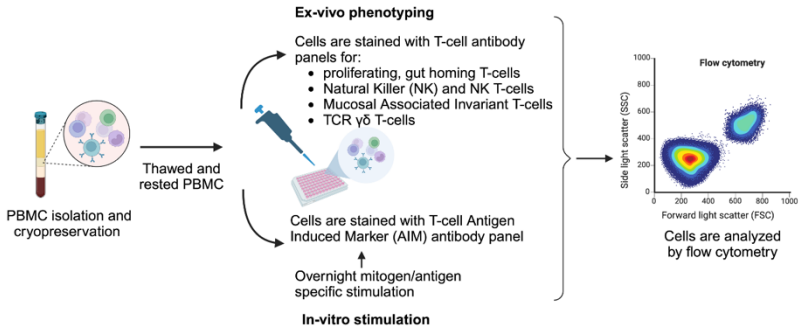


**Figure 6-1. Illustration of PBMC collection and flow cytometric analysis timepoints. Created with BioRender.com.**

PBMC samples were collected from the PBMC cohort (n=113) over a total of eight timepoints corresponding to baseline before vaccination (T1), 7 days post rotavirus dose 1 (T2), before rotavirus dose 2 (T3), 7 days post rotavirus dose 2 (T4), one month post rotavirus dose 2 (T5), before rotavirus dose 3 or measles vaccine (T6), 7 days post rotavirus dose 3 or measles vaccine (T7) and three months post rotavirus dose 3 or measles vaccine. Ex-vivo flow cytometry analysis was performed on cells collected at all timepoints. In-vitro stimulation was performed on cells collected at T1, T5, T6 and T8.

## Laboratory procedures

PBMC isolation: PBMC were isolated by density gradient centrifugation of whole blood on Histopaque® 1.077 g/ml media (Sigma-Aldrich). The PBMC were then washed and resuspended in Roswell Park Memorial Institute (RPMI) – 1640 media (Sigma Aldrich) supplemented with 1% Penicillin-Streptomycin-L-Glutamine (PSG, Gibco) and cryopreserved in media containing 20% dimethyl sulfoxide (DMSO) (Sigma Aldrich) in fetal calf serum (Gibco, Paisley, UK) in Liquid Nitrogen. For flow cytometry experiments, thawed PBMC were washed twice and resuspended to a minimum 5x10^6 PBMC/ml and up to 1x10^7 PBMC/mL in 1% PSG supplemented RPMI media. PBMC collected from each infant across different timepoints were assayed in the same experiment.

Immunofluorescent antibodies: Five immunofluorescent antibody staining panels were used in the ex-vivo immunofluorescent staining to phenotype activated or regulatory (CD3 FITC/CD4 PE/CD8 APC/CD25 PE-Cy7) and intestinal homing (CD3 FITC/CD4 PE/CD8 APC/CCR9 PE-Cy7/β7 PerCP-Cy5.5) conventional CD4 and CD8 T cells, mucosal associated invariant T (MAIT) cells (CD3 FITC/CD8 PE/ Vα7.2TCR APC/CD161 PE-Cy7), TCR gamma delta (TCR $\gamma\delta^+$) T cells (CD3 FITC/CD8 PE/ γδTCR APC/ Vδ1TCR PE-Vio770),

and Natural Killer T (NKT) and innate NK cells (CD3 FITC/CD56 PE-Cy7/CD57 APC/ NKG2C PE) (Supplementary Table S6-1). Cell proliferation was determined by staining with Ki67 PerCP-Cy5.5 antibody for all cell phenotype panels except the intestinal homing panel. A single immunofluorescent antibody staining panel comprising αβTCR FITC, CD4 PerCP-Cy5.5, CD69 PE/CD134 PE-Cy7, CD137 APC, CD14 APC-Cy7 and CD19 APC-Cy7 was used in the immunofluorescent staining for the activation induced marker (AIM) assay to phenotype activated CD4+ CD134+ CD137+ and CD8+ CD69+ CD137+ T cells excluding monocytes and B cells. All panels included Fixable Viability Dye- eFluor780 for exclusion of dead cells. Titration was done for all antibodies and optimal concentrations were used. All antibodies were sourced from Biolegend, USA except CD56 PE-Cy7 (BD Biosciences, USA), Fixable Viability Dye- eFluor780 and Ki67 PerCP-Cy5.5 (ThermoFisher eBioscience, USA).

Antigens: The rotavirus antigen was a peptide pool generated from the human rotavirus Group A VP6 sequence described by Kaufhold and colleagues (13). This human rotavirus VP6 protein made up of 397 amino acid residues (GenBank protein accession number AAB46985) was sequenced from an Indian rotavirus strain 116E, genotype G9P8[11] detected in asymptomatic neonatal infections in India (14, 15). The peptide pool was made of 39 individual peptides of 20 mer length overlapping by 10 amino acid residues and were generated by Mimotopes Pty Ltd (Australia). The individual peptides were reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich) to 20,000μg/ml stock and stored as aliquots at -80° Celsius. A peptide pool stock concentration of 500μg/ml per peptide was prepared by pooling the individual stock peptides diluted 40-fold in DMSO. A working peptide pool concentration of 20μg/ml per peptide was obtained by a 25-fold dilution of the peptide pool stock in PBS. An HCMV pp65 peptide pool (Catalogue number ARP-11549) stock at 20μg/ml concentration in PBS was used to detect HCMV specific T-cells as an antigen positive control. This HCMV peptide pool comprised 138 peptides of 15mer length overlapping by 11 amino acids spanning the entire HCMV pp65 protein. PHA mitogen (Sigma Aldrich, Germany) was used as a positive control and 1% PSG supplemented RMPI with a DMSO concentration equivalent to that in the rotavirus VP6 peptide pool final concentration was used as the vehicle control (DMSO background).

Ex-vivo immunophenotyping of T cells: Thawed cells (2x10^5 cells) were washed once with FACS buffer, incubated with Fc blocking reagent (Miltenyi Biotech, UK) at room temperature and then stained with the five different ex-vivo immunofluorescent antibody panels in a 96-well U-bottomed culture plate (ThermoFischerScientific, USA). After incubation at 4° Celsius for 30 minutes, the cells were washed and then fixed and permeabilised in a dark room at room temperature for 30 minutes. Cells were then washed and for all staining panels except

that for intestinal homing T cells, incubated with Fc blocking reagent and Ki67 antibody for intracellular staining in the dark for 30 minutes. After the staining incubation, cells were washed and resuspended in 250μL FACS buffer in microtubes. Stained and fixed cells were stored at 4° Celsius for a maximum of two days before acquisition of data by flow cytometry.

In-vitro stimulation of PBMC with antigens: Preliminary titration experiments using adult donor and child PBMC established the optimal antigen concentrations to be used for rotavirus VP6 and HCMV pp65 peptides and PHA antigens. All antigens were diluted in 1% PSG RPMI prior to use in in-vitro PBMC stimulation. On day one, thawed PBMC at 1x10^6 (or 5x10^5) cells were incubated in the presence of rotavirus VP6 peptide pool (1μg/ml per peptide), HCMV pp65 peptide pool (1μg/ml), PHA (positive control, 5μg/ml) or DMSO control in a 96 well U-bottomed plate at 37°Celcius 5% $CO_2$ for 20 hours. On day two, cells were washed once with FACS buffer, incubated with Fc blocking reagent at room temperature and then incubated with the AIM antibody staining cocktail at 4° Celsius for 30 minutes. After this incubation, stained cells were washed once before fixation and permeabilization at room temperature for 15 minutes in the dark. Fixation of the cells after surface staining was done using commercial Cytofix/Cytoperm solution (51-2090KZ, BD Biosciences, USA) containing about 4.2% formaldehyde for the purpose of stabilising the cells in overnight storage prior to flow cytometry acquisition. Finally, cells were washed and then resuspended in 250μL FACS buffer in microtubes. Stained and fixed cells were acquired by flow cytometry immediately or stored at 4° Celsius and acquired the next day.

Acquisition was performed using a 6-colour FACSVerse flow cytometer (Beckton Dickinson, USA) equipped with violet, blue and red lasers. Frequencies of the different innate and T-cell phenotypes (Supplementary Figure S6-1) and antigen stimulated AIM+ T-cells (Supplementary Figure S6-2) were analysed using FlowJo™ version 10 software (Beckton Dickinson, Belgium)

Analysis of antigen specific T-cell responses: Infants with CD4 T cell count below 500 cells in antigen stimulated wells were excluded from analysis at each visit. Analysis was conducted on infants with an average acquired CD4 T cell count of 19748 for the DMSO background, 21513 for rotavirus VP6, 20942 for HCMV pp65 and 12318 for PHA stimulated wells, and an average count of 6244, 6742, 6758 and 4599 CD8 T cells in the DMSO, rotavirus VP6, HCMV pp65 and PHA stimulated wells respectively overall across the four timepoints. Antigen-specific CD4+CD134+CD137+ (CD4+AIM+) and CD8+CD69+CD137+ (CD8+AIM+) T-cell frequencies were determined after subtraction of the corresponding DMSO background frequency for each infant sample at the specific timepoint. A detectable antigen specific CD4+ or CD8+ AIM+ T-cell frequency was defined as a net CD4+ or CD8+ AIM+ T-cell frequency

greater than zero. In parallel, a stimulation index (SI) was also calculated by dividing the antigen specific AIM+ CD4+ or CD8+ T-cell frequency by the corresponding DMSO background frequency for each infant sample at the specific timepoint. Antigen specific AIM+ CD4+ or CD8+ T-cell responders were defined as infants with a detectable net antigen-specific AIM+ CD4+ or CD8+ T-cell frequency and a CD4+ CD8+ AIM+ SI≥1.5. Antigen specific AIM+ CD4+ or CD8+ T-cell non-responders were defined as infants without a detectable net antigen-specific AIM+ CD4+ or CD8+ T-cell frequency or a CD4+ CD8+ AIM+ SI<1.5. Comparison of rotavirus VP6 specific AIM+ CD4+ or CD8+ T-cell frequencies between vaccine seroconverters and non-seroconverters was done by Mann-Whitney U test. The change in RV-IgA antibody titres among T-cell responders and non-responders across the four timepoints T1, T5, T6 and T8 was assessed by mixed effects model repeated measures analysis with multiple comparison adjustment done by Tukey's posttest.

Assessment of differences in ex-vivo immunophenotypes: Comparison of ex-vivo immunophenotypes between vaccine seroconverters and non-seroconverters at 7 days and one month post second ORV was done using the Mann-Whitney U test. Differences in the ex-vivo immunophenotypes at the seven timepoints post-vaccination in comparison to baseline were assessed using a mixed model repeated measures analysis with Dunnett's post-test to report p-values adjusted for multiple comparison. The mixed model repeated measures analysis was performed on log transformed ex-vivo T-cell frequencies where the ex-vivo T-cell frequencies with a value of zero were set to half the lowest frequency value observed at the specific timepoint prior to log transformation. Data transformation, analysis and presentation were performed using GraphPad Prism (GraphPad Software, LLC) software version 9.0 and Stata software version 17.0 (StataCorp, USA). Statistical significance was set at p-value of less than 0.05 and were denoted as * ($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$) throughout.

## 6.4 Results

Of the total 214 infants enrolled in the rotavirus vaccine RCT study, a total of 113 (52.8%) infants who were in the second half of prospectively enrolled infants had PBMC samples collected and comprised the PBMC cohort. The 101/214 infants did not have PBMC samples because they were not among the subset of infants from which PBMC collection was done. Among the PBMC cohort, 84/113 (74%) infants had data on RV-IgA seroconversion status one month after the second dose of Rotarix. The remaining 29 infants did not have RV-IgA seroconversion data nor PBMC at one month post second dose Rotarix due to missed visits or loss to follow up. Of the 84 that had PBMC collected and RV-IgA seroconversion data, 18

(21.4%) were vaccine seroconverters and 66 (78.6%) were non-seroconverters. All (18/18) vaccine seroconverters and 17/66 non-vaccine seroconverters, giving a total of 35 infants, were included for T cell phenotyping by flow cytometry in this study (Figure 6-2).

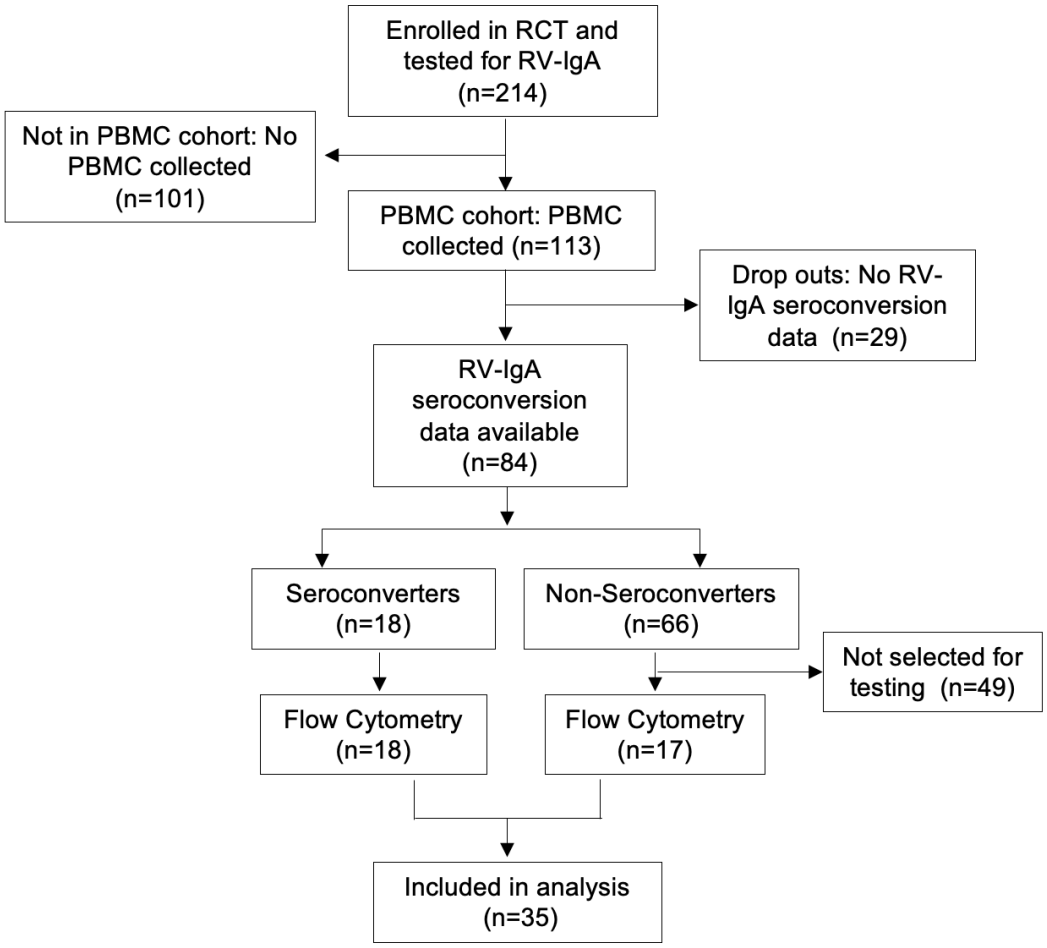


**Figure 6-2. Flow chart on selection of infants for the flow cytometry analysis.**

Infant baseline characteristics

A total of 35 infants comprising vaccine seroconverters (n=18) and non-seroconverters (n=17) were included in this study and baseline characteristics are shown in Table 6-1. All infants (n=35) received two doses of the ORV and 14/35 (40%) had also received a third ORV dose at 9 months old. Infants had a median age of 6 weeks at vaccination, most were born full-term (91.4%) with normal birthweight and growth (85.7%) and were well-nourished (97.1%). Over half of the infants were maternally HIV-exposed to HIV (51.4%) and among the HIV-exposed most infants were uninfected (93.3%). All infants were exclusively breastfed, and majority (88.6%) came from households with poor sanitation facility. The distribution of baseline

characteristics between vaccine seroconverters and non-seroconverters were similar (Table 6-1).

**Table 6-1. Distribution of infants by baseline characteristics**

| Characteristic | Total N (%) | Non-seroconverter n (%) | Seroconverter n (%) | p-value |
|---|---|---|---|---|
| Age at vaccination in weeks, median (IQR) | 6 (5,6) | 6 (6,6) | 6 (5,6) | 0.144 |
| Sex | | | | |
| Female | 16 (45.7) | 7 (41.2) | 9 (50.0) | 0.738 |
| Male | 19 (54.3) | 10 (58.8) | 9 (50.0) | |
| Gestation | | | | |
| Full-term | 32 (91.4) | 15 (88.2) | 17 (94.4) | 0.603 |
| Pre-term | 3 (8.6) | 2 (11.8) | 1 (5.6) | |
| Birthweight (kg) | | | | |
| Low <2.5kg | 5 (14.3) | 2 (11.8) | 3 (16.7) | 1.000 |
| Normal ≥2.5kg | 30 (85.7) | 15 (88.2) | 15 (83.3) | |
| Malnourished (WLZ) | | | | |
| No ≥ -2 | 34 (97.1) | 16 (94.1) | 18 (100.0) | 0.486 |
| Yes <-2 | 1 (2.9) | 1 (5.9) | 0 (0.0) | |
| Stunted (LAZ) | | | | |
| No ≥ -2 | 30 (85.7) | 15 (88.2) | 15 (83.3) | 1.000 |
| Yes <-2 | 5 (14.3) | 2 (11.8) | 3 (16.7) | |
| Maternal HIV exposure | | | | |
| Exposed | 18 (51.4) | 6 (35.3) | 12 (66.7) | 0.094 |
| Unexposed | 17 (48.6) | 11 (64.7) | 6 (33.3) | |
| Infant HIV status (n=15) | | | | |
| HIV-exposed-uninfected | 14 (93.3) | 10 (100.0) | 4 (80.0) | 0.333 |
| HIV-exposed-infected | 1 (6.7) | 0 (0.0) | 1 (20.0) | |
| Feeding mode | | | | |
| exclusive breastmilk | 35 (100.0) | 17 (100.0) | 18 (100.0) | n/a |
| breastmilk + formula | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| Toilet facility | | | | |

| | | | | |
|---|---|---|---|---|
| Not shared | 4 (11.4) | 3 (17.7) | 1 (5.6) | 0.338 |
| Shared | 31 (88.6) | 14 (82.3) | 17 (94.4) | |
| Total | 35 (100) | 17 (48.6) | 18 (51.4) | |

Higher frequency of CD4+CD25+ T-cells in vaccine seroconverters 7 days post second dose.

We assessed fluctuations in the ex-vivo NK and T-cell phenotypes at post vaccination timepoints (T2-T8) in comparison to baseline (T1). We observed minimal to absent circulating NKT cells and therefore did not analyse this subset (See Supplementary Figures S6-3, S6-4, S6-5, and S6-6). Here we report the ex-vivo CD4+ and CD8+ and intestinal homing T-cells frequencies among infants at 7 days and one month post the second dose (T4 and T5 respectively) by vaccine seroconversion status.

At baseline (n=27), there was no significant difference in CD4+CD25+ (p=0.148), CD4+Ki67+ (p=0.913) or CD4+CD25+Ki67 (p=0.990) T-cell frequencies between vaccine seroconverters and non-seroconverters. As shown in Figure 6-3A, vaccine non-seroconverters had lower frequencies of CD4+CD25+ T-cells (p=0.004) than seroconverters at 7 days post second dose (n=26) but there was no significant difference in CD4+Ki67+ (Figure 6-3B, p=0.241) and CD4+CD25+Ki67 (Figure 6-3C, p=0.427) T-cell frequencies by vaccine seroconversion. We found no significant difference in frequencies of CD4+CD25+ (Figure 6-3D, p=0.088), CD4+Ki67+ (Figure 6-3E, p=0.166) or CD4+CD25+Ki67 (Figure 6-3F, p=0.859) by vaccine seroconversion at one month post second dose (n=22).

We found no significant difference in CD8+CD25+ (p=0.458), CD8+Ki67+ (p=0.865) or CD8+CD25+Ki67 (p=0.825) T-cell frequencies between vaccine seroconverters and non-seroconverters at baseline (n=27). Similarly, there was no significant difference in CD8+CD25+, CD8+Ki67+ or CD8+CD25+Ki67 T-cell frequencies 7 days (n=26) (Figure 6-3G: p=0.097, Figure 6-3H: p=0.897 and Figure 6-3I: p=0.348 respectively), and one month (n=22) post second dose (Figure 6-3J: p=0.593, Figure 6-3K: p=0.217 and Figure 6-3L: p=0.505 respectively)

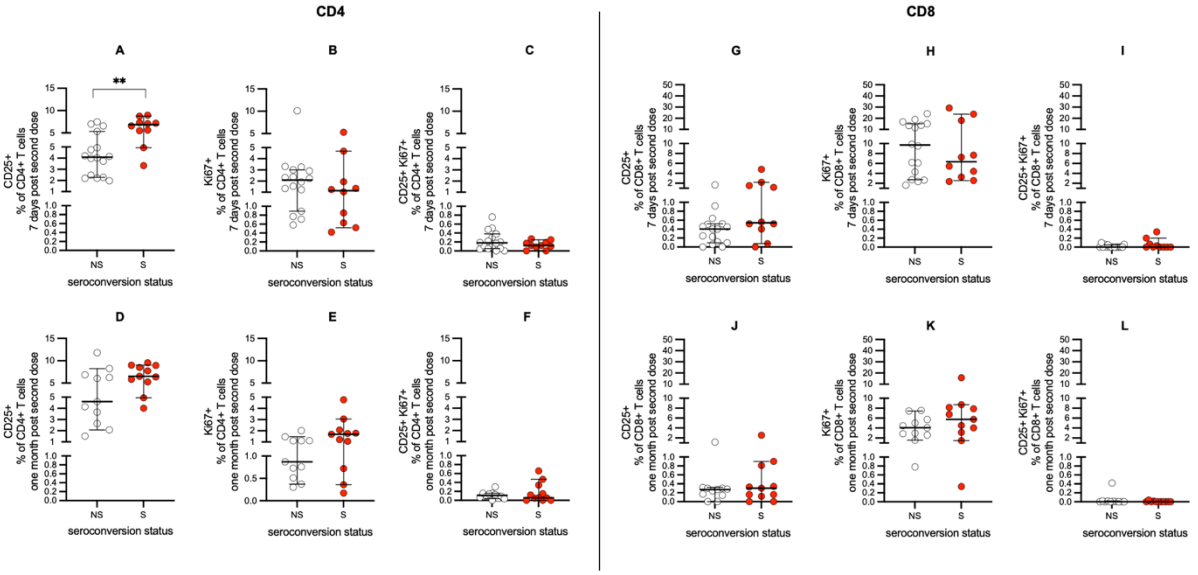


**Figure 6-3. Frequencies of CD4+ and CD8+ T-cells post second dose by vaccine seroconversion status**

The frequencies of CD25+, Ki67+ and CD25+ Ki67+ T-cells as a percentage of CD4+ T-cells at 7 days (n=26, 6-A-C) and one month (n=22, 6-D-F) post second dose and CD8+ T-cells at 7 days (n=26, 6-G-I) and one month (n=22, 6-J-L) post second dose. Each circle represents the frequency for a single infant among vaccine non-seroconverters ( NS, white and seroconverters (S, red). Solid horizontal bar indicates the median value with 95% confidence intervals. Comparison between groups assessed by Mann Whitney U test.

To explore the potential for intestinal T-cell homing and association with vaccine seroconversion, we compared frequencies of β7 integrin and CCR9 receptor expressing CD4+ and CD8+ T-cells between vaccine seroconverters and non-seroconverters. At baseline (n=30), there was no significant difference in β7+ (p=0.173), CCR9+ (p=0.902), or β7+CCR9+ (p=0.712), CD4+ T-cell frequencies by vaccine seroconversion. We did not find any statistically significant difference in the frequencies of intestinal homing β7+ (Figure 6-4A, p=0.147), CCR9+ (Figure, 6-4B, p=0.560) or β7+CCR9+ (Figure 6-4C, p=0.232) CD4+ T-cell subsets at 7 days post second dose (n=29) by vaccine seroconversion status. Similarly, no significant differences in frequencies were observed at one month post second dose (n=25) between vaccine seroconverters and non-seroconverters for β7+ (Figure 6-4D, p=0.758), CCR9+ (Figure, 6-4E, p=0.429) and β7+CCR9+ (Figure 6-4F, p=0.864) CD4+ T-cell subsets.

There was no difference in frequencies of β7+ (p=0.712), CCR9+ (p=0.943), or β7+CCR9+ (p=0.773) CD8+ T-cells by vaccine seroconversion at baseline (n=30). Likewise, no significant differences in frequencies of β7+, CCR9+ or β7+CCR9+ CD8+ T-cells were observed by vaccine seroconversion at 7 days post second dose (n=29, Figure 6-4G: p=0.619, Figure 6-4H: p=0.430 and Figure 6-4I: p=0.612 respectively) or at one month post second dose (n=25, Figure 6-4J: p=0.966, Figure 6-4K: p=0.574 and Figure 6-4L: p=0.563 respectively)

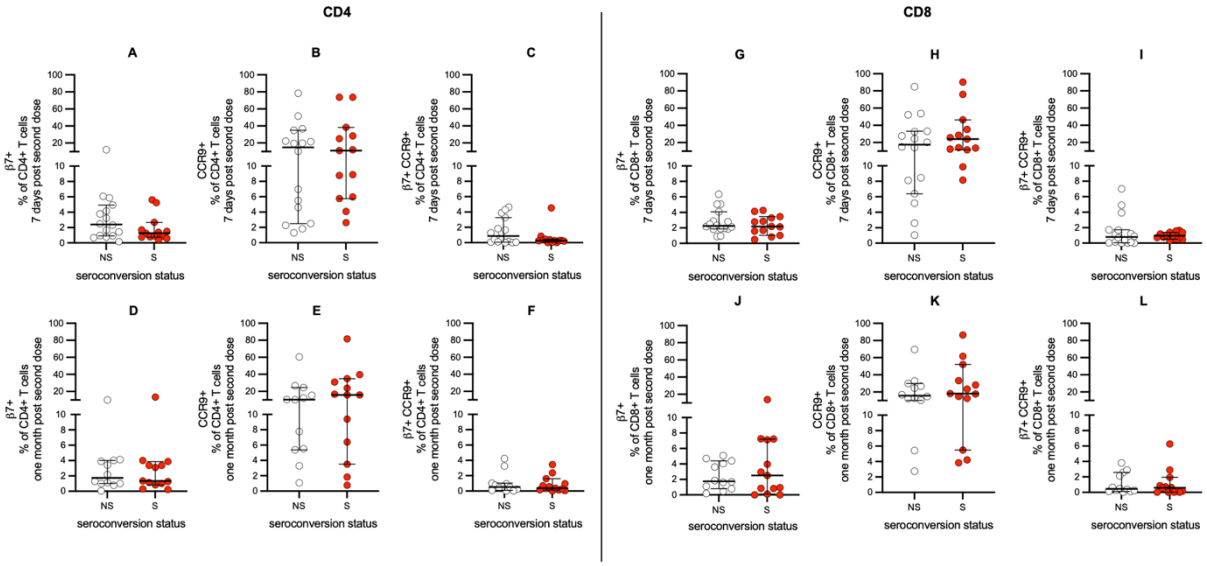


**Figure 6-4. Frequency of intestinal homing CD4+ and CD8+ T-cell by vaccine seroconversion.**

The frequencies of β7+, CCR9+, and β7+ CCR9+ T-cells as a percentage of CD4+ T-cells at 7 days (n=29, 6-4-A-C) and one month post second dose (n=25, 6-4D-F) and CD8+ T-cells at 7 days (n=29, 6-4G-I) and one month post second dose (n=25, 6-4J-L). Each circle represents the frequency for a single infant among vaccine non-seroconverters ( NS, white) and seroconverters (S, red). Solid horizontal bar indicates the median value with 95% confidence intervals. Comparison between groups assessed by Mann Whitney U test.

Circulating innate NK and unconventional T-cell subsets in rotavirus vaccinated infants

We also explored kinetics of innate NK cell and unconventional T-cell phenotypes at post vaccination timepoints (T2-T8) compared to baseline (See Supplementary Figures S6-5 and S6-6). Here we report total and proliferating frequencies of NK, MAIT and TCRγδ T-cells 7 days and one month post second dose by vaccine seroconversion status.

We found no significant difference in total NK cells at baseline (n=10, p=0.533), 7 days post (n=13, Figure 6-5A: p=0.534) and one month post (n=14, Figure 6-5G: p=0.142) the second dose. Similarly, there were no significant differences in total MAIT frequencies by vaccine seroconversion at baseline (n=24, p=0.776), 7 days post (n=28, Figure 6-5B: p=0.429) and one month post (n=20, Figure 6-5H: p=0.130) the second dose. The frequencies of total TCRγδ+, Vδ1+TCR γδ+, Vδ1-TCR γδ+ T-cells and the Vδ1+TCR γδ+/Vδ1-TCR γδ+ T-cell ratio was similar in vaccine seroconverters and non-seroconverters at baseline (n=24, p=0.799, p=0.932, p=0.755, p=0.887 respectively). Interestingly, we observed higher frequencies of Vδ1+TCR γδ+ T-cells (Figure 6-5D: p=0.024) and conversely, lower frequencies of Vδ1-TCR γδ+ T-cells (Figure 6-5E: p=0.029) corresponding to a higher Vδ1+TCR γδ+/Vδ1-TCR γδ+ T-cell ratio (Figure 6-5F: p=0.024) in infants who did not seroconvert compared to vaccine seroconverters at 7 days post the second dose (n=23). However, frequencies of these Vδ1+TCR γδ+, Vδ1-TCR γδ+ T-cells and the Vδ1+TCR γδ+/Vδ1-TCR γδ+ T-cell ratio were

similar between the two groups (Figure 6-5J: p=0.372, Figure 6-5K: p=0.360, Figure 6-5L: p=0.360 respectively) at one month post second dose (n=18).

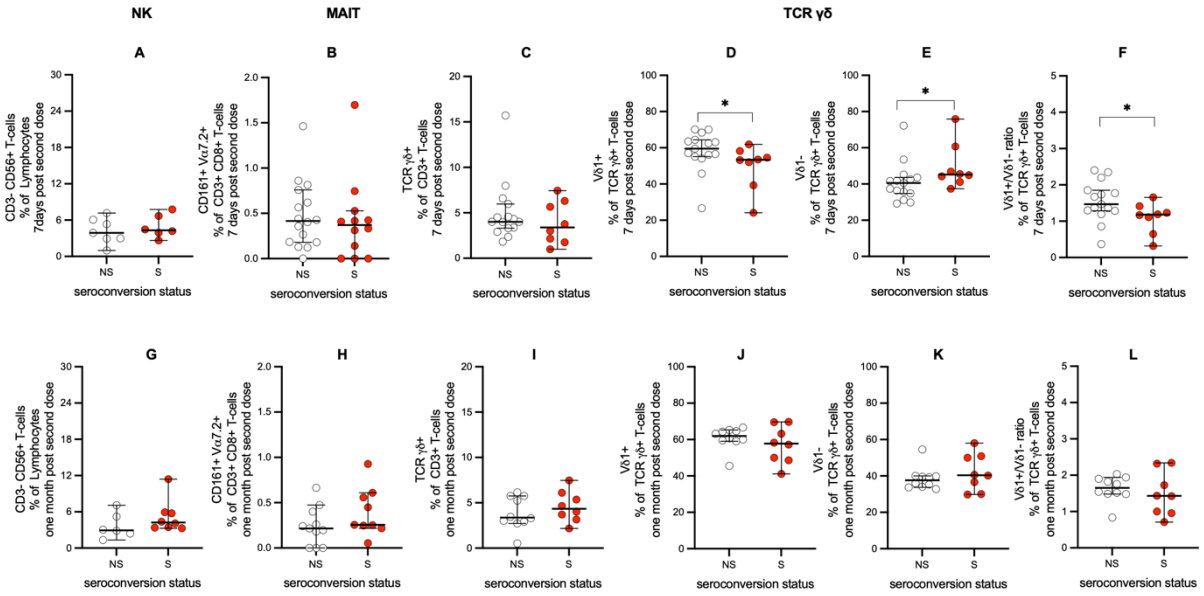


**Figure 6-5. Total NK, MAIT and TCR gamma delta T-cell frequencies post second dose by vaccine seroconversion status.**

Each circle represents the T-cell frequency for a single infant. Total NK (A, G) , MAIT (B, G) and TCR γδ (C-F and I-L) T-cell frequencies at 7 days and one month post the second dose among vaccine non-seroconverters (NS, white) and vaccine seroconverters (S, red). Solid horizontal bar indicates the median value with 95% confidence intervals. Comparison between groups assessed by Mann Whitney U test.

At baseline, vaccine seroconverters and non-seroconverters had comparable frequencies of proliferating NK cells (n=10, p=0.267), total TCRγδ+ T-cells (n=24, p=0.671), Vδ1+TCR γδ+ T-cells (n=24, p=0.173), Vδ1-TCR γδ+ T-cells (n=24, p=0.854) and proliferating Vδ1+TCR γδ+/Vδ1-TCR γδ+ T-cell ratio. We did not observe any significant differences in the proliferating frequencies of NK cells 7 days post (n=10, Figure 6-6A: p=0.703) and one month post the second dose (n=14, Figure 6-6F: p=0.216). Similarly, no significant differences in proliferating TCRγδ+, Vδ1+TCR γδ+, Vδ1-TCR γδ+ T-cells and Vδ1+TCR γδ+/Vδ1-TCR γδ+ T-cell ratio were observed at 7 days post the second dose (n=23, Figure 6-6B: p=0.400; Figure 6-6C: p=0.437; Figure 6-6D: p=0.693; Figure 6-6E: p=0.649 respectively) and one month post the second dose (n=18, Figure 6-6G: p=0.762; Figure 6-6H: p=0.633; Figure 6-6I: p=0.697; Figure 6-6J: p=0.192 respectively).

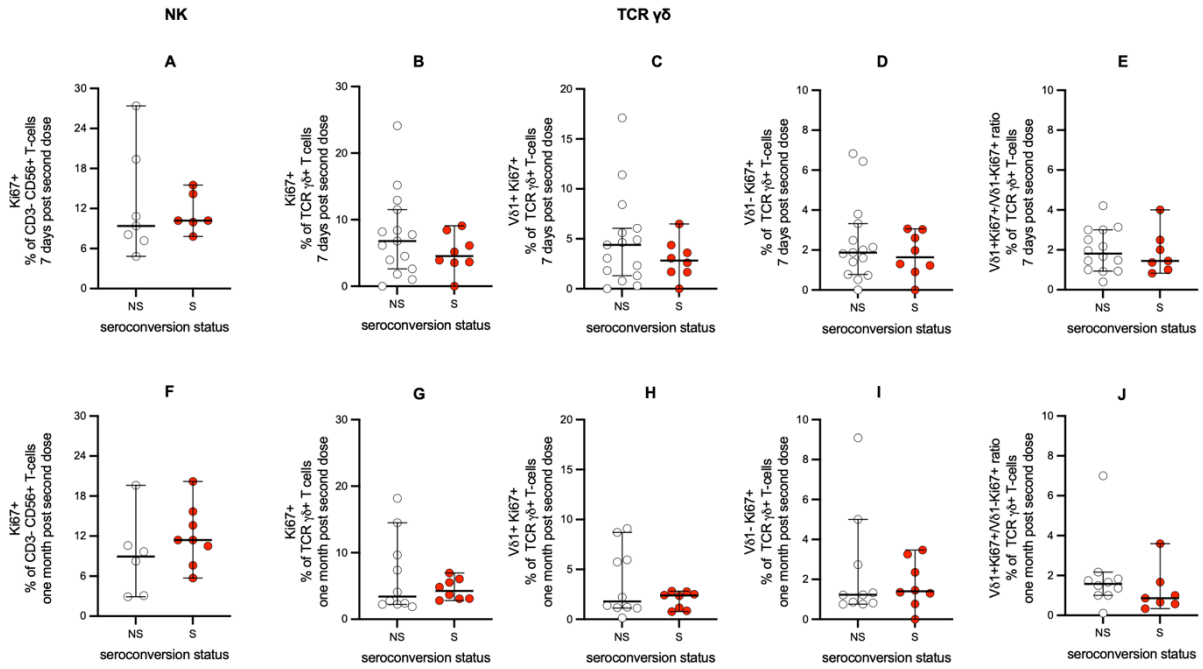


**Figure 6-6. Proliferating NK and TCR gamma delta T-cell frequencies post second dose by vaccine seroconversion status.**

Each circle represents the T-cell frequency for a single infant. Proliferating NK (A, F) and TCR γδ (B-E, G-J) T-cell frequencies at 7 days and one month post the second dose among vaccine non-seroconverters (NS, white) and vaccine seroconverters (S, red). Solid horizontal bar indicates the median value with 95% confidence intervals. Comparison between groups assessed by Mann Whitney U test.

## Limited number of rotavirus VP6-specific T-cell responders

Flow cytometry plots are shown in Figure 6-7 for a representative infant who was a VP6-specific AIM+ CD4+ T-cell responder (RV2174).

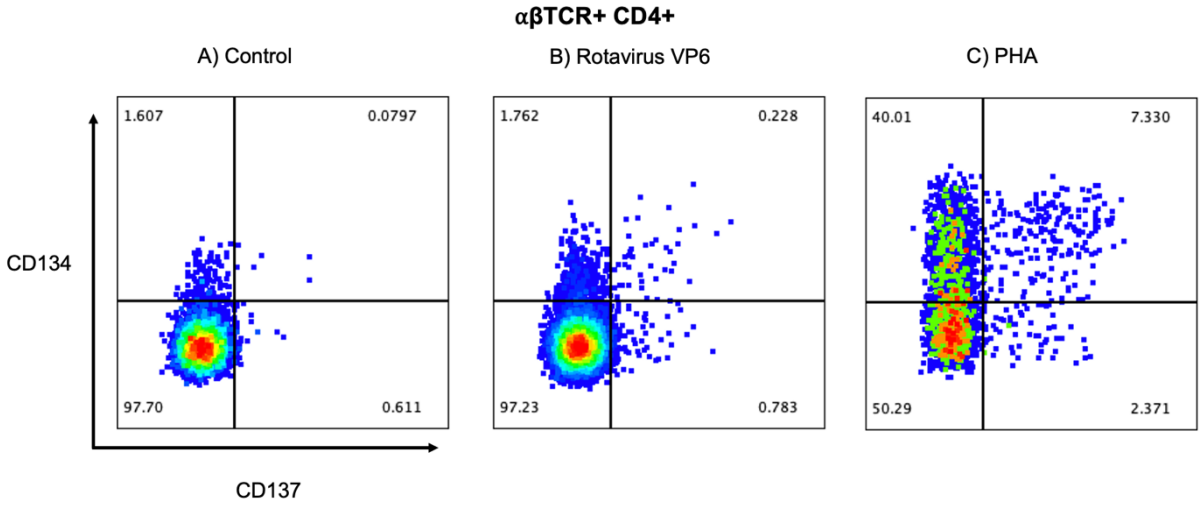


**Figure 6-7. Flow cytometry plots for a representative rotavirus VP6-specific AIM+ CD4+ T-cell responder.**

Flow cytometry acquisition plots are shown for a rotavirus VP6-specific AIM+ CD4+ T-cell responder (RV2174). Frequencies are shown for (A) DMSO control, (B) rotavirus VP6 peptide stimulated and (C) PHA stimulated wells.

Across all four visits, the average AIM+ frequencies in the DMSO background were 0.17% for CD4+ T cells and 0.58% for CD8 T cells. All infants were AIM+ CD4+ and CD8+ T-cell responders against the PHA positive control among total tested (n=24) except for a single infant (RV2106) at baseline who was removed from subsequent analysis. We observed few rotavirus VP6-specific AIM+ CD4+ T-cell responders among infants (n=29). As shown in Table 6-2, there were four rotavirus VP6-specific AIM+ CD4+ T-cell responders before vaccination, two infants at one month post the second dose, three infants before the third dose and a single infant at three months post the third dose. One infant (RV2162) had a rotavirus VP6-specific AIM+ CD4+ T-cell response at both one-month post second dose and before the third dose. Similarly, there were limited AIM+ CD8+ T-cell responding infants. Three infants were rotavirus VP6-specific AIM+ CD8+ T-cell responders before vaccination, two infants at one month post the second dose, three infants prior to the third dose and a single infant at three months post the third dose. We observed more HCMV specific AIM+ CD8+ T-cell responders than HCMV specific AIM+ CD4+ T-cell responders among infants (n=28) (Table 6-2).

**Table 6-2. Number and proportion of antigen-specific AIM+ CD4+ and CD8+ T-cell responders**

| | CD4+CD134+CD137+ T-cell responders n of Total (%) | | | | CD8+CD69+CD137+ T-cell responders n of Total (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-d1 | 1mopost-d2 | Pre-d3/MR | 3mopost-d3/MR | Pre-d1 | 1mopost-d2 | Pre-d3/MR | 3mopost-d3/MR |
| Rotavirus VP6 | 4/17 (23.5) | 2/18 (11.1) | 3/23 (13.0) | 1/11 (9.1) | 3/17 (17.7) | 3/18 (16.7) | 2/22 (9.1) | 1/12 (8.3) |
| HCMV | 1/10 (9.1) | 1/16 (6.3) | 2/21 (9.5) | 0/10 (0) | 2/11 (18.2) | 2/16 (12.5) | 7/20 (35.0) | 8/11 (72.7) |
| PHA | 13/14 (92.9) | 16/16 (100.0) | 16/16 (100.0) | 9/9 (100.0) | 14/14 (100.0) | 16/16 (100.0) | 16/16 (100.0) | 10/10 (100.0) |

Abbreviations: MR (measles-rubella), pre-d1 (before first dose), 1mopost-d2 (one month post second dose), Pre-d3/MR (before third dose and/or measles rubella vaccination), 3mopost-d3/MR (three months post third dose and/or measles rubella vaccination)

Low circulating rotavirus VP6-specific T-cell frequencies among vaccinated infants.

We observed low circulating T-cell frequencies among the limited number of rotavirus VP6-specific CD4+AIM+ and CD8+AIM+ T-cell responders. Among the total rotavirus VP6-specific CD4+AIM+ T-cell responders (n=10), the median net T-cell frequencies were 0.060% (IQR 0.047%, 0.194%) before vaccination (n=4), 0.321% (IQR 0.026%, 0.615%) one month post second dose (n=2) and 0.018% (IQR 0.010%, 0.368%) before third dose (n=3). The single rotavirus VP6-specific CD4+AIM+ T-cell responder at three months post third dose had a frequency of 0.148%. Among the total rotavirus VP6 specific CD8+AIM+ T-cell responders (n=9), the median net T-cell frequencies were 0.117% (IQR 0.043%, 0.509%) before vaccination (n=3), 0.360% (IQR 0.341%, 3.065%) one month post second dose (n=3) and 0.324% (IQR 0.303%, 0.344%) before third dose (n=2). The single rotavirus VP6-specific CD8+AIM+ T-cell responder at three months post third dose had a frequency of 0.256%. Robust AIM+ T-cell responses were observed against the positive control PHA in all infants tested (n=24) with overall median frequency of 12.7% (IQR 9.3%, 19.3%) for CD4+ and 32.0% (IQR 21.5%,44.4%) for CD8+ T-cells across all timepoints (T1-T8)

We assessed the relationship between detected rotavirus VP6-specific T-cell responses and rotavirus immunoglobulin A (RV-IgA) titres among infants with available data for both types of responses (n=29). As shown in Figure 6-8, rotavirus immunoglobulin A (RV-IgA) titres significantly increased at one month post- second dose (p=0.019), before third dose (p=0.0001) and 3 months after the third dose (p<0.0001) compared to baseline. Increased RV-IgA titres were also observed between one month post second Rotarix dose and before the third dose/MR (p=0.023) and 3 months after the third dose (p=0.005). We observed rotavirus VP6-specific CD4+ and CD8+ T-cell responses in infants with and without detectable RV-IgA at each timepoint (Figure 6-8A).

We also assessed post-vaccination rotavirus VP6-specific AIM+ CD4+ and CD8+ net T-cell responses among infants (n=26) by vaccine seroconversion status irrespective of whether they were T-cell responders or non-responders by our criteria. We observed higher post-vaccination rotavirus VP6-specific AIM+ CD4+ net T-cell frequencies among vaccine seroconverters (n=13) than non-seroconverters (n=13) (p=0.046, Figure 6-8B). Similarly, vaccine seroconverters had higher post vaccination rotavirus VP6-specific AIM+ CD4+ T-cell SI (Figure 6-8C, p=0.0181) compared to non-seroconverters. In contrast there was no difference in post vaccination rotavirus VP6-specific AIM+ CD8+ net T-cell frequencies (Figure 6-8D, p=0.346) and SI (Figure 6-8E, p=0.2614) between vaccine seroconverters and non-seroconverters.

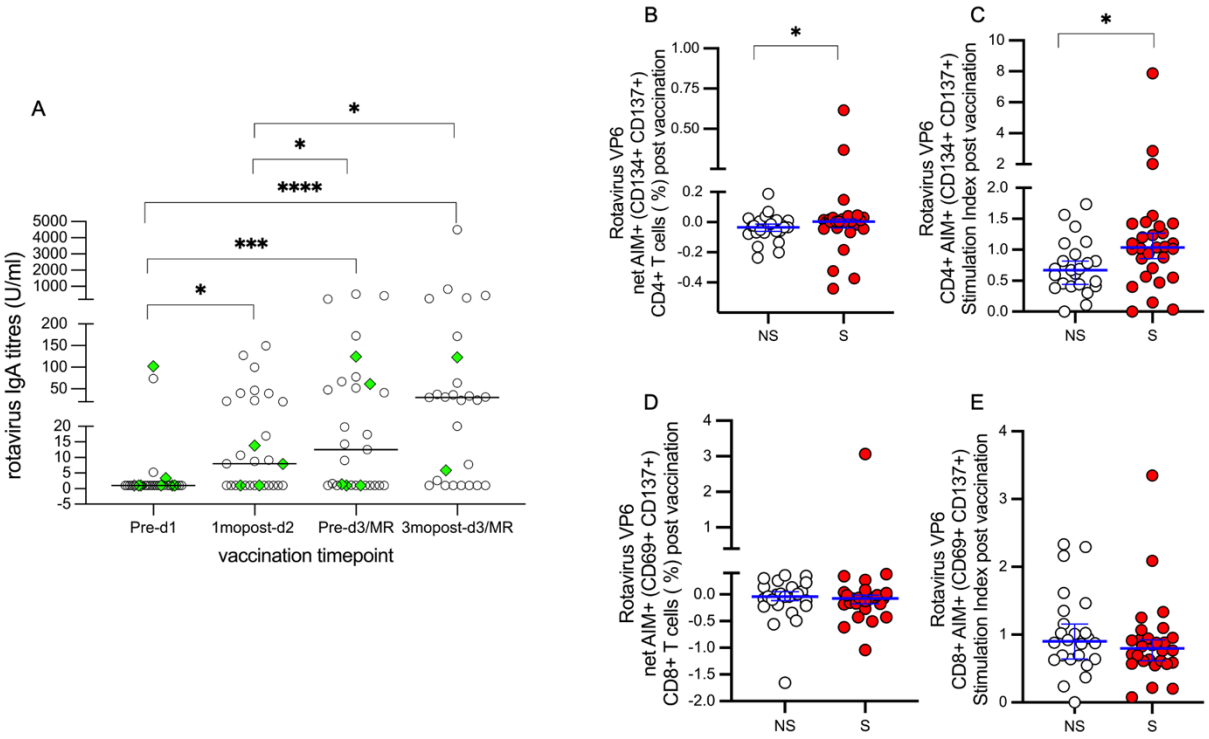


**Figure 6-8. Relationship between rotavirus VP6-specific T-cell responses and rotavirus antibody titres and seroconversion**

(A) Each data point represents the antibody titre in units/millilitre for a single infant at Pre-d1 (before first dose), 1mopost-d2 (one month post second dose), Pre-d3/MR (before third dose and/or measles rubella vaccination) and 3mopost-d3/MR (three months post third dose and/or measles rubella vaccination) among infants that were categorized into rotavirus VP6 CD4+ and/or CD8+ T-cell responders (n=29). At each timepoint, the white circles indicate infants who did not have a rotavirus VP6 CD4+ and/or CD8+ T-cell response and the green diamonds represent infants who had a rotavirus VP6 CD4+ and/or CD8+ T-cell response. Horizontal black line represents the median antibody titre. Net rotavirus VP6 specific CD4+ T-cell frequencies (B) and SI (C) between vaccine seroconverters and non-seroconverters. Net rotavirus VP6 specific CD8+ T-cell frequencies (D) and SI (E) between vaccine seroconverters and non-seroconverters. Comparison between vaccine seroconverters and non-seroconverters was done Mann Whitney U test. Comparison of difference in rotavirus antibody titres across timepoints was done by mixed effects analysis for repeated measures with Tukey's multiple comparison adjustment test. * ($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$).

## 6.5 Discussion

This study investigated T-cell responses after two and three doses of an ORV in Zambian infants. We measured the peripheral circulating frequency of rotavirus VP6 specific T-cells and ex-vivo NK and T-cell phenotypes post-vaccination and examined any association with vaccine seroconversion. We report limited number of rotavirus VP6-specific CD4+ and CD8+ T-cell responders and low frequencies of rotavirus VP6-specific CD4+ and CD8+ T-cells in the vaccinated infants. We also found that rotavirus VP6-specific T-cell responses were detected

in infants with and without rotavirus specific IgA antibodies but observed an enrichment of rotavirus VP6-specific CD4+ T-cell responses among vaccine seroconverters. In our ex-vivo analysis, we observed lower frequency of CD25+ CD4+T-cells and higher V$\delta$1+ TCR$\gamma\delta$ T-cells within a week after the second vaccine dose in vaccine non-seroconverters.

The VP6 antigen is the most abundant, highly conserved and immunogenic rotavirus protein (16) but we observed few infants that had a rotavirus VP6-specific T cell response which may have reflected the low number of vaccine seroconverters in our study. Majority of the infant RV-IgA antibody responses to live oral rotavirus vaccines measured by the standard rotavirus capture ELISA are directed against the VP6 protein which have been reported to provide protection via an intracellular neutralisation function (17). We saw an increase in these RV-IgA responses after each vaccine dose although a combination of natural rotavirus infections and the booster dose may also have contributed to the increase in RV-IgA observed between one month post second dose and 12 months as was seen for the overall infant study population of the parent study (12). Detection of breakthrough rotavirus infections in the parent RCT (18) and reported elsewhere (19) lends support to this.

Unlike the RV-IgA responses, we did not observe an increase in T-cell responders after each vaccine dose which suggests that induced memory rotavirus-VP6 specific T-cell are transient in circulation differing from antibody responses that remain elevated longer as reported by others (7). Interestingly we also observed T-cell responses among infants that were RV-IgA seronegative. Rotavirus specific lymphoproliferation and CD4 T-cell responses are reported predominantly in seropositive vaccinated children (8) and supports our findings of higher CD4 T-cell frequencies among vaccine seroconverters. However, these T-cell responses are also detected, albeit at lower levels, among seronegative children (7, 20). In the latter case, antibody responses below limit of detection for the ELISA assay or impairment in follicular CD4 T cell help important for B cell differentiation (21) are possible explanatory factors. Rotavirus specific CD4 T cells can protect against rotavirus infection in mice in the absence of virus specific antibodies (22) and whilst murine studies may not be generalizable to humans, presence of this memory rotavirus VP6-specific CD4 T-cell immunity in RV-IgA seronegative infants may similarly confer a level of protection against severe rotavirus diarrhoea but this protective effect was not determined in this study.

The rotavirus VP-6 specific CD4 and CD8 T cell frequencies detected in our study were low, generally below 0.1%, which is within similar range of frequencies reported in other published studies among children with rotavirus diarrhoea or post rotavirus vaccination within developing countries (8, 23-25). Notably, the results with antigen specific AIM+ T cells in this study need to be interpreted with caution since very few cells were analysed particularly for the CD8+ T cells (6244, 6742, 6758 and 4599 in the DMSO, rotavirus VP6, HCMV pp65 and PHA stimulated wells respectively) and high levels of background (average 0.58) were observed.

The bias of early life T cells towards generation of short-lived effector T-cells as suggested in a recent comprehensive review of early T-cell development (26) may contribute to these low-level responses in circulation. Sequestration of rotavirus-VP6 specific T-cells in intestinal sites supported by the fact that majority of rotavirus specific T-cells express intestinal homing markers (8, 27) is another potential reason for this observation. While we observed spikes of β7 integrin and CCR9 chemokine receptor expression on circulating CD4 and CD8 T cells across the time course of vaccination which may potentially reflect this migration, we were unable to determine specificity of these cells for rotavirus as done in other studies in vaccinated infants (8) nor did we find any difference in these intestinal homing phenotypes by vaccine seroconversion status. Additionally, the local intestinal immune response may not necessarily be reflected by these intestinally primed circulating T cells.

Elevation of CD25+CD4+ T cell frequency in vaccine seroconverters could have reflected activation of rotavirus vaccine specific T cells but could also reflect expansion of regulatory T cells. Upregulation of CD25, an IL-2 receptor that promotes differentiation, on CD4 T cells in vaccine seroconverters may reflect the higher rotavirus VP6 specific AIM+ CD4 T cell frequencies we observed in this group of children. However, higher RV-IgA and RV-IgG antibody levels have also been associated with higher frequencies of regulatory FOXP3+CD25+ CD4+ and CD8+ T cells in rotavirus vaccinated children which could be driven by protection from B-cell exhaustion by this regulatory mechanism. In contrast, lower antibody titres are observed with higher frequencies of CD4+IL-10+ regulatory T-cells which could inhibit proliferation of virus specific T-cells. (28) CD4+CD25+ with IL-10 secretion is reduced during rotavirus infection and may allow for effector T-cell proliferation for viral clearance (29) whereas other studies have found no effect on rotavirus specific CD4 T cell responses in children by CD4+ CD25+ regulatory T cells using a TGF-β mechanism (24). Distinguishing between these types of regulatory T-cells after live rotavirus vaccines can help elucidate the association with and subsequent impact on vaccine induced effector CD4 and CD8 T cell responses. The expansion of Vδ1+ TCRγδ T cells in vaccine non-seroconverters was likely

driven by HCMV which expands these unconventional T cells in primary infections (30) and whilst findings may not have equivalence in humans, knockout and depletion studies in mice show no impact of TCR$\gamma\delta$ T cells on RV-IgA antibody responses (31).

This study had some limitations. The sample size was small and likely impacted by low statistical power. The rationale for using rotavirus VP6 20mer peptides was based on their previous use to elicit rotavirus specific T-cell responses in published literature (13). Due to presentation of 20mer peptides via MHC II, the detected T-cell responses in this study were biased for helper CD4 T cells response. The 20mer peptide pool used in this study spanned the 1 to 397 amino acid residues of the VP6 from group A Rotavirus (14). CD4 T cell epitopes in 18mer and 20mer VP6 peptides in the regions AA242-259 and AA240-259 respectively in VP6 immunized mice (32) and 15mer peptides in region AA281-331 in human rotavirus infected rhesus macaques (33) have been detected. The VP6 peptide pool utilised in this study includes these regions but whether similar CD4 T cell epitope regions induced the CD4 T cell responses observed in these vaccinated infants remain to be determined and may be addressed in future experiments. The CD8+ T cell responses observed from activation via MHC class I would have required reprocessing of the 20mer peptides to smaller peptide lengths by antigen presenting cells such as dendritic cells. VP6 epitopes for cytotoxic CD8 T-cells have similarly been reported in murine studies utilizing 15mer or 10mer (34) and 9mer peptides (35) thus assessment with such appropriately size peptides for MHC class I presentation may have been more optimal to detect the CD8 T cell responses among vaccinated infants. Thus, use of 20mer peptide is likely to have reduced the detected number of CD8 T cell responders.

T-cell epitopes on other rotavirus structural proteins have been experimentally documented and reported using prediction models (35, 36) but were not studied here. The investigation of rotavirus T-cell responses using a more comprehensive peptide pool could well have increased the frequency of responding infants. The MHC class restriction of the presented VP6 peptides among infants may also have contributed to the low number of T-cell responders observed. Newer approaches that allow pooling a wide range of peptides spanning several antigens or the entire organism's proteome have been developed and employed for detecting T-cell responses to important viral pathogens in combination with the AIM assay (37) and may be of great benefit for rotavirus T-cell research. These MegaPools include diverse peptides of 15mer lengths which could be designed based on predicted or experimentally confirmed T-

cell epitopes giving an advantage of detecting both CD4 and CD8 T cell responses for wider range of epitopes and unbiased for MHC types (38).

We acknowledge that some biases may potentially have been introduced in the way the PBMC cohort was selected. Though unlikely, there may have been some subtle characteristic differences in the first half versus the second half of infants enrolled and between infants retained during follow up in the study compared to those that dropped out. We did not observe any significant differences in key baseline characteristics between vaccine seroconverters and non-seroconverters, however, the lack of matched selection may have introduced biases that we could not assess for. A sizeable proportion of infants in this study where HIV-exposed-uninfected and whilst no difference in vaccine seroconversion distribution was observed, there may have been a potential impact on the rotavirus T-cell response rate observed. Recent study indicates limited TCRβ chain clonality after vaccination in HEU infants (39) and we have certainly seen impacts on RV-IgA responses in interaction with human cytomegalovirus infection (HCMV) (40). Further studies with larger cohort size needed to resolve this possibility. Oral polio vaccine (OPV) which has been associated with lower rotavirus vaccine responses (41) was among co-administered vaccines and in this context, we cannot rule out its potential impact on blunting the rotavirus specific T cell response but also on observed trends in intestinal homing T cell phenotypes. Staggered administration of OPV and Rotarix vaccination has been associated with increased likelihood of RV-IgA seroconversion as opposed to concomitant administration (42) but this was not studied here. By using the surface marker-based AIM assay, we were not able to determine the effector subset of the detected CD4 and CD8 T-cell responses.

## 6.6 Conclusion

We detected limited rotavirus VP6-specific T-cell responses in response to oral rotavirus vaccination in Zambia but demonstrate enrichment of VP6-specific CD4 T-cells in vaccine seroconverters.

Author contributions

N.L conceived the study. NL and RC secured funding. N.L and M.G designed the experimental strategy. N.L performed the PBMC processing and flow cytometry analysis. N.L and S.B conducted the statistical analysis. N.L and M.G interpreted the data. N.L wrote the initial draft manuscript with review and editing from all authors. All authors reviewed and approved the final version of the manuscript.

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Data availability

Additional underlying data and information can be made available through the corresponding author upon reasonable request.

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| **First Name(s)** | Natasha Makabilo | | |
| **Surname/Family Name** | Laban | | |
| **Thesis Title** | Antibody and T-cell responses in rotavirus vaccinated Zambian infants: impact of human cytomegalovirus | | |
| **Primary Supervisor** | Martin Goodier | | |

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| Stage of publication | **Submitted** |

## Chapter 7: A longitudinal study of antibody responses to common cold HCoV and novel SARS-CoV-2 among mother-child pairs in Zambia

This chapter contains a research article submitted to BMC Infectious Diseases

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## 7.1 Abstract

The common cold human coronaviruses (HCoV) and novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infect children but seroprevalence estimates in Africa are limited. Association between cross-reactive HCoV and SARS-CoV-2 immunity is controversially debated and there is a research gap in Zambia. We investigated HCoV and SARS-CoV-2 antibody responses and cross-reactivity to elucidate seroepidemiology in Zambian children.

We measured HCoV OC43, HKU1, NL63 and 229E and SARS-CoV-2 Spike protein subunit 1 specific immunoglobulin G (IgG) in sequential plasma samples collected from Zambian mother-child dyads pre- and post the coronavirus disease 2019 pandemic over the first three years of life using enzyme-linked immunosorbent assay. We correlated maternal and child antibody levels to determine contribution of maternal immunity and within and between the human coronavirus to assess cross-reactivity. We additionally investigated the persistence of antibody response in children to assess durability.

HCoV OC43, HKU1, NL63, 229E and SARS-CoV-2 antibodies were detected among mothers and children. Maternal and child HCoV IgG levels were positively correlated and seroconversion to HCoV occurred following waning of maternal IgG antibodies in the first year of life. Children mounted durable but incompletely protective SARS-CoV-2 IgG antibodies post natural infection and HCoV HKU1 and 229E seropositivity was associated with boosted SARS-CoV-2 IgG antibody response.

There is broad circulation and early seroconversion of HCoV OC43, HKU1, NL63 and 229E in Zambia. Boosting of SARS-CoV-2 immunity by the common cold HCoV HKU1 and 229E types may have implications for SARS-CoV-2 vaccine immunity. Waning SARS-CoV-2 immunity and occurrence of re-infections necessitates continued surveillance inclusive of children to control prevailing and emergent variants that pose threats to public health.

## 7.2 Introduction

Human coronaviruses (HCoV) are a significant cause of respiratory infections in humans, particularly in children, and primarily associated with common cold and gastrointestinal disease (1, 2). Approximately 4-6% of children with acute respiratory illness are infected with common cold HCoV such as OC43, HKU1, NL63, and 229E, often as co-infections with other respiratory pathogens (3-6). In more recent years, highly pathogenic coronaviruses namely severe acute respiratory syndrome coronavirus (SARS-CoV), middle eastern respiratory syndrome coronavirus (MERS-CoV) and the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have emerged and associated with severe and highly fatal respiratory disease (2, 7). Moreover, SARS-CoV-2, which causes coronavirus disease 2019 (COVID-19), responsible for over 7 million deaths globally (8), has shown different infection patterns in children compared to adults, often resulting in milder symptoms but still contributing to community transmission and posing risks of severe outcomes (9, 10). Despite the occurrence of these viruses in pediatric populations, seroprevalence estimates in African children remain limited, creating a gap in understanding the epidemiology and immune responses to these pathogens.

The immune response to HCoVs is an important area of research due to the arguable yet potential cross-protection against SARS-CoV-2. Antibody responses during acute HCoV infections are rare but robust increments in antibody responses within 2-3 weeks after illness onset have been observed in individuals with common cold HCoV and SARS-CoV-2 infections with comparatively more durable IgG responses than other antibody classes (11). The coronavirus structural spike (S) protein is the major inducer of the host neutralizing antibodies targeted against its subunit 1 (S1) that mediates viral attachment and the subunit 2 (S2) involved in fusion and entry during infection (12, 13). Studies in children have indicated that prior exposure to common cold HCoV can influence the immune response to SARS-CoV-2 and alter the course of clinical presentation (14). It has been shown that Betacoronaviruses (OC43, HKU1, MERS-CoV, SARS-CoV and SARS-CoV-2) and Alphacoronaviruses (NL63 and 229E) exhibit higher viral sequence homology within their respective genera than across them (15, 16). This pattern also applies to T-cell epitopes, with HCoV Betacoronaviruses showing greater homology with SARS-Co-V-2 than Alphacoronaviruses (15). Consequently, natural and experimental studies have shown stronger antibody reactivity within each genus rather than between them (11, 16). Further studies suggested that antibody responses to the common cold HCoV Betacoronaviruses were boosted after SARS-CoV-2 infection as well as

detection of SARS-CoV-2 cross-reactivity in pre-pandemic sera (17-19). This cross-reactivity can provide insights towards observed SARS-CoV-2 immunity and is especially relevant in the context of pan-coronavirus vaccine development, and as it can impact the effectiveness of COVID-19 vaccines and inform strategies for booster vaccinations (20).

The aim of this study was to investigate the seroepidemiology of common cold HCoV and SARS-CoV-2 in mother-child pairs in Zambia. We examined the virus specific antibody responses and cross reactivity during the first three years of life in Zambian children. The results from our study can provide useful insights for developing effective public health strategies and vaccination programs to mitigate the impact of current and future coronavirus outbreaks.

## 7.3 Methods

### Study design and participants

This was a longitudinal study nested under an existing rotavirus vaccine trial. Briefly, the rotavirus vaccine trial enrolled mothers and their children aged 6-12 weeks old (n = 214) who were followed up to when the child was 36 months old. We made use of available participant data and stored plasma from both mothers and children sampled over the follow up period between September 2018 and November 2021 for HCoV testing. As illustrated in Figure 7-1, Plasma samples used in this study were collected at baseline when the child was 6-12 weeks old between September-November 2018 and during scheduled visits when the child was 14-20 weeks old (November 2018-February 2019), 9 months old (April 2019-July 2019), 12 months old (August 2019-October 2019) and 24 months old (August 2020-December 2020).

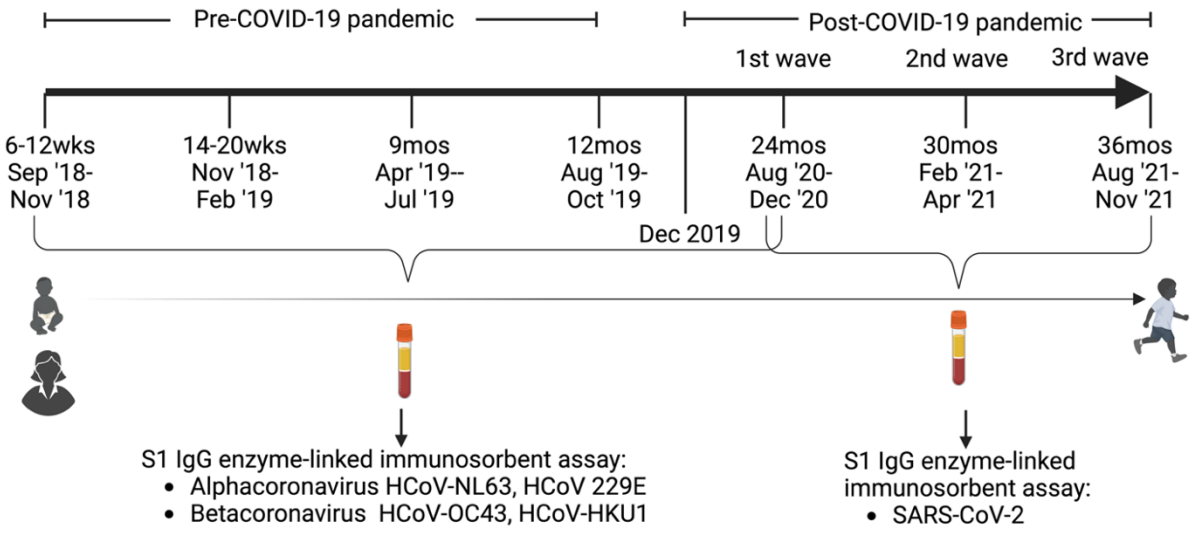


**Figure 7-1. Parent study sampling schema.**

Illustration of sampling timepoints under the parent rotavirus trial utilised for the nested longitudinal study. Created with BioRender.com

We included all children with known SARS-CoV-2 S1 IgG serostatus (n=150) in the first year of the COVID-19 pandemic (2020) as previously published (21) and their mothers for the measurement of HCoV-OC43, HCoV-HKU1, HCoV-NL63 and HCoV-229E antibodies (Figure 7-2). Among the 150 children, 148 (98.7%), 142 (94.7%), 145 (96.7%), 143(95.3%), and 146 (97.3%) had sufficient plasma available for HCoV testing at ages 6-12 weeks, 14-20 weeks, 9, 12 and 12 months respectively. Of the total mothers available 144/150 (96%) were tested for HCoV. We also explored SARS-CoV-2 S1 IgG serostatus within the subsequent 12 months among the SARS-CoV-2 S1 IgG seropositive (n=9) and seronegative counterparts matched for sex and age at a ratio of 2:1 (n=18) using plasma collected at the 30 months old (February 2021-April 2021) and 36 months old (August 2021-November 2021) timepoints.

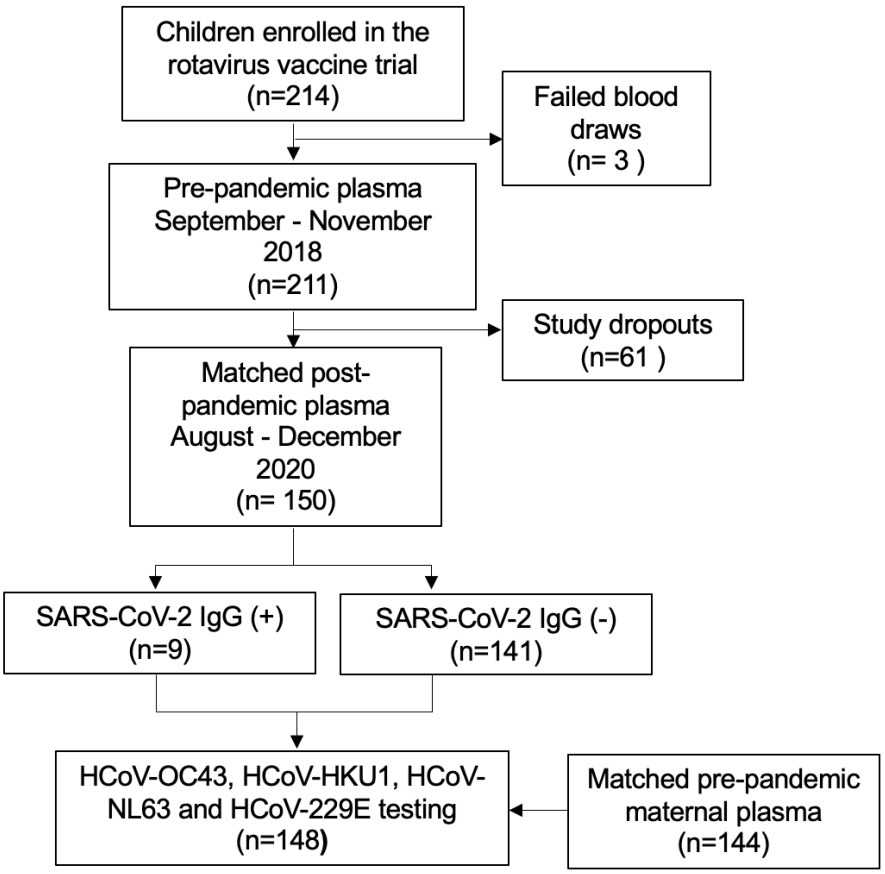


**Figure 7-2. Participant flow chart.**

Illustration of the selection process for participant samples included in the study and used in the laboratory analysis from a population of mothers and children enrolled in the rotavirus vaccine clinical trial.

Measurement of HCoV and SARS-CoV-2 specific antibodies

We tested plasma samples using an in-house indirect enzyme-linked immunosorbent assay (ELISA). We used commercially available antigens (Sino Biologicals Inc) for both the HCoV and SARS-CoV-2 ELISA: HCoV-NL63 Spike/S1 Subunit, (40600-V08H), HCoV-OC43 Spike/S1 Protein (40607-V08H1), HCoV-229E Spike/S1 Protein Subunit(40601-V08H), HCoV-HKU1 Spike/S1 Protein Subunit (40021-V08H) and SARS-CoV-2 Spike/S1-His recombinant protein (40591-V08H). Test plasma samples, diluted 1:100, were incubated at 4°Celsius overnight in duplicate wells of a 96-well microtiter plate (Greiner Bio-One) pre-coated with 0.5μg/ml HCoV or 1μg/ml SARS-CoV-2 antigen. Addition of 1:15000 diluted peroxidase conjugated anti-human IgG (Sigma-Aldrich) to the plate and incubation for 3 hours at room temperature was used to detect HCoV and SARS-CoV-2 antigen specific antibody responses. ELISA assays were developed by enzymatic reaction with o-Phenylenediamine dihydrochloride substrate (Sigma-Aldrich), development halted by addition of 1M Sulphuric

acid and absorbance measured at a 492nm wavelength using a microplate reader (Agilent, South Africa). We included adult plasma with known exposure to SARS-CoV-2 and HCoV in each experiment to generate relative absorbance units (rAU) as concentration readouts.

Data analysis

Background characteristics were summarized using frequency and proportion for categorical variables while interquartile interval was used for continuous variables. The S1 IgG seropositivity against HCoV-OC43, HCoV-HKU1, HCoV-NL63 and HCoV-229E was determined using a cut-off calculated by regression finite mixture model of the rAU readouts. The SARS-CoV-2 IgG seropositivity cut-off was calculated as the arithmetic mean of logarithm-transformed rAU of all pre-COVID-19 pandemic samples plus 3*standard deviation. Mann-Whitney U test was used to determine significance between groups and Wilcoxon signed rank test was used for matched pair. Spearman r was used to evaluate correlations. All analyses were performed in Stata 17 (StataCorp, College Station, TX, USA) and GraphPad Prism v9 (GraphPad Software, LLC). P-values <0.05 were considered significant and denoted as * ($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$) in figures.

7.4 Results

Among the total children with SARS-CoV-2 serostatus available (n=150) median age at baseline was 6 weeks and there no significant differences in general baseline characteristics between children who were SARS-CoV-2 IgG seropositive (n=9) and those that were seronegative (n=141) (Table 7-1).

**Table 7-1. Baseline characteristics of children.**

| Characteristic | All n (% of Total) | SARS-CoV-2 IgG - | SARS-CoV-2 IgG + | p-value |
|---|---|---|---|---|
| Child Age (weeks) | | | | |
| Median (IQR) | 6 (6,6) | 6 (6,6) | 6 (6,6) | 0.771 |
| Sex | | | | |
| female | 66 (44.0) | 64 (97.0) | 2(3.0) | 0.300 |

| | | | | |
|---|---|---|---|---|
| male | 84 (56.0) | 77 (91.7) | 7 (8.8) | |
| Gestation | | | | |
| Full-term | 143 (95.3) | 134 (93.7) | 9 (6.3) | 1.000 |
| Pre-term | 7 (4.7) | 7 (100.0), | 0 (0.0) | |
| Birth weight, (n=149) | | | | |
| <2.5kg | 15 (10.1) | 15 (100.0) | 0 (0.0) | 0.599 |
| ≥2.5kg | 134 (89.9) | 125 (93.3) | 9 (6.7) | |
| HIV unexposed | 103 (68.7) | 44 (93.6), | 6 (5.8) | 1.000 |
| HIV exposed | 47 (31.3), | 44 (93.6), | 3 (6.4), | |
| Total | 150 (100.0) | 141 (94.0) | 9 (6.0) | |

Seroprevalence of common cold HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1

Based on the statistical finite mixture regression models, calculated S1 IgG seropositivity cut-off antibody values among mothers (n=144) were 980.4 rAU for HCoV-NL63, 1072.2 rAU for HCoV-OC43, 936.8 rAU for HCoV-229E and 945.3 for HCoV-HKU1 (See Supplementary Figure S7-1, Additional File 1). For children, the calculated antibody cut-off values for the four coronavirus types ranged from 464.2 to 940.0 rAU at ages 6-12 weeks (n=148), 209.3 to 421.3 rAU at 14-20 weeks (n=142), 46.3 to 71.7 at 9 months (n=145), 41.0 to 80.0 rAU at 12 months (n=143) and 97.0 to 290.9 rAU at 24 months (n=146) (See Supplementary Figure S7-2, Additional File 2). We found evidence of exposure to all four common cold HCoV in mothers and children as shown in Figure 3. Among mothers at baseline, S1 IgG seropositivity was highest for HCoV-OC43 with 59/144 (41%) mothers that were seropositive, followed by 39/144 (27.1%) for HCoV-NL63, 29/144 (20.1%) for HCoV-229E and 22/144 (15.3%) for HCoV-HKU1 (Figure 7-3A). Among children, the trends in HCoV S1 IgG seropositivity at baseline were comparable to that observed in mothers with 37/148 (25%) children that were S1 IgG positive for HCoV-OC43, 25/148 (16.9%) for HCoV-NL63, 11/148, 7.4% for HCoV-HKU1 and 8/148 (5.4%) for HCoV-229E. At 14-20 weeks old (n=142), 9 (n=145), 12 (n=143) and 24 months (n=146) old seroprevalence was 8.5%, 28.3%, 35.7% and 57.5% for HCoV-NL63, 10.6%,

33.1%, 32.9% and 33.6% for HCoV-OC43, 12.0%, 25.5%, 66.4% and 42.5% for HCoV-HKU1 respectively (Figure 7-3B).

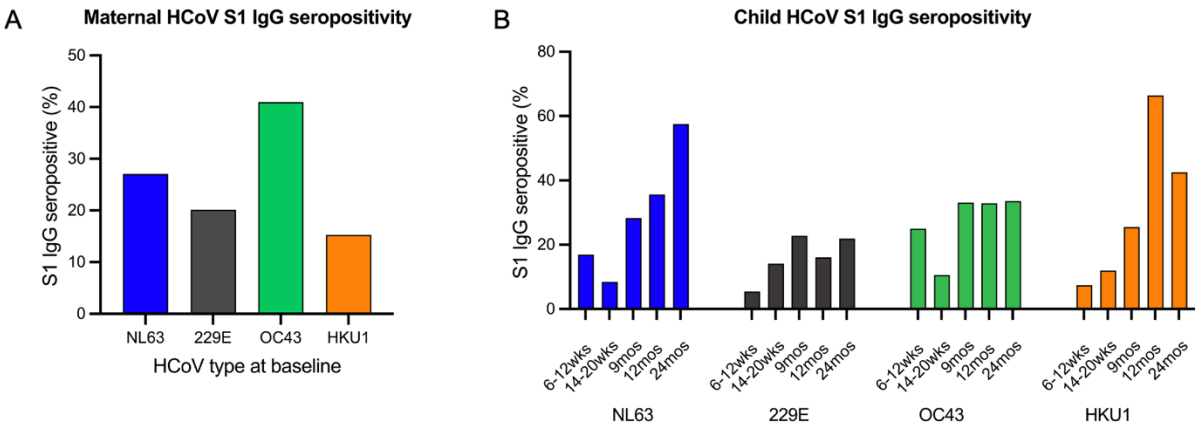


**Figure 7-3. Seroprevalence of common cold HCoV.**

Percent of S1 IgG seropositivity against HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 in mothers at baseline and longitudinally sampled children at ages 6-12 weeks (baseline, n=148), 14-20 weeks (n=142), 9 months (n=145), 12 months (n=143) and 24 months (n=146). The percent of S1 IgG seropositive individuals out of total number tested at timepoint are plotted as bars for each HCoV type.

<u>Maternal and child common cold HCoV antibody responses are correlated in early life.</u>

We assessed whether the similarities observed between maternal and child HCoV S1 IgG seroprevalence at baseline were reflective of transplacental HCoV S1 IgG transfer. As shown in Figure 7-4, a statistically significant relationship between maternal and child (n=142) S1 IgG antibodies at baseline was observed for all four HCoV with strong to moderate positive correlations for Alphacoronaviruses HCoV-NL63 (Figure 7-4A $r_s$=0.649, p<0.0001), HCoV-229E (Figure 7-4B $r_s$ =0.578, p<0.0001 n=141) and Betacoronaviruses HCoV-OC43 (Figure 7-4C $r_s$ =0.471, p<0.0001) and HCoV-HKU1 (Figure 7-4D $r_s$ =0.658, p<0.0001).

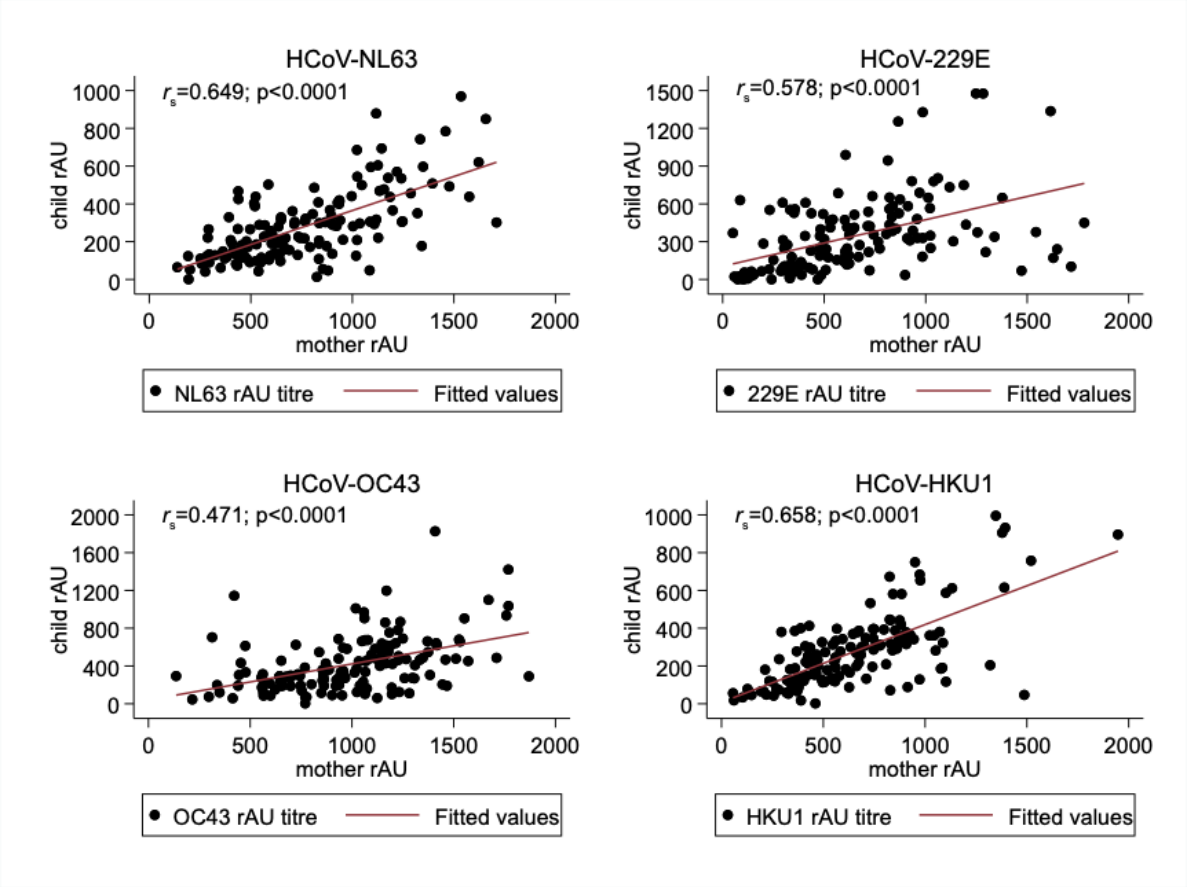


**Figure 7-4. Association of HCoV S1 IgG antibody titres in mothers and children at baseline.**

Spearman correlation coefficient ($r_s$) and statistical significance of the relationship between mother and child HCoV-NL63 (A), HCoV-229E (B), HCoV-OC43 (C) and HCoV-HKU1 (D) S1 IgG titres at baseline are shown. Each datapoint represents the HCoV specific S1 IgG titre in relative absorbance units in mother-child pairs.

Early seroconversion to all four endemic HCoV in children

To investigate the timing of initial seroconversion to the four HCoV in children we studied individual level trends of HCoV specific S1 IgG titres in serially collected plasma limited to children that had data on S1 IgG titres available at all sampling timepoints during the first two years of life (n=134). Overall, there was a waning of S1 IgG titres between baseline and 9 months old for HCoV-NL63 (Figure 7-5A), HCoV-229E (Figure 7-5B), HCoV-OC43 (Figure 7-5C), and HCoV-HKU1 (Figure 7-5D), after which an increase in titres was observed although in some children the increase in titres were evident by 14-20 weeks old (See Supplementary Figure S7-3, Additional File 3).

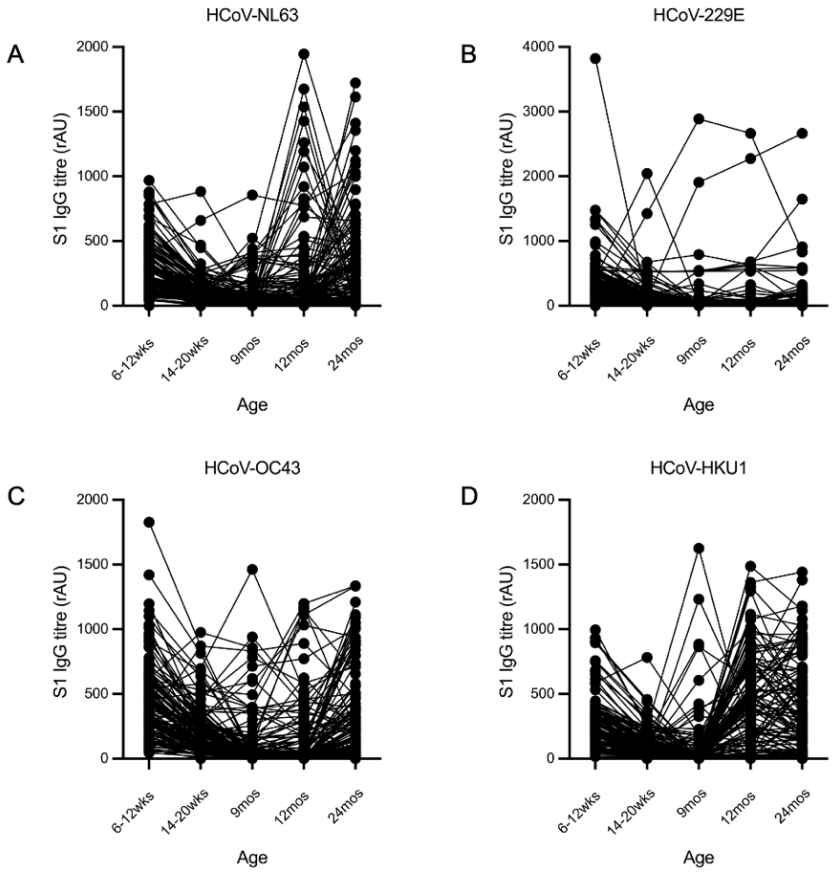


**Figure 7-5. Trajectory of S1 IgG titres against the common cold HCoV in children.**

Individual level trends of S1 IgG titres against HCoV-NL63 (A), HCoV-229E (B), HCoV-OC43 (C) and HCoV-HKU1 (D) in childrenwithin the first two years of life. Connected datapoints represent the trajectory of HCoV-specific S1 IgG antibody titres measured in relative absorbance units (rAU) for a single child among children that had data available at all five sampling timepoints (n=134).

## Cross-reactivity within the HCoV Alpha- and Betacoronavirus genus

Among children that were categorized as seropositive to any of the different endemic HCoV at 24 months during the first wave of pandemic (n=117), we assessed the relationship of S1 IgG antibody tires within HCoV-NL63 and HCoV-229E seropositive children, HCoV-OC43 and HCoV-HKU1 seropositive children and between the two genera. We also compared the relationship of SARS-CoV-2 S1 IgG titres within and between Alpha- and Betacoronavirus seropositive children. As shown in Figure 7-6, HCoV-NL63 and HCoV-229E S1 IgG titres were positively correlated ($r_s$ =0.44, p<0.0001). Similarly, positive correlation was observed for HCoV-OC43 and HCoV-HKU1 S1 IgG titres ($r_s$=0.40, p<0.0001). A significant but weak positive correlation was observed for HCoV-HKU1 and SARS-CoV-2 S1 IgG titres ($r_s$ =0.24,

p=0.01). There was no statistically significant correlation observed in S1 IgG titres when Alphacoronaviruses HCoV-NL63 and HCoV-229E were compared to Betacoronaviruses HCoV-OC43 and HCoV-HKU1. However, a significant but weak positive correlation was seen for HCoV-229E and SARS-CoV-2 S1 IgG titres ($r_s$=0.19, p=0.038).

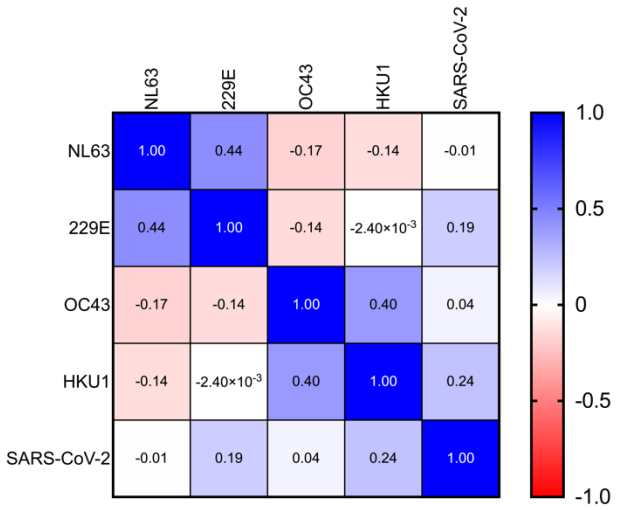


**Figure 7-6. Correlation between HCoV and SARS-CoV-2 IgG titres.**

Correlation matrix and Spearman correlation coefficients of S1 IgG titres measured in relative absorbance units within and between Alphacoronaviruses HCoV-NL63 and HCoV-229E and Betacoronaviruses HCoV-OC43, HCoV-HKU1 and SARS-CoV-2 among children (n=117) at 24 months old.

In a comparison of SARS-CoV-2 S1 IgG antibody titres at 24 months old by HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 S1 IgG serostatus at 12 and 24 months old, we observed higher SARS-CoV-2 S1 IgG antibodies among HCoV-HKU1 seropositive infants (p=0.0182) at 12 months (Figure 7-7A). Interestingly, both HCoV-HKU1 (p=0.0171) and HCoV-229E (p=0.0239) seropositive infants had higher SARS-CoV-2 S1 IgG antibodies at 24 months than their seronegative counterparts (Figure 7-7B).

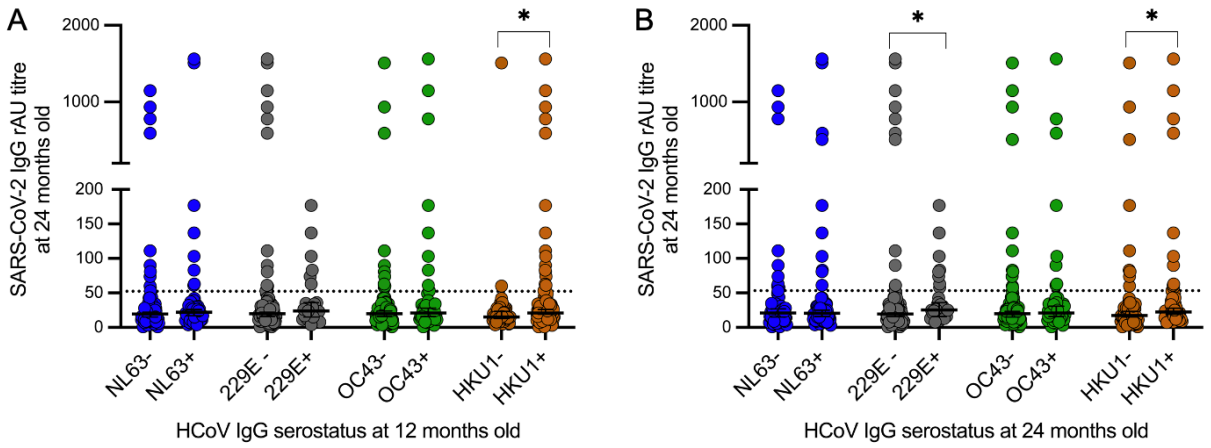


**Figure 7-7. SARS-CoV-2 S1 IgG by HCoV serostatus.**

SARS-CoV-2 S1 IgG antibody responses at 24 months old in HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 seronegative (-) and seropositive (+) children at 12 months (A) and 24 months (B) old. Each datapoint represents a singlechild. Median is shown as solid horizontal line with 95% confidence intervals. Dotted line shows the cut-off value for SARS-CoV-2 S1 IgG seropositivity.

Kinetics of S1 IgG antibodies after SARS-CoV-2 infection in children

We had previously report a seroprevalence of 9/150 (6%) among the children aged 24 months during the first wave of COVID-19 (21). Here, we further investigated the SARS-CoV-2 S1 IgG seroprevalence at 36 months old after the second and third waves of the COVID-19 pandemic among children with available samples (n=125) and observed a total of 33/125 (26.4%) of children that were SARS-CoV-2 S1 IgG seropositive by this timepoint.

In a subset of children, we longitudinally assessed SARS-CoV-2 S1 IgG in 8/9 children previously reported as SARS-CoV-2 S1 IgG seropositive at 24 months and their SARS-CoV-2 S1 IgG seronegative matched controls (n=18) at 30 months and 36 months old corresponding to 6 and 12 months post initial seroconversion to study kinetics of the antibody responses over time. As shown in Figure 7-8A, except for a single child, all children that were seropositive at 24 months old remained seropositive by 36 months old. We observed waning of SARS-CoV-2 IgG titres within 6 months and 12 months post initial seroconversion among these seropositive children (Figure 7-8A) but there was no significant difference in median SARS-CoV-2 IgG titres between 24 and 36 months old (p=0.167). Increase in SARS-CoV-2 S1 IgG titres was seen among previously seronegative children during the periods when the second and third COVID-19 waves occurred (Figure 7-8A). Significantly increased SARS-CoV-2 S1 IgG titres during the third COVID-19 wave compared to baseline was observed in

the mothers of children in this subset (Figure 7-8B) and a total of 16/21 (76.2%) were SARS-CoV-2 S1 IgG seropositive.

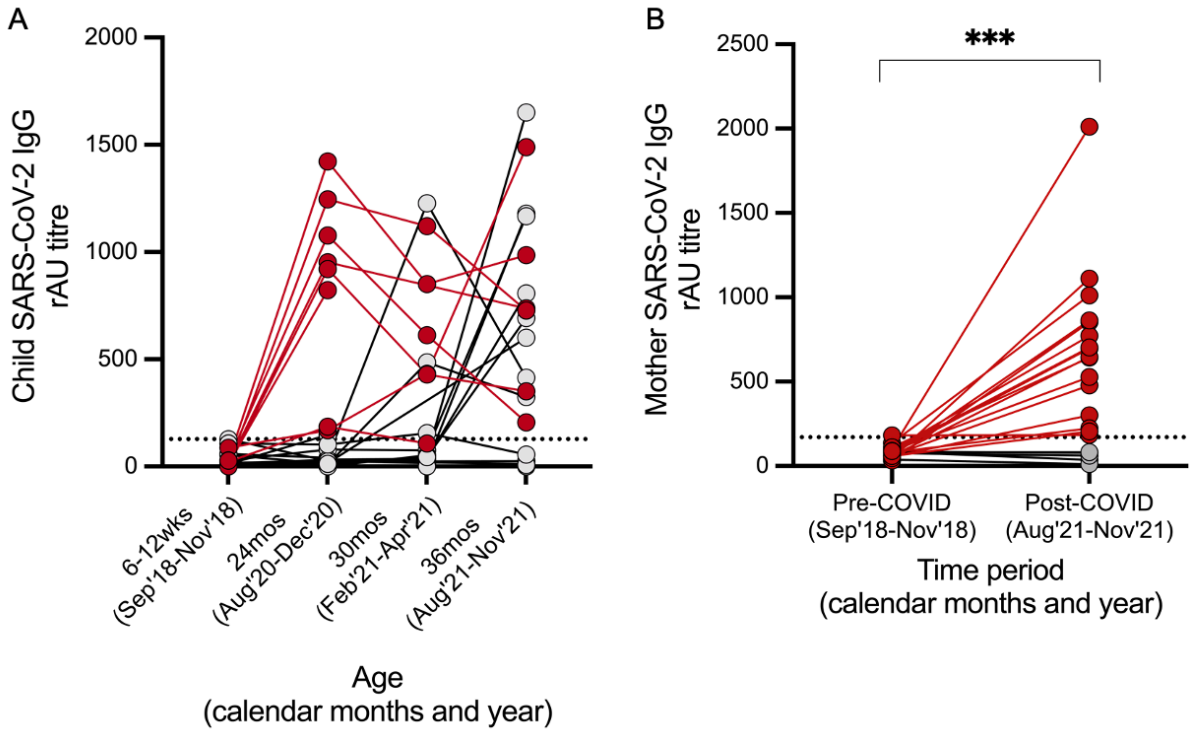


**Figure 7-8. Kinetics of SARS-CoV-2 S1 antibody responses pre- and post-COVID-19 pandemic in mothers and children.**

Child SARS-CoV-2 S1 IgG (A) are shown before COVID-19 and at three timepoints post COVID-19 pandemic during the first, second and third waves in Zambia. Each data represents a single child among those that were SARS-CoV-2 S1 IgG seropositive (n=8, red) and seronegative (n=18, grey) at 24 months old. SARS-CoV-2 S1 IgG titres are shown for matched mothers (n=21) among those that were SARS-CoV-2 S1 IgG seropositive (n=16, red) and seronegative (n=5, grey) at post-COVID-19 during the third wave (B). Dotted line shows the cut-off value for SARS-CoV-2 S1 IgG seropositivity.

## 7.5 Discussion

This study described the common cold HCoV, and SARS-CoV-2 spike specific IgG antibody profiles among mothers and children in Zambia. We found that NL63, 229E, OC43 and HKU1 HCoV were prevalent among mothers and children. Our findings suggest an efficient transplacental transfer of spike specific IgG for all four common cold HCoV as there was a strong correlation between mother and child HCoV IgG levels in the first 6-12 weeks of life. We provide evidence that this passively acquired maternal HCoV immunity wanes to low levels over the first 9 months of life for all the four HCoV types after which most seroconversions occur. In our assessment of cross-reactivity between HCoV, we observed higher SARS-CoV-2 S1 IgG titres among children with pre-existing antibodies to common cold HCoV-HKU1 and

HCoV-229E suggesting additive cross-reactive effects or a boosting effect of exposure to these HCoV on SARS-CoV-2 antibody titres. We also show that children mount durable SARS-CoV-2 S1 IgG responses that are sustained over one year post infection and document a further increase in SARS-CoV-2 S1 IgG seroprevalence during the second and third waves COVID-19 in Zambia.

To the best of our knowledge this is the first HCoV seroprevalence study that attempted to determine the kinetics of transplacental HCoV immunity and timing of seroconversion among children in Zambia. Seroprevalence estimates in our study differed from those reported in other similar studies in China (22, 23) the Netherlands (24) and the Philippines (25) which may be attributed to variation in methodologies used, seasonal HCoV distributions and inclusion of children with acute or severe respiratory illness in the study population. Nevertheless, we showed that all four HCoV are commonly circulating in Zambian children with initial infections occurring within the first year around 9 months old. Our observation of waning immunity is consistent with reports noting significant drop in in HCoV IgG antibodies or seropositivity within the first year of life (23, 25) and which most likely reflects the gradual loss of transplacental maternal immunity. This was supported by the observation of a positive correlation between the mother and child's HCoV IgG antibody levels at 6-12 weeks old. Correlations in neutralizing SARS-CoV-2 IgG have been reported in seropositive mothers and cord blood in Ghana (26). The observed kinetics of this maternal HCoV immunity can be insightful for understanding durability of protection against SARS-CoV-2 by maternal vaccination and timing of potential SARS-CoV-2 vaccination strategies in infants.

Our observed weak correlation between SARS-CoV-2 and Betacoronavirus HKU1 may be explained by the low 29% sequence homology identity for the S protein, however, we did not find association between SARS-CoV-2 and OC43 despite also having an approximately similar S protein sequence homology (16). Interestingly, we also found weak positive correlations between SARS-CoV-2 and Alphacoronavirus 229E. This suggests evidence of shared antibody epitopes across HCoV Alpha and Betacoronaviruses. Our finding that pre-existing HKU1 and 229E seropositivity was associated with increased SARS-CoV-2 IgG titres is supported by the weak correlations observed and reports in studies elsewhere where increase in HCoV-229E IgG antibodies were associated with increased SARS-CoV-2 IgG antibodies (27). However, it contrasts with some studies that found no correlation between pre-infection HKU1 antibodies and post infection SARS-CoV-2 IgG titres (28). We cannot speculate on the significance of these cross-reactive associations on SARS-CoV-2 protective

immunity from this data which require further study to assess any possible effect on neutralization functions. It is also clear that T cell responses contribute to cross-protection between circulating HCoV strains and SARS-CoV-2 (29). It will also be important to replicate our studies in the context of SARS-CoV-2 vaccination considering that distinct cross-reactivity profiles are observed between native and stabilized S proteins from natural infection and vaccination respectively (17).

Nearly all SARS-CoV-2 S1 IgG seropositive children in our study remained seropositive over the course of the 12 month period post initial seroconversion suggesting persistence of immunity like what has been reported elsewhere (28, 30). However, caution must be taken in the interpretation of these results as durable antibody titres may not imply persistent protection. We observed re-boosting of SARS-Co-V2 S1 antibody titres among the SARS-CoV-2 seropositive infants suggesting immune escape infections and new seroconversions among previously seronegative children. These may have been due to emergent SARS-Co-V2 variants which caused a sharp increase in seroprevalence in Africa during the second and third COVID-19 waves (31). Whilst S1 primarily induces neutralizing antibodies we were not able to ascertain this protective function against different SARS-CoV-2 variants in our study. At the very least our data provides information on trends of SARS-CoV-2 S1 IgG antibody responses post infection which may be helpful in informing the dosing for sustaining vaccine-induced immunity.

Key strengths of our study included our longitudinal design spanning both pre and post COVID-19 periods which was advantageous for temporal profiling of both the HCoV and SARS-CoV-2 antibody responses. We measured IgG against the immunodominant S protein allowing us to effectively capture prior exposure. Our sampling of mother-child pairs also permitted separation of maternal contribution to HCoV seropositivity. This study had limitations. The actual HCoV or SARS-CoV-2 infection in seropositive mothers and children was not confirmed by molecular methods and the neutralizing function of measured antibody responses was not studied here. The IgG responses peaks later than IgM or IgA and we may not have detected individuals with more recent infection (11). The HCoV seroprevalence estimates were based on samples collected at timepoints defined under the parent study aims. In Zambia, surveillance of respiratory pathogens in patients with influenza-like illnesses reported seasonal peaks for HCoV, whereby NL63 and 229E are reported to peak between November and January and OC43 and HKU1 between May and (6, 32). Our HCoV estimates

may therefore have been biased for or underestimated specific HCoV types depending on circulation patterns before or at sample collection.

### 7.6 Conclusion

HCoV widely circulate among children in Zambian children and adults with exposure occurring in the first year of life that is associated with waning of maternal immunity. In the post COVID-19 era, circulation of SARS-CoV-2 may follow similar patterns which can guide control measures. Boosting of SARS-CoV-2 immune responses by common cold HCoV may be exploited for improvement of vaccine-induced responses or in the design of pan-coronavirus vaccines. Persistent SARS-CoV-2 IgG responses from natural infection in children contribute to herd immunity in Zambia, but the evident waning of responses and re-infections emphasized the need for continued SARS-CoV-2 surveillance in children complemented by such seroprevalence studies.

### 7.7 Declarations

#### Ethics approval and consent to participate.

This study was approved by the National Health Research Authority of Zambia based on the ethical approval obtained by the University of Zambia Biomedical Research Ethics (Reference number: 003-02-18).

#### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NML and MG conceived the study. NML acquired the funding from The Wellcome Trust. RC acquired the funding from EDCTP2. NML, HN and AC performed the laboratory testing. NML, MG and SB analyzed and interpreted the data. NML wrote the original manuscript draft. NML, MG, SB and RC contributed to writing the manuscript. All authors read and approved the final manuscript.

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## Chapter 8: Discussion

The overarching aim of this thesis was to address the underperformance of ORV within a low-income setting of Zambia by contributing knowledge towards elicited vaccine antibody and T-cell responses and investigating the influence of an important immunomodulating pathogen, HCMV, on ORV immunogenicity. In response to COVID-19 the thesis also addressed the need to understand the seroepidemiology of HCoV in Zambia. This thesis has described for the first time in Zambia the antibody responses to a three-dose regimen of ORV and contributed to the body of knowledge concerning booster ORV doses in Africa. I have evaluated the effect of exposure to HCMV on ORV immunogenicity which led to key findings of a previously unknown interaction between HCMV and HIV on reducing rotavirus antibody responses. I have detected rotavirus specific T-cell responses among children receiving vaccination and generated new information on unconventional T-cells within the context of rotavirus vaccination. I have also generated research evidence of the timing of common cold coronavirus infections and the cross-reactive responses to SARS-CoV-2 in Zambian children. In this chapter, I will summarise these findings and discuss future work that can be done.

### 8.1 Summary findings and future work

In the comparison of two versus three doses of ORV, I found no significant difference in the induced rotavirus antibody levels three months after the third dose was administered. The administration of the third dose when children were 9 months old was strategic in the sense that should there have been significant differences observed, the timepoint would be operationally feasible for inclusion in the national EPI program as measles vaccination is administered at the same time. Thus, mothers would have avoided an extra clinic visit. Additionally, by this age it was conceivable that the effect of maternal antibodies known to interfere with ORV responses (1, 2) may have waned. The study did not observe any differences in safety profile between infants who received a third dose of Rotarix and those who did not. In some meta-analysis of randomised placebo-controlled clinical trials and post-licensure comparisons, rotavirus vaccination has not been associated with an increased risk of intussusception (3, 4). However, a significant limitation was that the study was not powered to detect these rare events of intussusception which would require much larger sample sizes.

The lack of a boosted RV-IgA response may have been impacted by the time between the third dose administration and the assessment of immune boosting. Studies elsewhere showed boosted antibody responses from a third dose of ORV when the boosting effect was assessed

within shorter time periods of within a month to two months (5, 6). It is also plausible that due to the high rotavirus burden maternal antibodies would still have been at high enough levels in infants to inhibit vaccine viral replication which we did not account for. A future study would be to investigate the kinetics of rotavirus transplacental IgG which could reveal more insights to this and potentially more optimal booster dose vaccination timepoints. Additionally, with the high rotavirus burden, it is possible that natural rotavirus infection during the three-month period augmented rotavirus immunity in the control arm to comparable levels as those who received a third dose. Lending support to this notion, an increase in RV-IgA among control arm children was seen. I did detect few rotavirus-positive stool samples in the study cohort, however as collection was passive within the first year of life rotavirus diarrhoeal cases may have been missed. In the measurement of antibody response, I also found that overall, the seroconversion was low at approximately 27%. Comparable estimates were reported for Rotavac vaccine within a similar setting in Zambia (7). It has been argued that use of heterologous strains in the rotavirus ELISA assays may undermine antibody responses thus it would be interesting to conduct this comparison using Rotarix specific virus strain in this cohort (8).

Birth doses for rotavirus vaccines have been proposed as alternative schedules to improve protection in children (9). Indeed, in previous studies from our group, an estimated 25% of Zambian children are seropositive for rotavirus implying early infection with the first weeks of life (1) and so neonatal doses may combat these early infections. Future work should also include the investigation of the neutralization capacity of induced rotavirus antibodies. Majority of antibodies detected in the rotavirus ELISA are non-neutralizing targeted at VP6 antigen. However, VP6 elicited antibodies have intracellular neutralization capacity (10). Stratification of vaccinated children by VP6 specific neutralizing functional antibody profiles would also be useful future studies as it is possible that children termed non-seroconverters may have strong antibody neutralization function despite low levels of detected RV-IgA. Rotavirus VP4 and VP7 are targeted by neutralizing antibodies and work is underway in this cohort to assess these responses.

The high prevalence of HCMV in Zambia especially in relation to HIV exposed children was well appreciated and some studies had already reported associations with physiological impairments in children (11) and investigated effects on oral polio vaccine (12). Surprisingly, despite the known reduced immunogenicity, there had not been any study that attempted to assess the potential impact of HCMV for ORV. To the best of my knowledge, I was the first to

attempt to measure this effect and found an interaction of HCMV and HIV exposure in infants that was associated with reduced rotavirus antibodies. This finding is concerning in view of the high HIV prevalence in Zambia which increases this the size of the sub-population vulnerable for reduced immunity. It would be important to replicate this study in a larger sample size and investigate whether these effects were specific for ORV or would be seen for other EPI vaccines in Zambia. It will also be important to assess the impact of HCMV on the T-cell repertoire in these low-responders especially with the known immunomodulatory role for HCMV on T-cells and the observed reduced TCR diversity in HEU infants (13).

Information on infant HIV exposure was obtained from antenatal cards and/or children clinic cards presented by mothers at the 6-week enrolment visit. The Zambian guidelines on HIV testing during pregnancy recommend that HIV testing by serological methods is done at the first antenatal visit and that retesting is done every three months if the mother is HIV negative(14). While some studies have estimated that about one third of pregnant women do not return for retesting during subsequent antenatal visits (15), other HIV testing timepoints are also recommended during labour and delivery if HIV test is not done within preceding 6 weeks and during post-natal care for mothers with unknown HIV status and at first point of contact with health facility (14). The RCT study also specifically offered HIV rapid testing at this 6 week point of contact with the enrolled mothers in keeping with these national guidelines. Therefore, mothers whose HIV status was recorded based only on first antenatal visit without subsequent testing but who may have acquired HIV by delivery would have been tested at delivery or this 6 week visit at health facility or enrolment timepoint unless they opted out thus reducing odds of misclassifying HIV exposed infants. It is however likely that HIV exposed infants of mothers who may have seroconverted after enrolment would have been misclassified us unexposed.

Testing for HCMV-IgM seropositivity was done from 6 weeks of age onwards in this study and therefore may have comprised both congenitally and post-natally acquired HCMV. Recommended methods for confirmation of congenital infection is by testing of saliva, urine or blood within the first 3 weeks of life (16). Thus, whether the contribution to observed effects of HCMV on ORV RV-IgA vaccine responses were more from congenital or post-natal infections could not be determined in this study. HCMV-IgM rather than HCMV-IgG was measured to determine infant exposure status due to the latter's transplacental transfer which could have mostly reflected previous maternal infection. Performing HCMV-IgG avidity testing as done in studies elsewhere (17) would have been able to distinguish high avidity HCMV-IgG

of maternal origin in early life from low avidity infant specific HCMV-IgG, however I did not do this due to funding limitations. It was likely that testing for only HCMV-IgM may have missed infants whose HCMV-IgM responses had waned or who had only HCMV-IgG at the time of sampling. Cross-reactivity of HCMV-IgM with other herpesviruses like Epstein-Barr virus (18) may have also misrepresented HCMV-IgM seropositive infants. Another limitation in the HCMV study was also that I could not ascertain actual infection by molecular methods and as such future work may also include determination of HCMV strains and the potential association with these observations. It is likely that other infant and maternal factors could have influenced the reduced rotavirus antibody responses in HCMV seropositive infants. For example, our group has found that breastmilk innate glycoproteins (19), maternal antibodies (20), enteric dysfunction (21) and HBGA (22) all have some level of impact on rotavirus immunogenicity. About 21% of children remained HCMV IgM seronegative by 1 year of life. Future work would be to explore the characteristics to assess any protective mechanisms for instance whether maternal HCMV transplacental immunity had a role to play in delayed acquisition. To further this line of work, investigation of transplacental glycoprotein B and pentameric complex IgG responses that have been associated with protection (23) and association with HCMV acquisition by 12 months of age is underway.

Low level rotavirus VP6-specific T-cell responses were reported in this thesis agreeing with several other studies with similar findings (24) however this emphasizes the need to continue research to understand immune parameters that can be ideal biomarkers for assessing rotavirus immunogenicity. There is no correlate of protection for rotavirus vaccines which hampers speedy evaluation of vaccine candidates (25) and as such study of rotavirus vaccine induced immunity remains and important research area. A novelty of my work was the inclusion of innate and unconventional T-cells in studying the immune response to ORV in children. These unconventional T-cells have been linked to intestinal immune protection against viral infection (26) and some like the the $\gamma\delta$T-cells to have both innate and adaptive immune functions (27). In this study I found that vaccine non-seroconverters had higher V$\delta$1+ $\gamma\delta$T-cells and it would be interesting to explore these subsets further and assess whether this is related to HCMV infection which is known to expand these cell subsets and determine the mechanism of impact if any on rotavirus vaccine response as future work.

I found that Zambian children seroconverted to HCoV OC43, HKU1, NL63 and 229E in the first year of life. This was like the seroconversion timings in studies done elsewhere (28, 29)

and to my knowledge this was the first longitudinal HCoV serological study in children within Zambia. The data describe in the thesis shows evidence of pre-existing immunity to these HCoV prior to the COVID-19 pandemic which supports reports from others (30). Moreover, I documented strong correlation between maternal and child HCoV antibodies. In the post-COVID19 pandemic and vaccination era, future studies would be to investigate the kinetics of transplacental SARS-CoV-2 antibodies as done in other settings (31), and it would be interesting to determine whether this passively acquired SARS-CoV-2 immunity would follow similar trajectories to HCoV for Zambian children.

Whether this pre-existing HCoV immunity contributed to the COVID-19 transmission dynamics in Zambian children is unclear and could not be addressed in this thesis. Notably, I observed evidence of cross-reactivity by virtue of correlations in IgG titres between Alphacoronavirus 229E and Betacoronavirus HKU1 with SARS-CoV-2. Future work would be to measure the neutralizing ability of these cross-reactive sera on SARS-Cov-2 variants and investigate the cross-reactive T-cell responses in these children to confirm these findings. I also found that SARS-CoV-2 infections in children induced persistent IgG antibody responses, but waning over time and occurrence of re-infections emphasizes the need to include children who may be potential transmission reservoirs in the local surveillance strategies .

## 8.2 Conclusion

In conclusion, RV-IgA was not boosted by a third dose at 9 months in the Zambian setting. Other vaccine schedules such as birth doses prior to infection may need to be assessed for improved protection. HCMV seropositivity negatively impact rotavirus immunity by significant reduction of RV-IgA responses in HEU infants and there is a need for future research to confirm these findings in larger cohorts and determine the biological mechanisms for such effects. As they may have a role to play in the observed diminished rotavirus immunogenicity in poor resource areas. T-cells directed against the VP6 protein are induced by rotavirus vaccination but circulate at very low frequencies and there is evidence of an association between vaccine seroconversion and V$\delta$1+ $\gamma\delta$T-cells. HCoV antibodies are effectively transferred between mother and infants and evidence of cross-reactive HCoV antibodies was found in children which can inform local SARS-CoV-2 control strategies in Zambia.

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**Appendices**

1. Supplementary Tables

**Supplementary Table S 0-1. PRISMA Checklist for the systematic review**

| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| **TITLE** | | | |
| Title | 1 | Identify the report as a systematic review, meta-analysis, or both. | 60 |
| **ABSTRACT** | | | |
| Structured summary | 2 | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 61 |
| **INTRODUCTION** | | | |
| Rationale | 3 | Describe the rationale for the review in the context of what is already known. | 61-62 |
| Objectives | 4 | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). | 62 |
| **METHODS** | | | |
| Protocol and registration | 5 | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. | n/a |
| Eligibility criteria | 6 | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. | 62-63 |
| Information sources | 7 | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. | 62-63 |
| Search | 8 | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. | 247-250 (Supplementary Table S 2-3) |

| | | | |
|---|---|---|---|
| Study selection | 9 | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). | 63-64 |
| Data collection process | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. | 63-64 |
| Data items | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. | 63-64 |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. | 64 and 245-246 (Supplementary Table S 2-2) |
| Summary measures | 13 | State the principal summary measures (e.g., risk ratio, difference in means). | n/a |
| Synthesis of results | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$) for each meta-analysis. | n/a |
| Risk of bias across studies | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies). | n/a |
| Additional analyses | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. | n/a |
| **RESULTS** | | | |
| Study selection | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. | 64-65 |
| Study characteristics | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. | 67-72 (Table 2-1) |
| Risk of bias within studies | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). | 73 |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 73-105 (Table 2-2 to 2-5) |
| Synthesis of results | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency. | n/a |
| Risk of bias across studies | 22 | Present results of any assessment of risk of bias across studies (see Item 15). | n/a |

| | | | |
|---|---|---|---|
| Additional analysis | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]). | n/a |
| **DISCUSSION** | | | |
| Summary of evidence | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). | 106-110 |
| Limitations | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). | 110-111 |
| Conclusions | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research. | 111 |
| **FUNDING** | | | |
| Funding | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. | 111 |

**Supplementary Table S 0-2. Checklist used to assessment quality of included studies.**

| Domain | Quality appraisal checklist |
|---|---|
| **Introduction** | 1. Was the research problem clearly described? |
| | 2. Was the study well justified with rationale clearly stated? |
| | 3. Were the research questions and/or /hypothesis stated? |
| | 4. Were the research aims clearly stated? |
| **Method** | 5. Was the study design clearly stated? |
| | 6. Was the sample size provided? |
| | 7. Was the sample size calculation including assumptions used clearly described? |
| | 8. Was the study population clearly described? |
| | 9. Were the study population inclusion and exclusion criteria clearly stated? |
| | 10. Was the T-cell laboratory procedure well described? |
| | 11. Was the statistical analysis well described? |
| | 12. Were ethics procedures described? |

| Domain | Quality appraisal checklist |
|---|---|
| **Results** | 13. Was the participant flow clearly described? |
| | 14. Were background characteristics of study population reported? |
| | 15. Were the results linked to the research aim or methods? |
| **Discussion** | 16. Was a summary of key findings stated? |
| | 17. Was there a comparison and/or contrasting of findings to other relevant studies? |
| | 18. Were strengths and limitations of the study considered and reported? |
| **Conclusion** | 19. Were the conclusions logically based on the results? |

**Supplementary Table S 0-3. MEDLINE search strategy**

Database: Ovid MEDLINE(R) <1946 to February Week 4 2020>

Search Strategy:

---

1 exp T-Lymphocytes/ (322411)

2 ((thym* adj3 lymphocyt*) or (thym* adj3 cell*) or t-cell* or tcell* or t-lymphocyt* or tlymphocyt*).mp. (465331)

3 ((cd4-positive* adj3 cell*) or (cd4-positive* adj3 lymphocyte*) or (cd4-positive* adj3 t-cell*) or (cd4-positive* adj3 tcell*) or (cd4-positive* adj3 t-lymphocyt*) or (cd4-positive* adj3 tlymphocyt*) or (t4 adj3 cell*) or (t4 adj3 lymphocyt*) or (cd4+ adj3 cell*) or (cd4+ adj3 lymphocyt*)).mp. (122090)

4 ((helper adj3 cell*) or (helper adj3 lymphocyt*) or (t-helper adj3 cell*) or (t-helper adj3 lymphocyt*) or (helper-inducer adj3 t-cell*) or (helper-inducer adj3 tcell*) or (helper-inducer adj3 t-lymphocyt*) or (helper-inducer adj3 tlymphocyt*) or (helper adj3 t-cell*) or (helper adj3 tcell*) or (helper adj3 t-lymphocyt*) or (helper adj3 tlymphocyt*) or (inducer adj3 cell*) or (inducer adj3 lymphocyt*)).mp. (36567)

5 ((th-1 adj3 cell*) or (th1 adj3 cell*) or (th-1 adj3 lymphocyt*) or (th1 adj3 lymphocyt*) or (t-helper adj3 type-1) or (thelper adj3 type-1) or (type-1 adj3 t-cell*) or (type-1 adj3 tcell*) or (type-1 adj3 t-lymphocyt*) or (type-1 adj3 tlymphocyt*)).mp. (29030)

6 ((th-2 adj3 cell*) or (th2 adj3 cell*) or (th-2 adj3 lymphocyt*) or (th2 adj3 lymphocyt*) or (t-helper adj3 type-2) or (thelper adj3 type-2) or (type-2 adj3 t-cell*) or (type-2 adj3 tcell*) or (type-2 adj3 t-lymphocyt*) or (type-2 adj3 tlymphocyt*)).mp. (23362)

7 ((th-17 adj3 cell*) or (th17 adj3 cell*) or (th-17 adj3 lymphocyt*) or (th17 adj3 lymphocyt*) or (t-helper adj3 type-17) or (thelper adj3 type-17) or (type-17 adj3 t-cell*) or (type-17 adj3 tcell*) or (type-17 adj3 t-lymphocyt*) or (type-17 adj3 tlymphocyt*)).mp. (11110)

8 ((suppressor adj3 t-cell*) or (suppressor adj3 tcell*) or (suppressor adj3 t-lymphocyt*) or (suppressor adj3 tlymphocyt*) or (regulatory adj3 t-cell*) or (regulatory adj3 tcell*) or (regulatory adj3 t-lymphocyt*) or (regulatory adj3 tlymphocyt*) or (th3 adj3 cell*) or (th3 adj3 lymphocyt*) or (tr1 adj3 cell*) or (tr1 adj3 lymphocyt*) or (treg adj3 cell*) or (treg adj3 lymphocyt*)).mp. (44525)

9 ((cd8-positive* adj3 cell*) or (cd8-positive* adj3 lymphocyt*) or (cd8-positive* adj3 t-cell*) or (cd8-positive* adj3 tcell*) or (cd8-positive* adj3 t-lymphocyt*) or (cd8-positive* adj3 tlymphocyt*) or (t8 adj3 cell*) or (t8 adj3 lymphocyt*) or (cd8+ adj3 cell*) or (cd8+ adj3 lymphocyt*)).mp. (71907)

10 ((killer adj3 t-cell*) or (killer adj3 tcell*) or (killer adj3 t-lymphocyt*) or (killer adj3 tlymphocyt*) or (nkt adj3 cell*) or (nkt adj3 lymphocyt*) or (inkt adj3 cell*) or (inkt adj3 lymphocyt*)).mp. (10220)

11 ((epitheli* adj3 t-cell*) or (epitheli* adj3 tcell*) or (epitheli* adj3 t-lymphocyt*) or (epitheli* adj3 tlymphocyt*) or (intra-epitheli* adj3 t-cell*) or (intra-epitheli* adj3 tcell*) or (intraepitheli* adj3 t-cell*) or (intraepitheli* adj3 tcell*) or (intra-epitheli* adj3 t-lymphocyt*) or (intra-epitheli* adj3 tlymphocyt*) or (intraepitheli* adj3 t-lymphocyt*) or (intraepitheli* adj3 tlympocyt*) or (iel adj3 cell*) or (mucosa* adj3 t-cell*) or (mucosa* adj3 tcell*) or (mucosa* adj3 t-lymphocyt*) or (mucosa* adj3 tlymphocyt*) or (gamma-delta adj3 t-cell*) or (gamma-delta adj3 tcell*) or (gamma-delta adj3 t-lymphocyt*) or (gamma-delta adj3 tlymphocyt*)).mp. (10397)

12 ((mait adj3 cell*) or (mait adj3 lymphocyt*) or (mucosal-associated adj3 t-cell*) or (mucosal-associated adj3 tcell*) or (mucosal-associated adj3 t-lymphocyt*) or (mucosal-associated adj3 tlymphocyt*)).mp. (419)

13 ((cytotox* adj3 t-cell*) or (cytotox* adj3 tcell*) or (cytotox* adj3 t-lymphocyt*) or (cytotox* adj3 tlymphocyt*) or (lympholy* adj3 cell*) or (cell-mediated adj3 lympholy*) or lympholy* or (tc1 adj3 cell*) or (tc1 adj3 lymphocyt*) or (tc2 adj3 cell*) or (tc2 adj3 lymphocyt*)).mp. (46608)

14 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 (518662)

15 exp Antigens, Differentiation, T-Lymphocyte/ (73399)

16 ((t-cell* adj3 different*) or (tcell* adj3 different*) or (t-lymphocyt* adj3 different*) or (tlymphocyt* adj3 different*) or (t-cell adj3 antigen*) or (tcell adj3 antigen*) or (t-lymphocyt* adj3 antigen*) or (tlymphocyt* adj3 antigen*)).mp. (74182)

17 ((cd4 adj3 antigen*) or (cd4 adj3 molecul*) or (cd4 adj3 receptor*) or (cd4 adj3 protein*) or (cd4 adj3 glycoprotein*) or (t4 adj3 antigen*) or (t4 adj3 molecul*) or (t4 adj3 receptor*) or (t4 adj3 protein*) or (t4 adj3 glycoprotein*) or (leu-3 adj3 antigen*) or (leu-3 adj3 molecule*) or (leu-3 adj3 receptor*) or (leu-3 adj3 protein*) or (leu-3 adj3 glycoprotein*)).mp. (23004)

18 ((cd8 adj3 antigen*) or (cd8 adj3 molecul*) or (cd8 adj3 receptor*) or (cd8 adj3 protein*) or (cd8 adj3 glycoprotein*) or (t8 adj3 antigen*) or (t8 adj3 molecul*) or (t8 adj3 receptor*) or (t8 adj3 protein*) or (t8 adj3 glycoprotein*) or (leu-2 adj3 antigen*) or (leu-2 adj3 molecule*) or (leu-2 adj3 receptor*) or (leu-2 adj3 protein*) or (leu-2 adj3 glycoprotein*)).mp. (13824)

19 ((t3 adj3 antigen*) or (t3 adj3 molecul*) or (t3 adj3 receptor*) or (t3 adj3 protein*) or (t3 adj3 glycoprotein*) or (t3 adj3 complex*) or (cd3 adj3 antigen*) or (cd3 adj3 molecul*) or (cd3 adj3 receptor*) or (cd3 adj3 protein*) or (cd3 adj3 glycoprotein*) or (cd3 adj3 complex*) or (leu-4 adj3 antigen*) or (leu-4 adj3 molecule*) or (leu-4 adj3 receptor*) or (leu-4 adj3 protein*) or (leu-4 adj3 glycoprotein*)).mp. (17501)

20 15 or 16 or 17 or 18 or 19 (136030)

21 exp Lymphocyte Count/ (39023)

22 ((cd4 adj3 count*) or (cd4+ adj3 count*) or (t4 adj3 count*) or (cd4 adj3 number*) or (cd4+ adj3 number*) or (t4 adj3 number*) or (leu-3 adj3 count*) or (leu-3 adj3 number*)).mp. (39676)

23 ((cd8 adj3 count*) or (cd8+ adj3 count*) or (t8 adj3 count*) or (cd8 adj3 number*) or (cd8+ adj3 number*) or (t8 adj3 number*) or (leu-2 adj3 count*) or (leu-2 adj3 number*)).mp. (4782)

24 ((cd4-cd8 adj3 ratio*) or (t4-t8 adj3 ratio*)).mp. (8108)

25 21 or 22 or 23 or 24 (60291)

26 exp Th1-Th2 Balance/ (940)

27 ((th1-th2 adj3 ratio*) or (th1-th2 adj3 balance*)).mp. (2847)

28 26 or 27 (2847)

29 exp Receptors, Antigen, T-Cell/ (38190)

30 ((t-cell* adj3 receptor*) or (tcell* adj3 receptor*) or (t-lymphocyt* adj3 receptor*) or (tlymphocyt* adj3 receptor*) or tcr).mp. (63057)

31 (gamma-delta adj3 tcr).mp. (1465)

32 (alpha-beta adj3 tcr).mp. (2145)

33 29 or 30 or 31 or 32 (64680)

34 exp Immunity, Cellular/ (165487)

35 ((cell-mediated adj3 immunit*) or (cell* adj3 respons*) or (cell* adj3 immunit*) or (t-cell* adj3 immunit*) or (tcell* adj3 immunit*) or (t-lymphocyt* adj3 immunit*) or (tlymphocyt* adj3 immunit*)).mp. (260891)

36 34 or 35 (350015)

37 14 or 20 or 25 or 28 or 33 or 36 (750569)

38 Rotavirus Vaccines/ (2264)

39 ((rotavirus* adj3 vaccin*) or (rotavirus* adj3 immuni#ation) or rotarix or (rv1 adj3 vaccin*) or rotateq or (rv5 adj3 vaccin*) or rotavac or (rotavirus* adj3 116e) or (rotavirus* adj3 rix4414) or rotashield or rotasiil or (lanzhou adj3 rotavirus*) or (rotavirus* adj3 LLR) or rotavin-m1).mp. (3606)

40 38 or 39 (3606)

41 Rotavirus/ (8656)

42 (rotavirus* or (human* adj3 rotavirus*) or (rotavirus* adj3 antigen*) or (rotavirus* adj3 VP*) or (rotavirus adj3 NSP*) or (rotavirus* adj3 protein*) or (rotavirus* adj3 peptide*) or (rotavirus* adj3 particle*) or (rotavirus* adj3 pathogen*)).mp. (14416)

43 41 or 42 (14416)

44 Rotavirus Infections/ (7658)

45 ((rotavirus* adj3 infect*) or (rotavirus* adj3 enteri*) or (rotavirus* adj3 gastroenteri*) or (rotavirus* adj3 diarrh?ea) or (rotavirus* adj3 disease*) or (rotavirus* adj3 morbid*) or (rotavirus* adj3 mortal*)).mp. (9260)

46 44 or 45 (9260)

47 40 or 43 or 46 (14422)

48 37 and 47 (470)

49 limit 48 to (english language and yr="1973 -Current") (465)

**********

**Supplementary Table S 0-4. Flow cytometry antibodies**

| Antibody | Clone | Isotype | Antibody dilution per staining volume | Catalogue Reference | Vendor |
|---|---|---|---|---|---|
| CD3 FITC | SK7 | Mouse IgG1, κ | 1:20 | 344804 | Biolegend USA |
| CD4 PE | SK3 | Mouse IgG1, κ | 1:40 | 344606 | Biolegend USA |
| CD8 PE | SK1 | Mouse IgG1, κ | 1:20 | 344706 | Biolegend USA |
| CD8 APC | SK1 | Mouse IgG1, κ | 1:40 | 344722 | Biolegend USA |
| CD25 PE-Cy7 | BC96 | Mouse IgG1, κ | 1:10 | 302612 | Biolegend USA |
| CCR9 PE-Cy7 | L053E8 | Mouse IgG2a, κ | 1:10 | 358910 | Biolegend USA |
| β7 PerCP-Cy5.5 | FIB27 | Rat IgG2a, κ | 1:10 | 121008 | Biolegend USA |
| CD56 PE-Cy7 | | | 1:20 | 335826 | BD Biosciences, USA |
| CD57 APC | HNK-1 | Mouse IgM, κ | 1:10 | 359610 | Biolegend USA |

| | | | | | |
|---|---|---|---|---|---|
| NKG2C PE | | | 1:10 | FAB138P | Biotechne Ltd UK |
| Vα7.2TCR APC | 3C10 | Mouse IgG1, κ | 1:10 | 351708 | Biolegend USA |
| CD161 PE-Cy7 | HP-3G10 | Mouse IgG1, κ | 1:10 | 339918 | Biolegend USA |
| γδTCR APC | B1 | Mouse IgG1, κ | 1:10 | 331212 | Biolegend USA |
| Vδ1TCR PE-Vio770 | REA173 | | 1:20 | 130-137-697 | Miltenyi Biotec UK |
| Ki67 PerCP-Cy5.5 | 20Raj1 | | 1:40 | 46-5699-42 | ThermoFisher (eBioscience) USA |
| CD14 APC-Cy7 | QA18A22 | Mouse IgG1, κ | 1:20 | 398708 | Biolegend USA |
| CD19 APC-Cy7 | HIB19 | Mouse IgG1, κ | 1:20 | 302218 | Biolegend USA |
| αβTCR FITC | IP26 | Mouse IgG1, κ | 1:10 | 306706 | Biolegend USA |
| CD4 PerCP-Cy5.5 | SK3 | Mouse IgG1, κ | 1:10 | 344608 | Biolegend USA |
| CD69 PE | FN50 | Mouse IgG1, κ | 1:20 | 310906 | Biolegend USA |
| CD134 PE-Cy7 | Ber-ACT354B4-1 | Mouse IgG1, κ | 1:10 | 350012 | Biolegend USA |
| CD137 APC | | Mouse IgG1, κ | 1:10 | 309810 | Biolegend nd USA |

| | | | | | |
|---|---|---|---|---|---|
| Fixable Viability Dye eFluor780 | n/a | n/a | 1:10 | 65-0865-14 | ThermoFisher (eBioscience), USA |

**Supplementary Table S 0-5. Geometric mean RV-IgA titre at 12 months by baseline characteristics of infants**

| | **Number of infants,** | **RV-IgA (units/mL) at 12 months** | |
|---|---|---|---|
| **Characteristics** | n (% of total) | GMT (95% CI) | p-value |
| Sex | | | |
| Female | 73 (47.1) | 19.9 (11.3, 35.0) | 0.312 |
| Male | 82 (52.9) | 30.1 (17.0, 53.3) | |
| Gestation | | | |
| Full-term | 146 (94.2) | 26.1 (17.3, 39.3) | 0.290 |
| Pre-term | 9 (5.8) | 10.43 (1.3, 85.5) | |
| Mode of Delivery | | | |
| Caesarean | 8 (5.2) | 13.3 (2.1, 83.1) | 0.473 |
| Vaginal | 147 (94.8) | 25.6 (16.9, 38.7) | |
| Feeding | | | |
| Breastmilk | 145 (93.5) | 25.2 (16.6, 38.3) | 0.724 |

| | | | |
|---|---|---|---|
| Breastmilk + formula | 10 (6.5) | 18.8 (3.7, 95.5) | |
| Birth weight, kg (n=154) | | | |
| <2.5 | 16 (10.4) | 44.3 (10.9, 180.0) | 0.323 |
| ≥2.5 | 138 (89.6) | 22.9 (15.0, 34.9) | |
| Stunting (LAZ <-2) | | | |
| No | 129 (83.2) | 23.2 (15.1, 35.5) | 0.469 |
| Yes | 26 (16.8) | 34.4 (10.8, 109.2) | |
| Wasting (WLZ <-2) | | | |
| No | 152 (98.1) | 25.6 (17.1, 38.3) | 0.227 |
| Yes | 3 (1.9) | 4.3 (0.01, 2391.3) | |
| Maternal HIV (n=154) | | | |
| negative | 107 (69.5) | 20.6 (13.0, 32.5) | 0.148 |
| positive | 47 (30.5) | 39.0 (17.2, 88.5) | |
| Toilet facilityy sharing across households | | | |
| not shared | 32 (20.7) | 59.4 (23.2, 151.9) | 0.027 |

| | | | |
|---|---|---|---|
| shared | 123 (79.3) | 19.7 (12.7, 30.6) | |
| Water source | | | |
| piped into household | 55 (35.5) | 28.7 (15.1, 54.5) | 0.592 |
| wells/public taps and boreholes | 100 (64.5) | 22.8 (13.6, 38.3) | |
| Number of children in household | | | |
| 1-3 | 122 (78.7) | 22.7 (14.5, 35.4) | 0.665 |
| 4-6 | 29 (18.7) | 32.6 (11.6, 91.7) | |
| 7-9 | 4 (2.6) | 50.7 (1.9, 1334.1) | |
| **Total** | **155 (100)** | **24.76 (16.60, 36.92)** | |

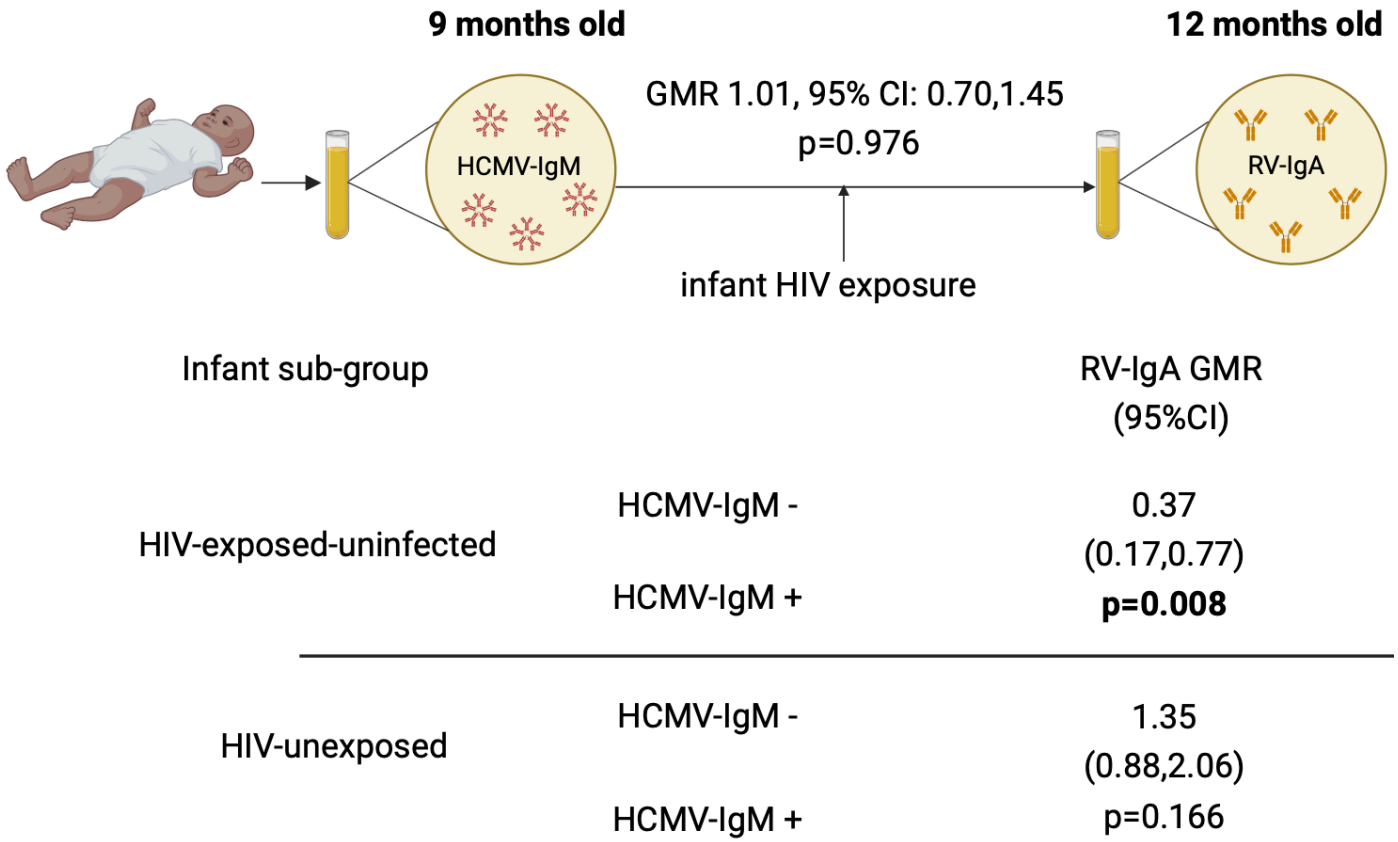
**Supplementary Table S 0-6. Percent of infant with four-fold increase in RV-IgA between 9 and 12 months by baseline characteristics.**

| | **Number of infants,** | **Four-fold increase in RV-IgA between 9 and 12 months** | |
|---|---|---|---|
| **Characteristics** | n (% of total) | n (%) | p-value |
| Sex | | | |
| Female | 73 (47.1) | 31 (50.8) | 0.511 |
| Male | 82 (52.9) | 30 (49.2) | |
| Gestation | | | |
| Full-term | 146 (94.2) | 58 (95.1) | 1.000 |
| Pre-term | 9 (5.8) | 3 (4.9) | |
| Mode of Delivery | | | |
| Caesarean | 8 (5.2) | 3 (4.9) | 1.000 |
| Vaginal | 147 (94.8) | 58 (95.1) | |
| Feeding | | | |
| Breastmilk | 145 (93.5) | 57 (93.4) | 1.000 |

| | | | |
|---|---|---|---|
| Breastmilk + formula | 10 (6.5) | 4 (6.6) | |
| Birth weight, kg (n=154) | | | |
| <2.5 | 6 (10.4) | 6 (9.8) | 1.000 |
| ≥2.5 | 138 (89.6) | 55 (90.2) | |
| Stunting (LAZ <-2) | | | |
| No | 129 (83.2) | 49 (80.3) | 0.511 |
| Yes | 26 (16.8) | 12 (19.7) | |
| Wasting (WLZ <-2) | | | |
| No | 152 (98.1) | 61 (100.0) | 0.279 |
| Yes | 3 (1.9) | 0 (0.0) | |
| Maternal HIV (n=154) | | n=60 | |
| negative | 107 (69.5) | 41 (68.3) | 0.858 |
| positive | 47 (30.5) | 19 (31.7) | |
| Toilet facilityy sharing across households | | | |
| not shared | 32 (20.7) | 16 (26.2) | 0.223 |

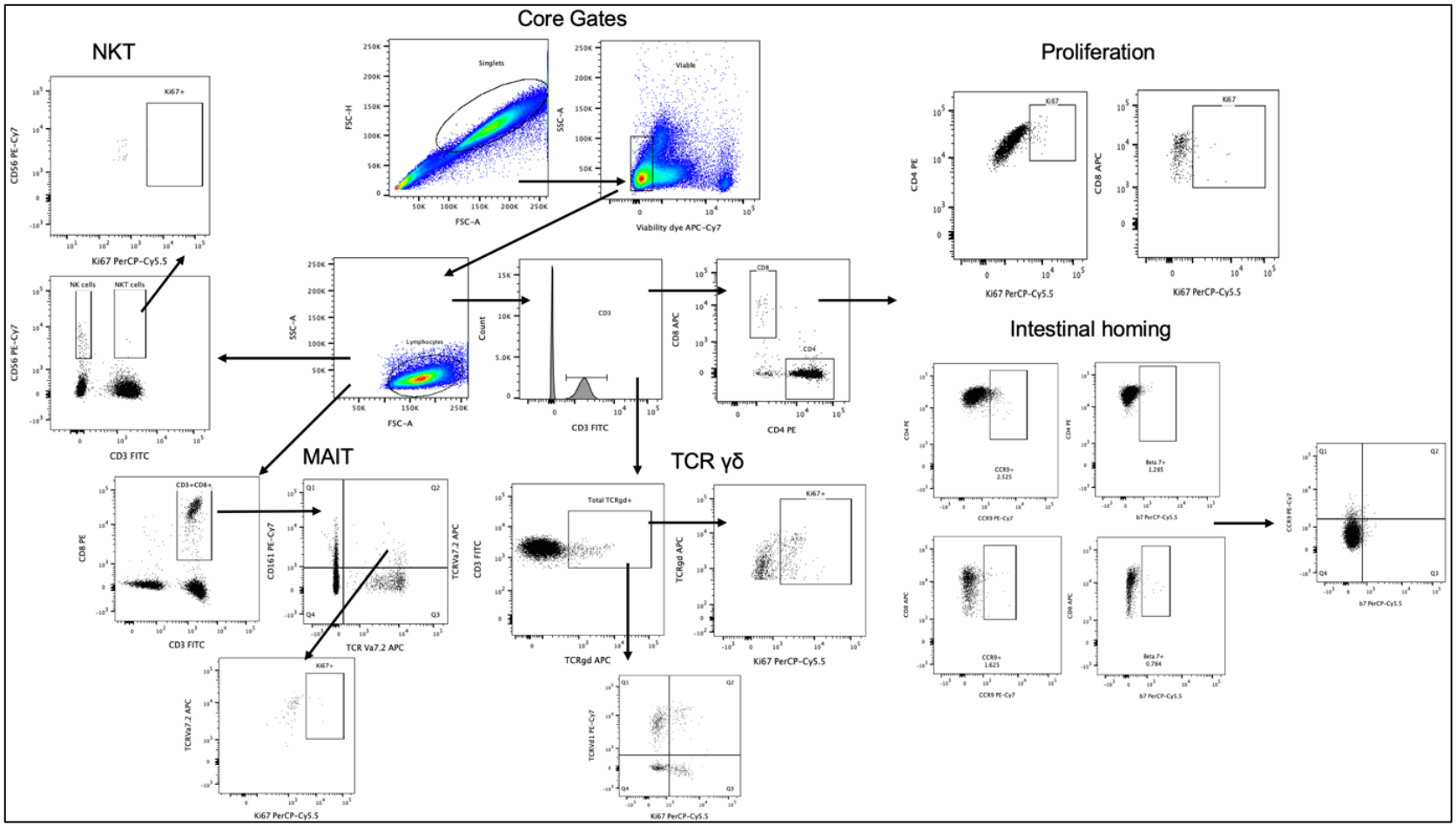
| | | | |
|---|---|---|---|
| shared | 123 (79.3) | 45 (73.8) | |
| Water source | | | |
| piped into household | 55 (35.5) | 22 (36.1) | 1 |
| wells/public taps and boreholes | 100 (64.5) | 39 (63.9) | |
| Number of children in household | | | |
| 1-3 | 122 (78.7) | 43 (70.5) | 0.825 |
| 4-6 | 29 (18.7) | 15 (24.6) | |
| 7-9 | 4 (2.6) | 3 (4.9) | |
| **Total** | **155 (100)** | **61 (100))** | |

2. Supplementary Figures



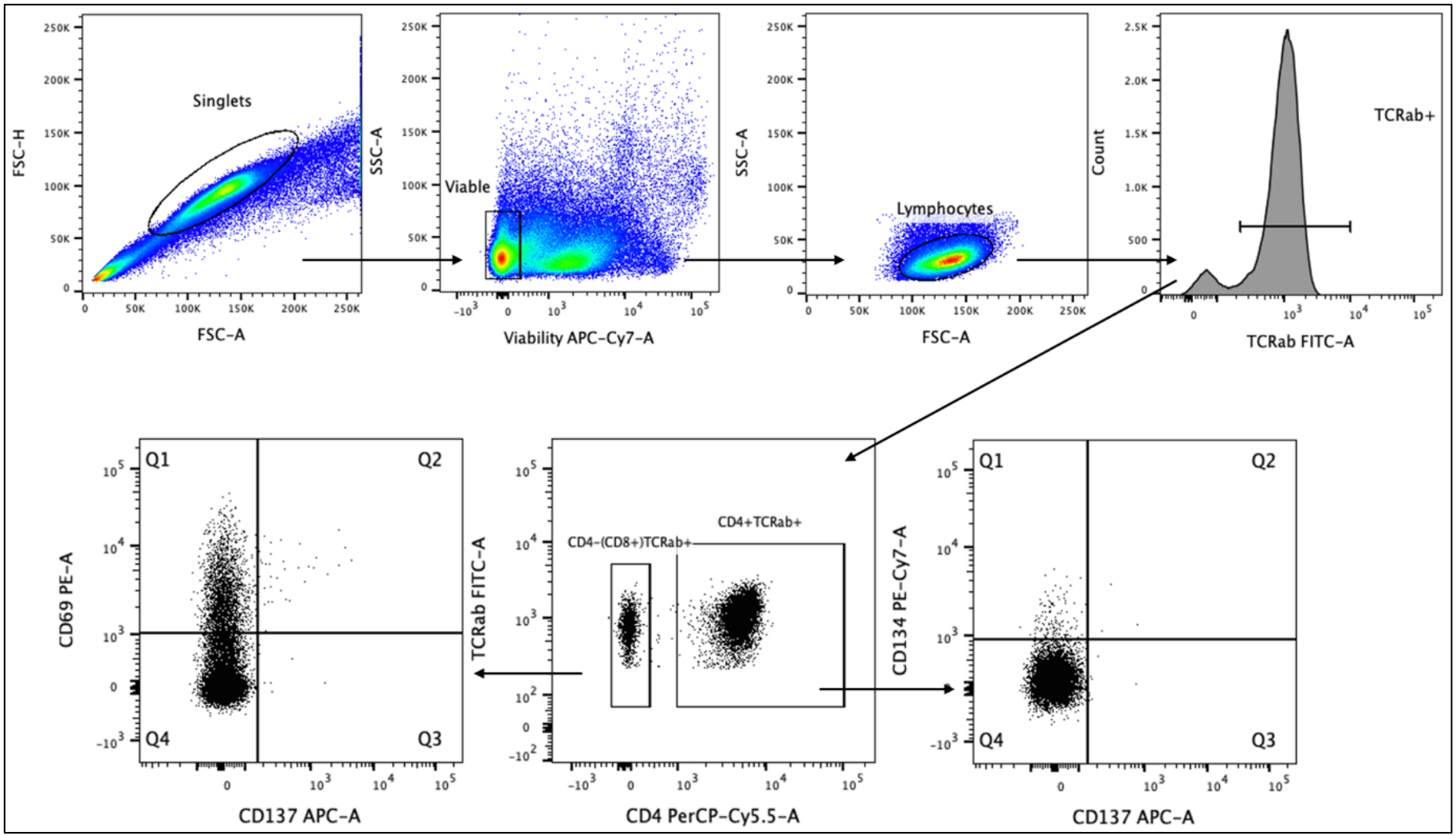

**Supplementary Figure S 0-1. Graphical abstract**

Among rotavirus vaccinated infants, there was no evidence of an association between HCMV-IgM serostatus at 9 months with RV-IgA titre at 12 months (GMR 1.01, 95%CI: 0.70,1.45; p=0.976). However, HIV-exposed-uninfected infants who were HCMV-IgM seropositive at 9 months old had a 63% reduction in RV-IgA geometric mean titres at 12 months compared to HIV-exposed-uninfected infants who were HCMV-IgM seronegative (geometric mean ratio 0.37, 95%CI: 0.17, 0.77; p=0.008). Created with BioRender.com



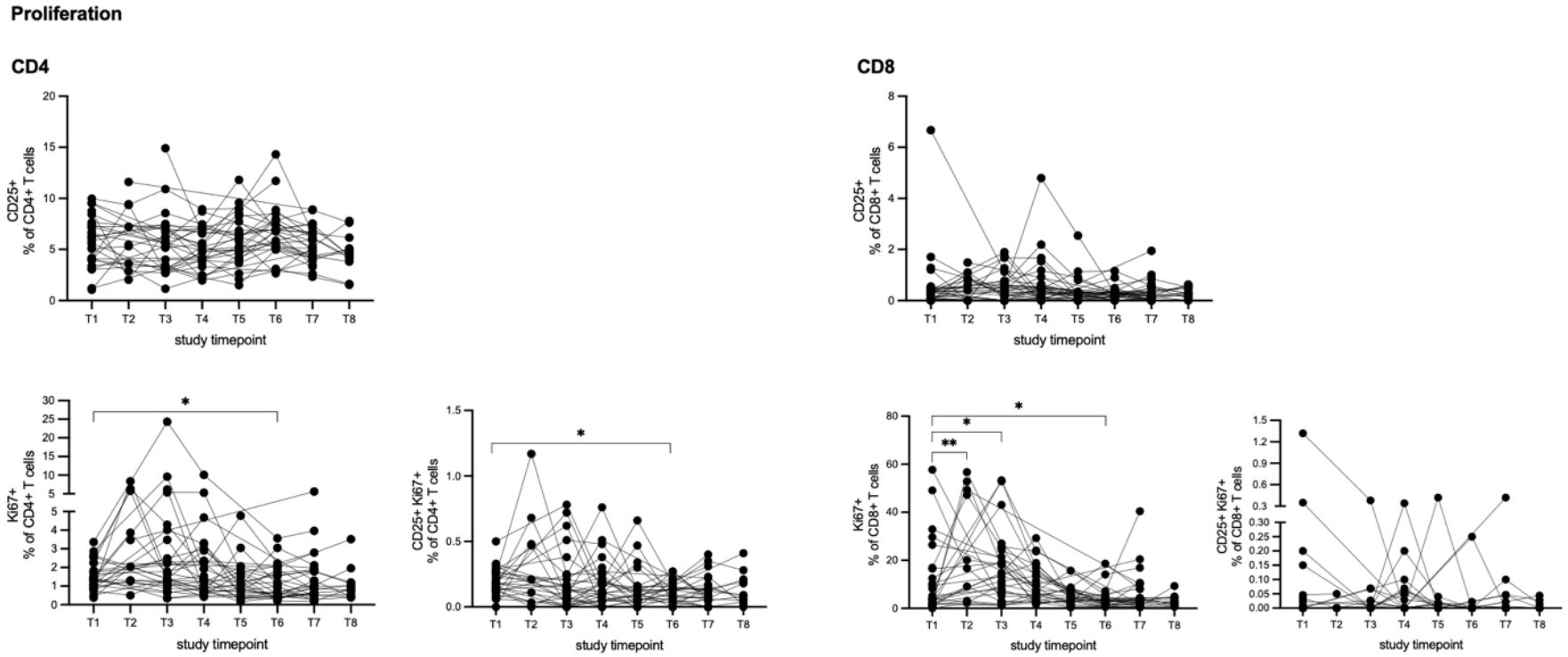

**Supplementary Figure S 0-2. Ex-vivo flow cytometry gating strategy.**

Gating strategy used in the determination of ex-vivo T cell frequencies in FlowJo



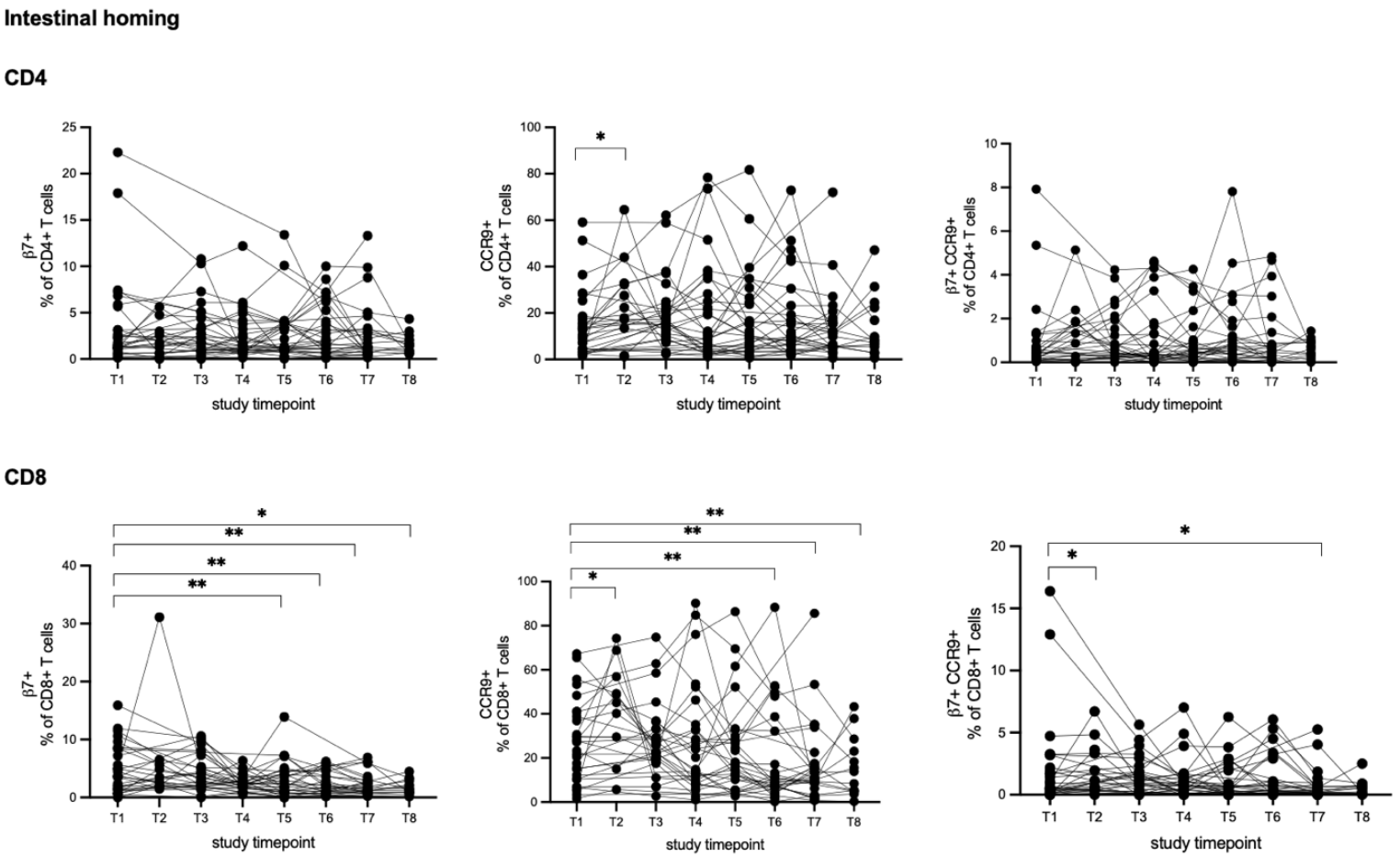

**Supplementary Figure S 0-3. In-vitro stimulation AIM assay flow cytometry gating strategy.**

Gating strategy used in the determination of antigen specific T cell frequencies detected using the AIM assay in FlowJo



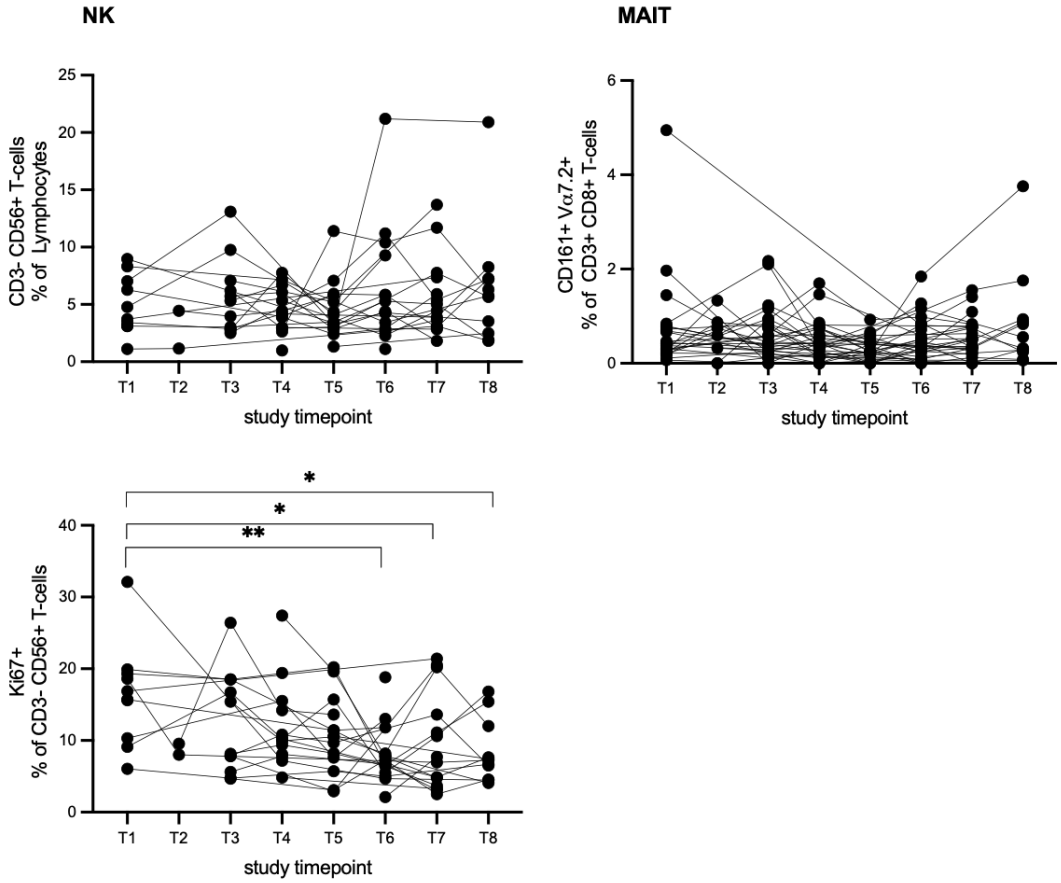

**Supplementary Figure S 0-4. Ex-vivo analysis of proliferating conventional CD4 and CD8 T-cells by study timepoint.**

The raw frequencies of CD25+, Ki67+ and CD25+ Ki67+ T-cells as a percentage of CD4+ and CD8+ T-cells across the eight study timepoints (n=31) irrespective of vaccine seroconversion status. Each connected circle represents the T cell frequency for a single infant. The differences in frequencies post-vaccination (T2 to T8) in comparison to baseline (T1) was assessed by mixed effects repeated measures analysis with Dunnett's test adjustment for multiple comparison on log transformed data.



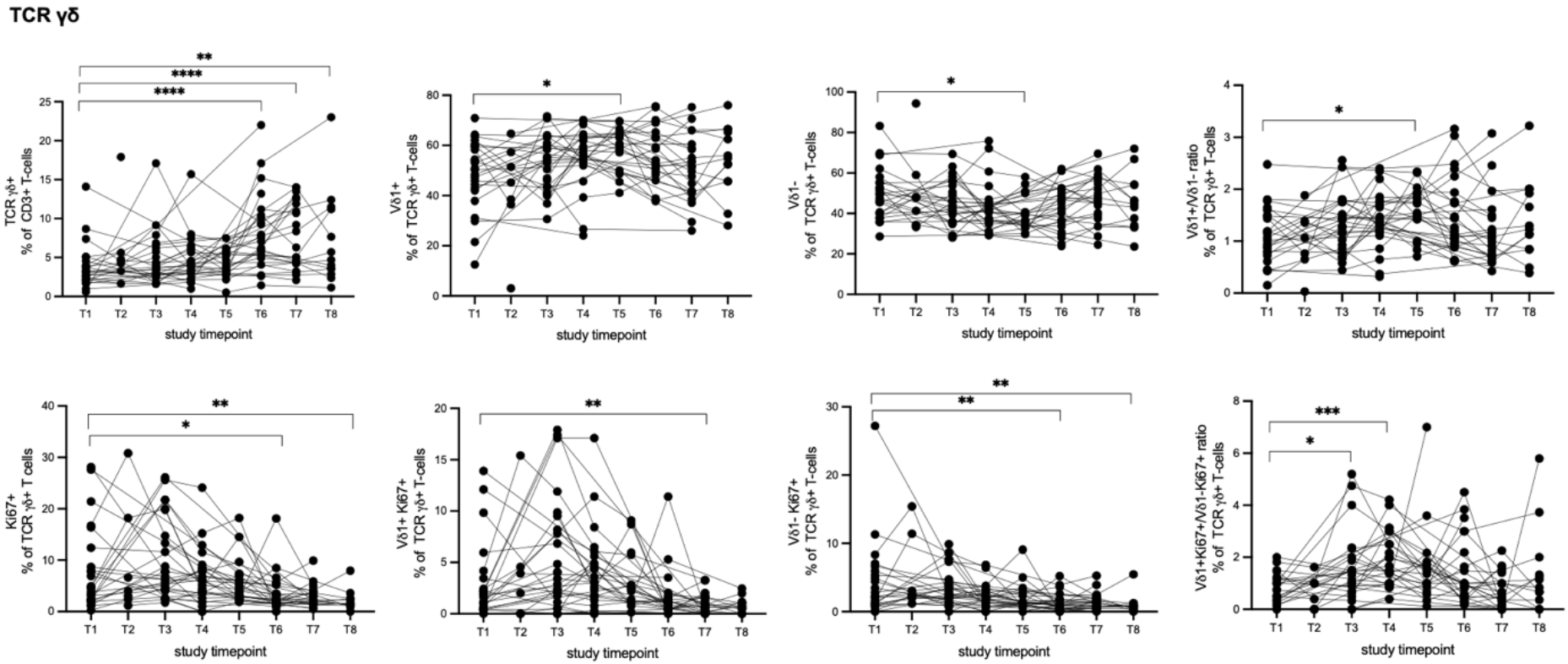

**Supplementary Figure S 0-5. Ex-vivo analysis of intestinal homing CD4 and CD8 T-cells by study timepoint.**

The raw frequencies of β7+, CCR9+ and β7+CCR9+ T-cells as a percentage of CD4+ and CD8+ T-cells across the eight study timepoints (n=34) irrespective of vaccine seroconversion status. Each connected circle represents the T cell frequency for a single infant. The differences in frequencies post-vaccination (T2 to T8) in comparison to baseline (T1) was assessed by mixed effects repeated measures analysis with Dunnett's test adjustment for multiple comparison on log transformed data.



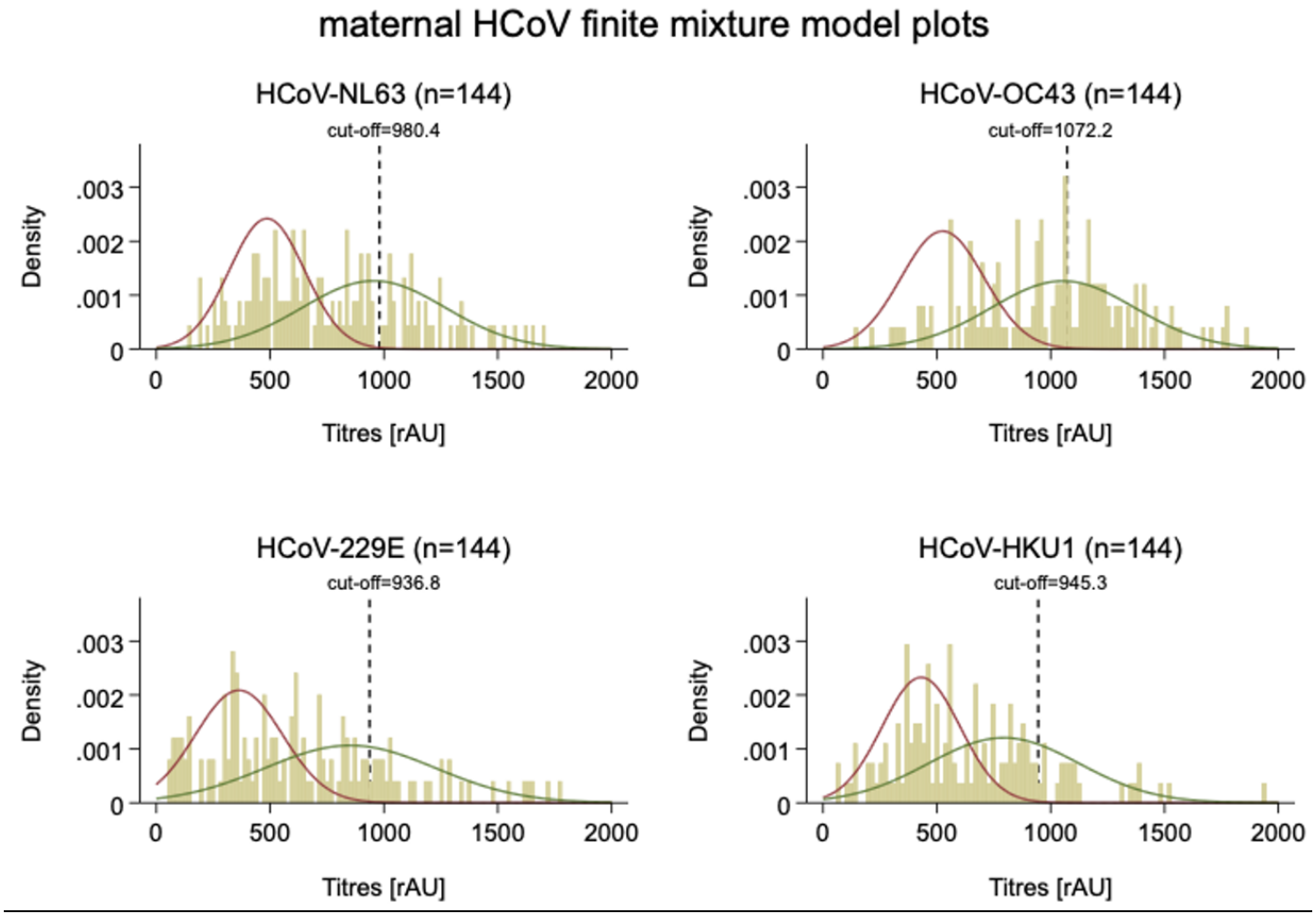

**Supplementary Figure S 0-6. Ex-vivo analysis of innate natural killer and adaptive mucosal associated invariant T-cells by study timepoint.**

The raw frequencies of total and proliferating (Ki67+) NK cells (n=26) as a percentage of lymphocytes and NK cells respectively and total MAIT cells as a percentage of CD8+ T cells (n=34) across the eight study timepoints irrespective of vaccine seroconversion status. Each connected circle represents the T cell frequency for a single infant. The differences in frequencies post-vaccination (T2 to T8) in comparison to baseline (T1) was assessed by mixed effects repeated measures analysis with Dunnett's test adjustment for multiple comparison on log transformed data



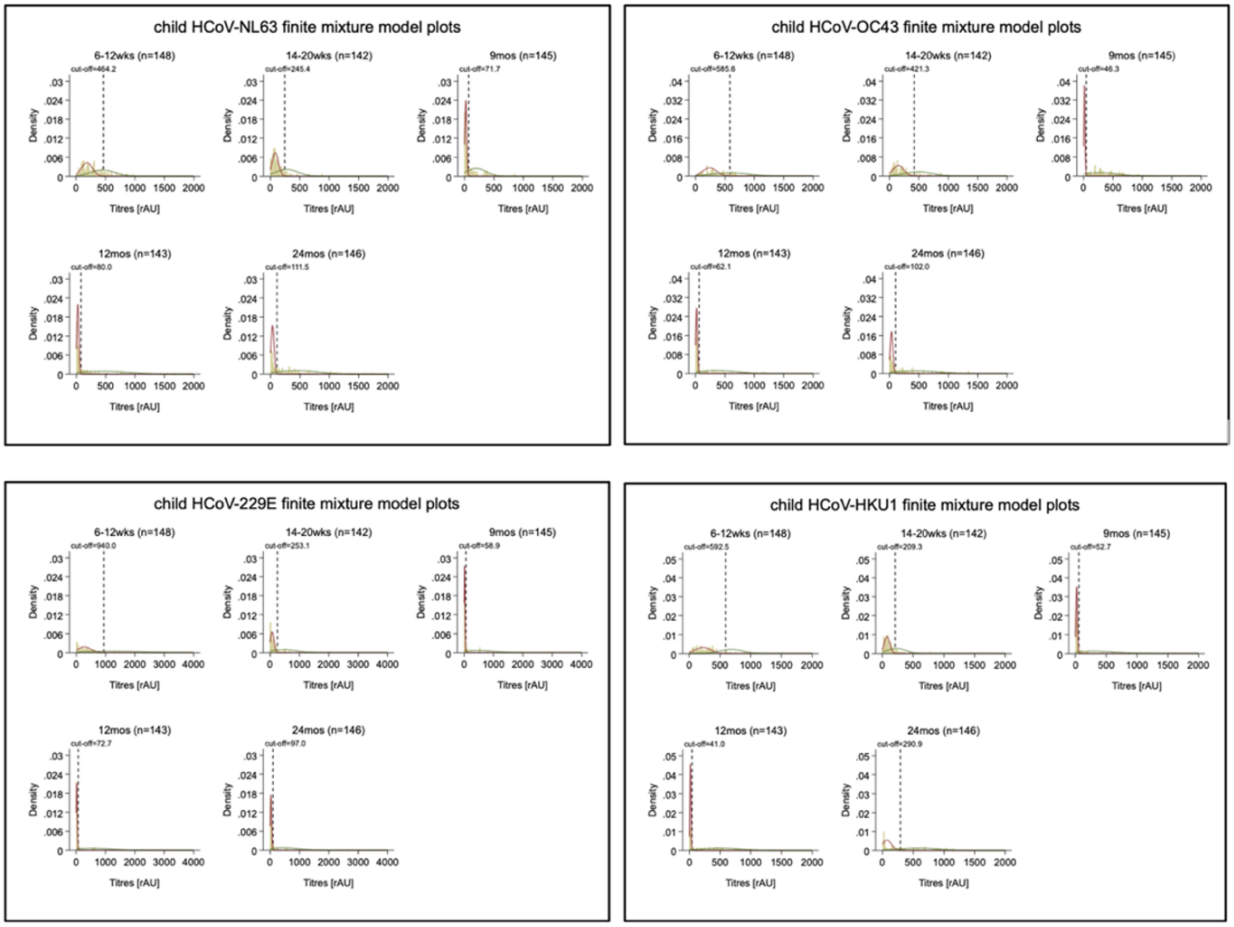

**Supplementary Figure S 0-7. Ex-vivo analysis of gamma delta T-cells by study timepoint.**

The raw frequencies of total TCRγδ+ as percentage of CD3+ T cells, total Vδ1+ and Vδ1- as a percentage of TCRγδ+ T cells and total, Vδ1+, Vδ1- proliferating (Ki67+) T cells and ratio as a percentage of TCRγδ+ T cells (n=34) across the eight study timepoints irrespective of vaccine seroconversion status. Each connected circle represents the T cell frequency for a single infant. The differences in frequencies post-vaccination (T2 to T8) in comparison to baseline (T1) was assessed by mixed effects repeated measures analysis with Dunnett's test adjustment for multiple comparison on log transformed data



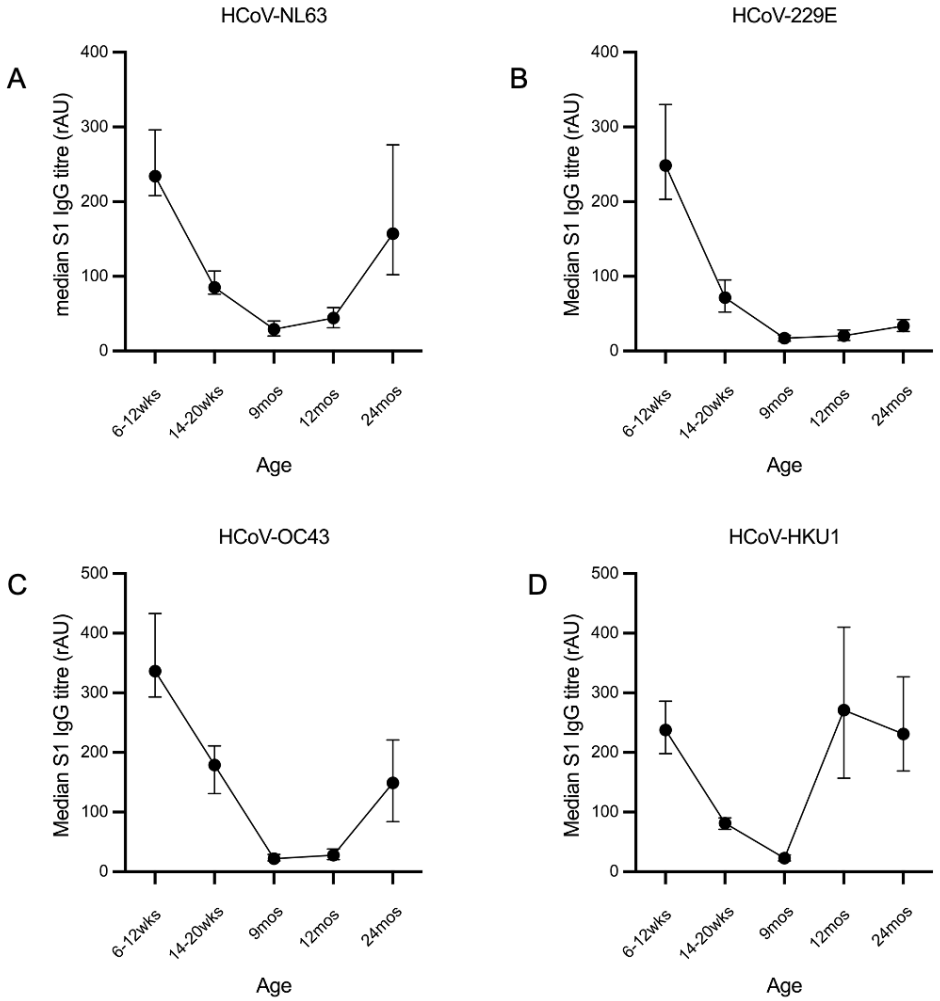

**Supplementary Figure S 7-1. Cut-off values for maternal coronavirus specific rAU titres calculated from finite mixture regression models.**

The predicted normal distributions of seronegative (red) and seropositive (green) populations are shown overlayed on histogram plots of the spike S1 IgG antibody titres for HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 among mothers (n=144) at baseline. The titre cut-off value (vertical dashed line) was calculated as the mean of the seronegative population plus 3x the standard deviation for each HCoV type.




**Supplementary Figure S 7-2. Cut-off values for child HCoV specific rAU titres calculated from finite mixture regression models.**

The predicted normal distributions of seronegative (red) and seropositive (green) populations are shown overlayed on histogram plots of the spike S1 IgG antibody titres for HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 among children aged 6-12 weeks (n=148) 14-20 weeks (n=142), 9 months (n=145), 12 months (n=143) and 24 months (n=146). The titre cut-off value (vertical dashed line) was calculated as the mean of the seronegative population plus 3 x the standard deviation for each HCoV type at each timepoint.




**Supplementary Figure S 7-3. Trajectory of S1 IgG titres against the common cold HCoV in children.**

Each data point represents the median S1 IgG titres measured in relative absorbance units and 95% confidence intervals for HCoV-NL63 (A), HCoV-229E (B), HCoV-OC43 (C) and HCoV-HKU1 (D) in children within the first two years of life

3. Ethical Approval Letter – UNZABREC

**THE UNIVERSITY OF ZAMBIA**

**BIOMEDICAL RESEARCH ETHICS COMMITTEE**

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm
**Assurance No. FWA00000338**
**IRB00001131 of IORG0000774**

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

19th April, 2018. Ref: 003-02-18.

Dr. Roma Chilengi,
CIDRZ,
P.O Box 34681,
**Lusaka.**

Dear Dr. Chilengi,

**RE: SUBMITTED RESEARCH PROPOSAL: "A RANDOMIZED CONTROLLED TRIAL OF TWO VERSUS THREE DOSES OF ROTARIX™ VACCINE FOR BOOSTING AND LONGEVITY OF VACCINE IMMUNE RESPONSES IN ZAMBIA" V1.1 DATED 13 MARCH 2018 (REF.003-02-18)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee (UNZABREC) on18th April, 2018. The proposal is approved. The approval is based on the following documents that were submitted for review:

a) **Study proposal**
b) **Questionnaires**
c) **Participant Consent Form**

**APPROVAL NUMBER : REF. 003-02-18**

**This number should be used on all correspondence, consent forms and documents as appropriate.**

- **APPROVAL DATE : 19th April 2018**
- **TYPE OF APPROVAL : Standard**
- **EXPIRATION DATE OF APPROVAL : 18th April 2019**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the UNZABREC Offices should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All SAEs and any other serious challenges/problems having to do with participant welfare, participant safety and study integrity must be reported to UNZABREC within 3 working days using standard forms obtainable from UNZABREC.
- **MODIFICATIONS**: Prior UNZABREC approval using standard forms obtainable from the UNZABREC Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the UNZABREC using standard forms obtainable from the UNZABREC Offices.
- **NHRA:** Where appropriate, apply in writing to the National Heath Research Authority for permission before you embark on the study.
- **QUESTIONS**: Please contact the UNZABREC on Telephone No. 256067 or by e-mail on unzarec@unza.zm
- **OTHER:** Please be reminded to send in copies of your research findings/results for our records. You're also required to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours sincerely,

[redacted]

Dr. S.H Nzala
**VICE-CHAIRPERSON**

CIDRZ CENTRE FOR INFECTIOUS DISEASE RESEARCH IN ZAMBIA
RECEIVED
26 APR 2018

# 4. Ethical Approval Letter – LSHTM REC 1

**London School of Hygiene & Tropical Medicine**
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

**www.lshtm.ac.uk**

**Observational / Interventions Research Ethics Committee**

Miss Natasha Laban
LSHTM

12 March 2019

Dear Natasha,

**Study Title:** Profiling innate and adaptive immune responses to rotavirus vaccination in Zambian infants

**LSHTM Ethics Ref:** 16168

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Conditions of the favourable opinion**

Approval is dependent on local ethical approval having been received, where relevant.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| Document Type | File Name | Date | Version |
|---|---|---|---|
| Investigator CV | Resume_Chilengi March 2017._1AP64 | 01/03/2017 | 1.0 |
| Local Approval | LETTER OF SUBMITTED RESEARCH PROPOSAL | 19/04/2018 | 1 |
| Local Approval | ROVAS 2 study NHRA approval | 11/06/2018 | 1 |
| Protocol / Proposal | Protocol_Edit_Final | 09/07/2018 | 1.2 |
| Consent form | ICF_Edit_Final | 09/07/2018 | 1.2 |
| Local Approval | ZAMRA Approval_ROVAS2 | 09/08/2018 | 1 |
| Investigator CV | CV. M. Goodier September_2018 | 12/09/2018 | 1 |
| Investigator CV | CV_Natasha Makabilo Laban | 09/10/2018 | 1.0 |
| Covering Letter | LEO Ethics Clarification_Cover Letter | 05/03/2019 | 1 |

**After ethical review**

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

**Professor John DH Porter**
**Chair**

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

**Improving health worldwide**

# 5. Ethical Approval Letter – LSHTM REC 2

**London School of Hygiene & Tropical Medicine**
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

**www.lshtm.ac.uk**

**Observational / Interventions Research Ethics Committee**

Miss Natasha Laban
LSHTM

22 November 2021

Dear Miss Natasha Laban

**Study Title:** Coronavirus seroepidemiology and immunology among adults and children pre- and post-COVID-19 pandemic in Zambia

**LSHTM Ethics Ref:** 26277

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

**Conditions of the favourable opinion**

Approval is dependent on local ethical approval having been received, where relevant.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| Document Type | File Name | Date | Version |
|---|---|---|---|
| Local Approval | ROVAS-2 initial BREC approval | 19/04/2018 | 1 |
| Local Approval | ROVAS 2 study NHRA approval | 11/06/2018 | 1 |
| Local Approval | Kalulushi hepatitis initial BREC study approval | 15/03/2019 | 1 |
| Local Approval | Kalulushi hepatitis NHRA approval | 07/06/2019 | 1 |
| Other | Human_Tissue_online_training_Certificate_MG_03_02_20 | 03/02/2020 | 1 |
| Protocol / Proposal | UNZABREC Ethics Proposal_CoV-Imm_final 22072020 | 22/07/2020 | 1 |
| Local Approval | CoV-Imm study initial NHRA study application | 24/07/2020 | 1 |
| Information Sheet | ICF ENGLISH V1.5_102020CLEAN | 31/07/2020 | 1.5 |
| Local Approval | CoV-Imm NHRA protocol approval | 05/08/2020 | 1 |
| Information Sheet | Kalulushi hepatitis_ Informed Consent Version 1.3 Final 04 May 2021_CLEAN | 04/05/2021 | 1.3 |
| Investigator CV | CV and publications_Goodier_May 2021 | 17/05/2021 | 1 |
| Investigator CV | Natasha Makabilo Laban_CV 2021signed | 04/06/2021 | 1 |
| Other | Human_Tissue_online_training_Certificate_NML | 04/06/2021 | 1 |
| Covering Letter | Cover Letter_NMLfinal | 15/11/2021 | 1 |
| Covering Letter | Support Letter_MGfinal | 18/11/2021 | 1 |

**After ethical review**

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project

by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

[redacted]

**Professor Jimmy Whitworth**
**Chair**

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

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**Improving health worldwide**

6. NHRA Approval Letter

**NATIONAL HEALTH RESEARCH AUTHORITY**
**Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA**
Tell: +260211 250309 | Email: znhrasec@gmail.com | www.nhra.org.zm

**Ref No:.....** **Date: 5th August, 2020**

CIDRZ CENTRE FOR INFECTIOUS DISEASE RESEARCH IN ZAMBIA
RECEIVED
11 AUG 2020

The Principal Investigator
Dr. Izukanji Sikazwe
CIDRZ
P.O. Box 34681
**LUSAKA.**

Dear Dr. Sikazwe,

**Re: Request for Authority to Conduct Research**

The National Health Research Ethics Board (NHREB) is in receipt of your request for authority to conduct research titled **"Pre and post pandemic assessment of coronavirus immunity and cross-protection against SARSCoV-2 in mother-infant pairs and adult healthcare workers in Zambia (CoV-Imm)."**

I wish to inform you that following submission of your request to the Board, its review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. **A Material Transfer Agreement is obtained and cleared by the National Health Research Ethics Board should there be any need for samples to be sent outside the country for analysis.**
2. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
3. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
4. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
5. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Prof. Patrick Musonda
Chairperson
**National Health Research Ethics Board**

*viruses*

MDPI

*Review*

# T-Cell Responses after Rotavirus Infection or Vaccination in Children: A Systematic Review

**Natasha Makabilo Laban [1,2,*], Martin Rhys Goodier [1,3], Samuel Bosomprah [2,4], Michelo Simuyandi [2], Caroline Chisenga [2], Obvious Nchimunya Chilyabanyama [2] and Roma Chilengi [2]**

[1] Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK; martin.goodier@lshtm.ac.uk
[2] Enteric Disease and Vaccine Research Unit, Centre for Infectious Disease Research in Zambia, Lusaka P.O. Box 34681, Zambia; samuel.bosomprah@cidrz.org (S.B.); michelo.simuyandi@cidrz.org (M.S.); caroline.chisenga@cidrz.org (C.C.); obvious.chilya@cidrz.org (O.N.C.); roma.chilengi@cidrz.org (R.C.)
[3] Flow Cytometry and Immunology Facility, Medical Research Council Unit, The Gambia at London School of Hygiene and Tropical Medicine, Fajara, Banjul P.O. Box 273, The Gambia
[4] Department of Biostatistics, School of Public Health, University of Ghana, Accra P.O. Box LG13, Ghana
* Correspondence: natasha.laban@cidrz.org

**Abstract:** Cellular immunity against rotavirus in children is incompletely understood. This review describes the current understanding of T-cell immunity to rotavirus in children. A systematic literature search was conducted in Embase, MEDLINE, Web of Science, and Global Health databases using a combination of "t-cell", "rotavirus" and "child" keywords to extract data from relevant articles published from January 1973 to March 2020. Only seventeen articles were identified. Rotavirus-specific T-cell immunity in children develops and broadens reactivity with increasing age. Whilst occurring in close association with antibody responses, T-cell responses are more transient but can occur in absence of detectable antibody responses. Rotavirus-induced T-cell immunity is largely of the gut homing phenotype and predominantly involves Th1 and cytotoxic subsets that may be influenced by IL-10 Tregs. However, rotavirus-specific T-cell responses in children are generally of low frequencies in peripheral blood and are limited in comparison to other infecting pathogens and in adults. The available research reviewed here characterizes the T-cell immune response in children. There is a need for further research investigating the protective associations of rotavirus-specific T-cell responses against infection or vaccination and the standardization of rotavirus-specific T-cells assays in children.

**Keywords:** T-cell; rotavirus; child; infection; vaccination



**Citation:** Laban, N.M.; Goodier, M.R.; Bosomprah, S.; Simuyandi, M.; Chisenga, C.; Chilyabanyama, O.N.; Chilengi, R. T-Cell Responses after Rotavirus Infection or Vaccination in Children: A Systematic Review. *Viruses* **2022**, *14*, 459. https://doi.org/10.3390/v14030459

Academic Editor: Jens H. Kuhn

Received: 15 January 2022
Accepted: 16 February 2022
Published: 23 February 2022



## 1. Introduction

Rotavirus is the leading cause of life-threatening diarrhea among young children, particularly in those below five years of age [1,2]. Globally, rotavirus has been responsible for approximately 258 million diarrhea episodes and an estimated 128,515 diarrhea deaths in this population with the largest burden within Sub-Saharan Africa [3]. Fortunately, rotavirus vaccines are widely available and have significantly contributed to reductions in rotavirus-associated diarrhea morbidity and mortality globally [4–6]. However, despite being discovered nearly half a century ago in 1973 and more than a decade since vaccine introduction, immune mechanisms, and correlates of protection against rotavirus remain poorly understood [7].

In humans, rotavirus is transmitted via a fecal-oral route and is known to predominantly infect and replicate in mature enterocytes of the intestinal epithelium inducing innate and adaptive humoral and cellular immune responses [8]. In children, repeated rotavirus infection leads to a lower likelihood of subsequent rotavirus infections and reduced occurrence of moderate to severe diarrheal disease suggesting the development of immune

memory [9]. This acquired, non-sterilizing immunity is derived from a combination of gut secretory and humoral antibody and cell-mediated immune effectors with neutralizing antibodies directed against the viral capsid proteins and viral epitope recognition by T-cells thought to play an important role in protection [8]. However, immune parameters correlating with protection against rotavirus in humans are yet to be demonstrated [10].

Rotavirus-specific antibodies are well documented and frequently studied in children as immune markers of previous infection or vaccination [11]. However, even though they are recognized as being important for protection, it is generally appreciated that these antibody markers are sub-optimal correlates of protection [12,13]. In contrast, there is sparse data on the underlying T-cell immune responses to rotavirus infection or vaccination, particularly in children, and even fewer still have studied the role of this T-cell immunity in protection against rotavirus. The current understanding of rotavirus T-cell mediated immunity has for the most part been achieved through studies in animal models which have shown that T-cells have crucial roles in suppression of rotavirus replication, clearance of infection, and generation of antibody responses associated with protection [10,14–16].

As rotavirus remains a cause of high morbidity and mortality in children, especially in the developing world [3], it is necessary to fully understand the immune mechanisms underlying protection. Improved knowledge on T-cell-mediated rotavirus immunity can inform vaccine development and is particularly important considering the sub-optimal antibody immune correlates and the consistent observation of markedly lower vaccine immunogenicity and efficacy in children within high rotavirus burden regions [17]. We, therefore, conducted a systematic review of literature on T-cell responses to rotavirus in children to consolidate currently available knowledge on the characteristics of T-cell immunity to rotavirus in this population including its association with the antibody responses.

## 2. Materials and Methods

### *2.1. Literature Search Strategy*

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) checklist (Table S1) in the preparation of the systematic review manuscript [18]. The literature search was conducted in Embase (1947 to March 2020), MEDLINE (1946 to March 2020), Web of Science (1970 to 2020), and Global Health (1910 to week 9 2020) electronic databases using a combination of "T-cell", "rotavirus" and "child" keywords to identify relevant articles (File S1).

### *2.2. Inclusion Criteria*

Studies included in this review were those that were primary research, were conducted among children or used child-derived samples in any region of the world, reported T-cell immune responses to rotavirus, had full English text available and had rotavirus as the main focus of the study. There was no restriction to study design, but we restricted selection to articles published after 1973, the year rotavirus was discovered.

### *2.3. Exclusion Criteria*

We excluded studies that did not include children or child-derived samples, did not report T-cell responses against rotavirus, or had no English full text available. Non-primary research including review articles and conference abstracts were also excluded.

### *2.4. T-Cell Responses*

The T-cell responses considered in the systematic review were T-cell quantity (counts, ratios, frequencies), phenotype (activation, cell surface markers, epitopes) function (cytokine secretion), activity (proliferation), and kinetics (pre and post-infection or vaccination, durability) for all CD4 and CD8 T-cells subsets.

### *2.5. Study Selection and Data Extraction*

EndNote reference manager software was used to remove duplicate articles identified from the search strategy. The resulting unique articles were imported into Rayyan web-tool software for additional duplicate identification and article selection. Three reviewers (NML, CC, MS) independently selected potentially eligible articles by screening the title and abstract of all unique articles for the keywords using the Rayyan web-tool software. Full texts of articles selected by all three reviewers combined were retrieved and assessed for eligibility using the inclusion and exclusion criteria. Articles concordantly selected as eligible by the three reviewers were included in the review and those concordantly rejected were excluded from the review. Discordance in selection was discussed and re-assessed together by all three reviewers until a consensus on inclusion or exclusion was made. Data were extracted into an excel sheet to capture information on the author, year of publication, study location, study design, characteristics of the child population, sample size, rotavirus context (rotavirus infection or vaccination), T-cell responses, and laboratory methods used for measures of T-cell immunity.

### *2.6. Quality Assessment and Data Synthesis*

We reviewed published articles of similar nature to our systematic review to identify potential appraisal tools and we adapted a recently published quality assessment tool [19] and quality level thresholds (0% to 39% = low, 40% to 69% = moderate, and 70% to 100% = high) [20] for our critical appraisal (Table S2). One author (NML) conducted the quality assessment which was reviewed by two other authors (SB and ONC). Due to the wide heterogeneity in laboratory methodology and reported T-cell response across the studies included in the systematic review, formal quantitative meta-analysis was not conducted, and results were presented in a thematic narrative format.

## 3. Results

### *3.1. Literature Search Results*

Articles retrieved from the literature search comprised 937 from Embase, 465 from MEDLINE, 574 from Web of Science, and 125 from Global Health electronic databases giving a total of 2101 articles identified. After the removal of 906 duplicate articles, a resulting total of 1195 articles were screened for eligibility based on title and abstract and an additional 1162 articles were excluded because they were non-primary research ($n$ = 710), were not about rotavirus in humans ($n$ = 288,) did not include children ($n$ = 96), did not report T-cell responses ($n$ = 72). The remaining 33 articles underwent further screening for eligibility by full text based on set inclusion criteria. After full-text screening, a further 16 articles were excluded because they did not have full text available to the reviewers ($n$ = 2), did not report T-cell responses for children ($n$ = 10), and rotavirus was not the main focus ($n$ = 4). This resulted in 17 articles that met the inclusion criteria and were included in the systematic review as summarized in Figure 1.

### *3.2. Characteristics of Articles Included in Systematic Review*

Among the seventeen studies included in the systematic review, the earliest study identified was published in 1988 and the latest in 2018. Most of the studies were conducted among children in the Americas (9/17) followed equally by Europe (3/17) and Asia (3/17) while the least number of studies (2/17) was conducted among African children. Ten studies reported T-cell immunity in the context of rotavirus infection, two studies reported T-cell responses to rotavirus vaccination, and five studies reported rotavirus-specific T-cell response in healthy children. Laboratory methods used to measure T-cell responses varied across studies and included flow cytometry, lymphoproliferation, microscopy, indirect fluorescence microscopy, gene microarray, and enzyme-linked immunospot (ELISpot) assays. Different types of T-cell outcomes in response to mitogen, human rotavirus, and non-human rotavirus antigens were reported across studies. More detailed characteristics of the studies included in the systematic review are as outlined in Table 1.

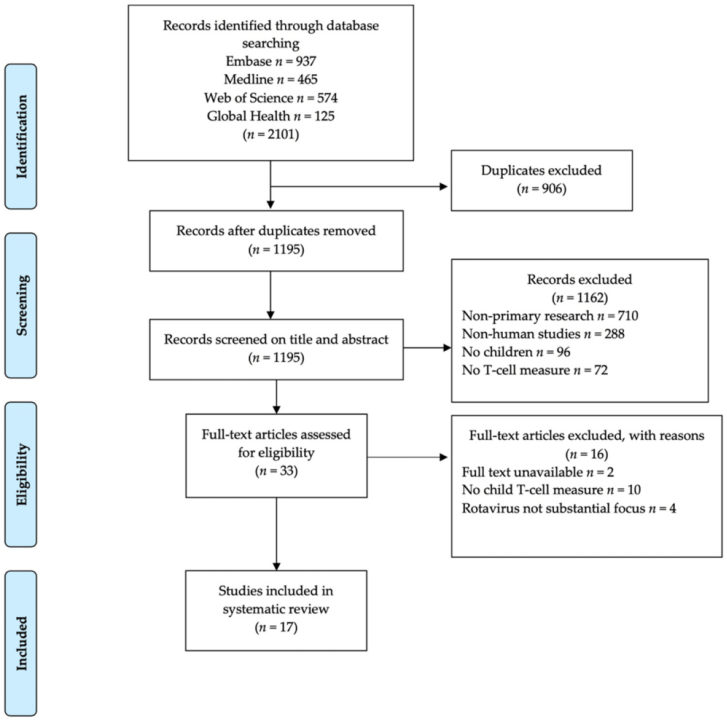


**Figure 1.** Flow chart of literature search results and article selection process.

**Table 1.** Characteristics of studies included in the systematic review.

| Author Year [Ref] | Country | Design | Child Population, *n* | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| Dong et al., 2015 [21] | China | Observational | RV-AGE, *n* = 102; Healthy, *n* = 30 | 3 mos to 3 yrs; 2 mos to 3 yrs | Rotavirus infection | PMA Ionomycin | Flow Cytometry | • Treg ($CD4^+CD25^+$)<br>• Th17 ($CD4^+IL\text{-}17^+$) |
| Elaraby et al., 1992 [22] | Egypt | Observational | RV-AGE, *n* = 6; Healthy, *n* = 50 | NR; Newborn, 1 to <12 mos, 12 to 24 mos, 24 to 60 mos | Rotavirus infection | Rotavirus antigen, PHA | Light microscopy, Indirect Fluorescent Microscopy | • Stimulation Index, positive > 1.5<br>• Helper/suppressor (CD4:CD8) ratio<br>• CD3, CD4, CD8 subsets |
| Iwasa et al., 2008 [23] | Japan | Observational | RV-AGE, *n* = 1 | 6 mos | Rotavirus infection | Nil | Flow Cytometry | • $CD4^+/CD8^+IFN\text{-}\gamma^+$<br>• $CD4^+/CD8^+$<br>• $CD4^+/CD8^+$ ratio |
| Jaimes et al., 2002 [24] | Colombia | Observational | RV-AGE, *n* = 12 | 6 mos to 7 yrs | Rotavirus infection | RRV, SEB, CD28, CD49d | Flow Cytometry | • $CD4/CD8\ CD69^+IFN\text{-}\gamma^+$,<br>• $CD4/CD8\ CD69^+IL\text{-}13^+$ |
| Makela et al., 2006 [25] | Finland | Observational | Healthy (T1D at risk), *n* = 183 | ≤15 yrs | N/A | HRV Wa, BRV NCD, CBV, PPD, TT, PHA, PCB | Proliferation assay, PCR | • Stimulation Index, positive ≥ 3<br>• $IFN\text{-}\gamma^+$, $IL\text{-}4^+$, $IL\text{-}10^+$ and $TGF\text{-}\beta^+$ PBMC gene expression |
| Makela et al., 2004 [26] | Finland | Observational | Healthy children (T1D at risk), *n* = 20 | 3 mos to 5 yrs | N/A | HRV Wa, BRV NCD, PPD, TT, PHA | Proliferation assay | • Stimulation Index, positive ≥ 3 |
| Mesa et al., 2010 [27] | Colombia | Observational | RV-AGE, *n* = 17; Non-RV-AGE, *n* = 36 | Median 14 mos (range 4 to 22 mos) | Rotavirus infection | HRV Wa, Simian RRV, SEB, CD28, CD49d | Flow Cytometry | • $CD4^+$ and $CD8^+$<br>• $CD4^+/CD8^+IL\text{-}2^+$, $IL\text{-}10^+$, $IL\text{-}13^+$, $IL\text{-}17^+$, $IFN\text{-}\gamma^+$<br>• $CD4^+CD25^+$,$CD4^+CD25^+CD127^{low}$, $CD4^+CD25^+CD127^{low}TGF\text{-}\beta^+$ |
| Offit et al., 1992 [28] | USA | Observational | Healthy, *n* = 48 | Newborn to 18 yrs old | N/A | HRV Wa, HRV HCR3a strains, Simian RRV, concanavalin A | Proliferation assay | • Stimulation Index, positive ≥ 3:1 |

**Table 1.** *Cont.*

| Author Year [Ref] | Country | Design | Child Population, *n* | Age | Rotavirus Exposure | T-Cell Stimulant | T-Cell Detection Method | T-Cell Response Markers Evaluated |
|---|---|---|---|---|---|---|---|---|
| Offit et al., 1993 [29] | USA | Observational | RV-AGE, *n* = 8 | <2 yrs old. | Rotavirus infection | HRV HCR3a, HRV W179 | Proliferation assay | • Stimulation Index, positive ≥ 3:1 |
| Parra et al., 2014 [30] | Colombia | Observational | Healthy, *n* = 5 | 2 to 8 yrs old | N/A | Simian RRV, Influenza vaccine, TT, SEB, CD28, CD49d | Flow Cytometry, Proliferation assay | • $CD4^+$ /$CD8^+$ $IL\text{-}2^+$ $IFN\text{-}\gamma^+$, $TNF\text{-}\alpha^+$<br>• IFN-γ, TNF-α, GM-CSF, RANTES MCP-1 and IL-10, IL-4, IL-6, IL-17A, IL-9, and IL-2 secreting PBMC<br>• $CD4^+$ and $CD8^+$ proliferation |
| Parra et al., 2014 [31] | Colombia | Randomised Controlled Trial | Vaccine, *n* = 35<br>Placebo, *n* = 24 | 2 to 4 mos | Rotavirus vaccination | RRV, NSP2, VP3-4, VP6-7, SEB, CD28, CD49d | Flow Cytometry | • VP6-7 $tetramer^+$ $CD62L^-CD45RA^+/^-$ and $CD62L^+CD45RA^-CD4^+$,<br>• Gut homing ($\alpha4\beta7^+$ and $CCR9^+$) VP6-7 $tetramer^+$ $CD4^+$ |
| Rojas et al., 2003 [32] | Colombia | Observational | RV-AGE, n= 15; Non-RV-AGE, *n* = 13 | 3 mos to 7 yrs | Rotavirus infection | RRV, SEB, CD28, CD49d | ELISpot | • $IFN\text{-}\gamma^+$, $IL\text{-}4^+$ secreting PBMC,<br>• $IFN\text{-}\gamma^+$, $IL\text{-}4^+$ secreting $CD4^+$ and CD8 |
| Rott et al., 1997 [33] | USA | Observational | RV-AGE, *n* = 1 | NR | Rotavirus infection | RRV, concanavalin A | Flow Cytometry, Proliferation assay | • $\beta7^+$ and $\beta7^-$ PBMC<br>• Stimulation Index |
| Wang et al., 2007 [34] | USA | Observational | RV-AGE, *n* = 10; Healthy, n= 8 | <3 yrs | Rotavirus infection | Nil | Flow Cytometry PCR | • $CD4^+$ / $\alpha\beta CD4^+$, and $CD8^+$ / $\alpha\beta CD8^+$,<br>• $CD4^+$ /$CD8^+CD69^+$, and $CD4^+$ /$CD8CD83^+$<br>• CD1C, CD2, CD3D, CD28, CD96, CD2, α β TCR, Lck and Lck substrate, LAT, SLP-76, IL-16, CD27, IL-17R, IL-27Rα, IL-7R, RP1, LIGHT, and MAL gene expression |
| Weinberg et al., 2018 [35] | Botswana, Tanzania, Zambia, Zimbabwe | Randomised Controlled Trial | Vaccine, *n* = 42; Placebo *n* = 47 | 2 to ≤15 wks | Rotavirus vaccination | Nil | Flow Cytometry | • $CD4^+$<br>• $CD4^+IL\text{-}10^+$<br>• $CD4^+FOXP3^+CD25^+CD8^+$ $FOXP3^+CD25^+$ |
| Wood et al., 1988 [36] | England | Observational | RV-AGE, *n* = 2 | Newborn and 11 mos | Rotavirus infection | Simian RRV SA11strain, PHA | Proliferation assay | • Stimulation Index, positive > 2, T-cell frequency |
| Yasukawa et al., 1990 [37] | Japan | Observational | Healthy child, *n* = 1 | Newborn | N/A | HRV Wa strain, BRV NCD strain | Proliferation assay | • Scintillation count/minute |

Abbreviations: α4β7 = alpha 4 beta 7. β7 = beta 7. BRV = bovine rotavirus. CBV = Coxsackie B4 virus. CCR9 = C-C motif chemokine receptor 9. CD1C = cluster of differentiation 1C. CD2 = cluster of differentiation 2. CD3 = cluster of differentiation 3. CD3D = cluster of differentiation 3D. CD4 = cluster of differentiation 4. αβCD4 = alpha beta cluster of differentiation 4. CD8 = cluster of differentiation 8. αβCD8 = alpha beta cluster of differentiation 8. CD25 = cluster of differentiation 25. CD27 = cluster of differentiation 27. CD28 = cluster of differentiation 28. CD45RA = cluster of differentiation 45RA. CD49d = cluster of differentiation 49d. CD62L = cluster of differentiation 62L. CD69 = cluster of differentiation 69. CD83 = cluster of differentiation 83. CD96 = cluster of differentiation 96. CD127 = cluster of differentiation 127. CD28 and CD49d were used as co-stimulators. ELISpot = enzyme linked Immunospot. FOXP3 = Forkhead box protein P3. GM-CSF = granulocyte-macrophage colony-stimulating factor. HCR3a = human cytopathic rotavirus 3a. HRV = human rotavirus. IFN-γ = interferon gamma. IL2 = interleukin 2. IL4 = interleukin 4. IL6 = interleukin 6. IL7R = interleukin 7 receptor. IL9 = interleukin 9. IL10 = interleukin 10. IL13 = interleukin 13. IL16 = interleukin 16. IL17 = interleukin 17. IL17R = interleukin 17 receptor. IL27R α = interleukin 27 receptor alpha subunit. LAT = linker for activation of T cells. Lck = lymphocyte-specific protein tyrosine kinase. LIGHT = tumor necrosis factor superfamily member 14. MAL = myelin and lymphocyte protein. MCP1 = monocyte chemoattractant protein 1. mos = months. N/A = not applicable. NCD = Nebraska Calf Diarrhoea. NR = not reported. NSP2 = non-structural protein 2. PBMC = peripheral blood mononuclear cells. PCB = purified Coxsackie B4 virus. PCR = polymerase chain reaction. PHA = phytohemagglutinin. NR = not reported. PMA = phorbol myristate acetate. PPD = tuberculin purified protein derivative. RANTES = regulated on activation, normal T-cell expressed and secreted. RP1 (synonym MAPRE2) = microtubule associated protein RP/EB family member 2. RRV = rhesus rotavirus. RV-AGE = rotavirus acute gastroenteritis. SEB = staphylococcal enterotoxin B. SLP-76 = Src homology 2 domain-containing leukocyte protein of 76 kilodalton. TCR = T-cell receptor. αβTCR = alpha beta T-cell receptor. T1D = type 1 diabetes. TGF-β = transforming growth factor beta. Th17 = T helper 17. TNF-α = tumor necrosis factor alpha. Treg = regulatory T-cell. TT = Tetanus Toxoid. USA = United States of America. yrs = years. wks = weeks. VP6-7 = viral protein 6-7.

### *3.3. Quality Assessment of Individual Studies*

Of the included studies, 15/17 (88.2%) were observational studies while only 2/17 (11.8%) made use of experimental designs in the form of randomized controlled trials (Table 1). Using our adapted appraisal tool and threshold definitions of study quality, most articles were of moderate quality 11/17 (65%). The remaining 6/17 (35%) articles were appraised as high-quality articles of which the majority 5/6 (83%) were published in more recent years (Table 1). Most studies included in the review provided adequate information on research gaps around immunity to rotavirus, including research questions and rationales for the study of T-cell-specific responses to rotavirus. However, there was generally poor methodological reporting for most studies with minimal to no detailed

information provided on the exact study design employed, calculations, and assumptions for stated samples sizes or specification of inclusion and exclusion criteria for children or child-derived samples included in the studies. In most studies, there was also a poor presentation of participant or sample flow from recruitment to laboratory testing results as well as little to no information on children's background characteristics (Table S2).

### *3.4. T-Cell Proliferation against Rotavirus Develops and Broadens Reactivity with Increasing Age*

Children can mount detectable T-cell proliferation to different strains of rotavirus after in-vitro stimulation which is associated with age. As shown in Table 2, six studies reported induction of T-cell proliferation against human and non-human rotavirus strains and its relationship with the child's age. Children with acute rotavirus diarrhea had more positive and significantly higher T-cell proliferation to rotavirus antigen compared to healthy children. Among healthy children, T-cell proliferation was absent in newborns, minimally present in children aged <6 months but became more commonly detected in older age groups of children [22,25,26,28,37]. In contrast to this, however, one study also reported evidence of detectable T-cell proliferation in newborn children [28]. In healthy children, although T-cell proliferation to a human rotavirus strain was observed to be stronger than that against a bovine rotavirus strain, a positive correlation of T-cell reactivity was observed between the strains [26]. By the age of 2 years old and beyond, most children had developed T-cell reactivity against two strains of human rotavirus and against rhesus rotavirus strains [28]. However, T-cell proliferation against two different human rotavirus strains has also been observed among children aged <2 years old with acute and convalescent rotavirus diarrhea caused by different infecting rotavirus strains [29].

**Table 2.** Relationship between rotavirus T-cell proliferation and child age.

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Elaraby et al., 1992 [22] | RV-AGE: ($n$ = 6), age NR<br>Healthy: birth ($n$ = 14), 1 to <12 mos ($n$ = 14), 12 to <24 mos ($n$ = 10), and 24 to <60 mos ($n$ = 12). | Lymphoproliferation against rotavirus antigen (strain NR) | • Lymphoproliferation in all 6/6 (100%) children with RV-AGE versus 18/50 (36%) in healthy children.<br>• No lymphoproliferation in newborns but increasing lymphoproliferation in older age groups from 2/14 (14%) in 1 to <12 mos, to 5/10 (50%) in 12 to <24 mos and up to 11/12 (92%) in 24 to <60 mos age groups.<br>• Mean (SD) lymphoproliferation lowest at birth 1.11 (0.16) and in the 1 to <12 mos age group 1.08 (0.22), increased to 1.5 (0.72) in the 12 to <24 mos age group and highest in the 24 to <60 mos age group at 3.58 (1.66) | • Rotavirus is an effective T-cell inducer<br>• T-cell immunity to rotavirus increases with age. |
| Makela et al., 2004 [26] | Healthy: ($n$ = 20), 3 months to 60 months age: sampled 3 mos to 6 mos ($n$ = 23), 9 mos to 12 mos ($n$ = 26), 15 mos to 24 mos ($n$ = 65), 27 mos to 36 mos ($n$ = 38), 39 mos to 48 mos ($n$ = 31), 51 mos to 60 mos ($n$ = 11) | Lymphoproliferation against bovine NCD (P serotype 6, G serotype 6) and human purified and lysate Wa (P serotype 1, G serotype 1A) rotavirus strain | • Lymphoproliferation against both human and bovine rotavirus antigens are more common with increasing age (NS, Fisher's exact test)<br>• Positive correlation between lymphoproliferation against bovine and human lysate rotavirus ($p$ < 0.0001, $r_S$ = 0.52, Spearman correlation test) and between bovine and purified human rotavirus $p$ < 0.0001, $r_S$ = 0.56, Spearman correlation test) | • T-cell immunity to rotavirus increases with age and is cross-reactive |
| Makela et al., 2006 [25] | Healthy: ($n$ = 183), age range 3.5 yrs to 11.3 yrs | lymphoproliferation against human Wa (P serotype 1, G serotype 1A) and bovine NCD (P serotype 6, G serotype 6) rotavirus strains | • Lymphoproliferation positively correlated with age for human ($r$ = 0.32, $p$ < 0.0001) and bovine ($r$ = 0.20, $p$ = 0.001) rotavirus | • T-cell immunity to rotavirus increases with age and is cross-reactive |
| Offit et al., 1992 [28] | Healthy: age groups newborns ($n$ = 11), 16 days to <6 mos ($n$ = 11), 6 mos to <2 yrs ($n$ = 8), 2 yrs to 5 yrs ($n$ = 8), 5 yrs to 18 yrs ($n$ = 10). | lymphoproliferation against human Wa (serotype 1) and HCR3a (serotype 3) and simian rhesus rotavirus strain 2 (serotype 3) antigens | • Few 1/11 (9%) children aged <6 mos had lymphoproliferation against human rotavirus but unexpectedly 4/11 (36%) newborns showed lymphoproliferation against both human and simian rotavirus antigens<br>• In contrast, 6/8 (75%) and 4/8 (50%) children aged between 6 mos to 2 yrs and 10/13 (77%) and 6/16 (38%) aged between 6 mos to 5 yrs had lymphoproliferation against human rotavirus and simian rotavirus antigens respectively.<br>• In children aged >5 yrs old, ~80% had lymphoproliferation against both human and simian rotavirus antigens | • T-cell immunity to rotavirus increases with age and is cross-reactive.<br>• T-cell immunity to rotavirus may occur at birth due to maternal transfer or in-utero rotavirus exposure |
| Offit et al., 1993 [29] | RV-AGE: $n$ = 8, <2 yrs caused by serotype 1 (P type 1, G type 1, $n$ = 2), serotype 3 (P type 1, G type 3, $n$ = 3) and serotype 4 (P type 1, G type 4, $n$ = 3) rotavirus strains and followed up in convalescence and late convalescence | Lymphoproliferation against human WI79 (P-type 1, G type 1) and HCR3a (P-type non-human, G type 3) rotavirus strain antigens | • During the acute stage, a few 1/8 (13%) children had lymphoproliferation against WI79 rotavirus antigen.<br>• In contrast during convalescence most 6/8 (75%) $p$ < 0.05 and 5/6 (83%) $p$ < 0.05 children had lymphoproliferation against WI79 and HCR3a rotavirus strains respectively; 4/6 (67%) had lymphoproliferation to both strains, 1/6 (17%) to only HCR3a, 2 to only WI79, while no reactivity to either strain was observed in 1/6 (17%) children studied. At late convalescence, all 4/4 (100%) children studied had lymphoproliferation.<br>• No proliferative response specific for the G type of the infecting rotavirus strain in either convalescent or late convalescent children was observed | • T-cell immunity is present during acute and convalescent rotavirus infection.<br>• T-cell immunity to rotavirus is not G-type specific and may recognize T-cell epitopes shared by different rotavirus strains |

**Table 2.** *Cont.*

| Author, Year [Ref] | Child Age | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Yasukawa et a., 1990 [37] | Healthy full-term newborn | Lymphoproliferation against human Wa (serotype 1) strain rotavirus antigen | • Lymphoproliferation against human rotavirus antigen absent in the newborn | • T-cell immunity to rotavirus occurs in an antigen-specific manner |

Abbreviations: HCR3a = human cytopathic rotavirus 3a. mos. = months. NCD = Nebraska Calf Diarrhoea. NR = not reported. NS = not statistically significant. *p* = probability value. RV-AGE = rotavirus acute gastroenteritis. *r* = Pearson's correlation coefficient. $r_s$ = Spearman's rank correlation coefficient. SD = standard deviation. yrs = years.

### *3.5. Rotavirus T-Cell Proliferation and Frequency Coincides with Antibody Responses but Is More Transient*

Six studies reported T-cell immunity in association with rotavirus antibody responses as shown in Table 3. T-cell responses were observed more frequently in rotavirus antibody seropositive than seronegative children and among secondary than primary infections indicating that both memory T-cell and antibody responses are induced by rotavirus exposure and built from repeated exposure [25,26,31]. The strength and magnitude of T-cell responses occurred in very close association with the antibody response. Makela et al. showed that generally, lower antibody titers to rotavirus were accompanied by minimal or absent T-cell responses while increased antibody responses were associated with stronger T-cell responses. However, strong T-cell immunity was also observed in the absence of increasing antibody responses in a single child in this study and although firm conclusions cannot be made based on this lone observation, it highlights the need to detect both antibody and cellular responses in assessing rotavirus immunity [26]. Compared to antibodies that persisted long after infection, T-cell responses were more transient, detectable two to eight weeks and three to five months post-infection but declining as early as 5 months to nearly undetectable levels within 12 months post rotavirus exposure [26,29]. However, both T-cell and antibody responses were minimal during acute rotavirus infection but more frequent during convalescence [29]. Unlike antibodies present at birth, T-cell immunity was generally absent in early infancy (<6 months) developing much later in infancy and may therefore be a better indicator of active infant immunity than antibodies and distinguish from passive maternal immunity in the very young infants [28]. Both T-cell and antibody responses can be mounted against different infecting rotavirus strains indicating an inability to clearly distinguish rotavirus P and G serotypes [29]. Rotavirus-specific CD4 T-cells are positively associated with antibody responses, while regulatory T-cells may either have a positive or negative association with the antibody response to rotavirus [35]. One study among T-cell deficient children further emphasized intimate associations between T-cell immunity and antibody response in the context of clearance of rotavirus infection. Wood et al. described chronic rotavirus infection in two children with congenital T-cell deficiency [36]. In a child with cartilage hair hypoplasia associated T-cell deficiency and acute rotavirus diarrhea, no serum antibody immune response to rotavirus was detected. Likewise, no significant proliferative response to rotavirus was observed ~1 year after the onset of diarrhea and diarrhea persisted over an 18-month period characterized by poor weight gain and failure for the child to thrive despite treatment. In the same study, a second child with CHARGE congenital abnormalities and DiGeorge syndrome associated T-cell deficiency who was infected with rotavirus, the rotavirus IgG antibody response was undetectable two months after rotavirus infection and despite treatment, this child failed to thrive and died at 5 months old.

**Table 3.** Rotavirus T-cell proliferation, frequencies, and phenotypes in relation to an antibody response.

| Author, Year [Ref] | Population and Antibody Response | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Makela et al., 2006 [25] | Healthy: rotavirus IgA and/or IgG seropositive ($n$ = 112) or rotavirus seronegative ($n$ = 41) | Lymphoproliferation and IFN-γ producing PBMC against purified and lysate human and bovine rotavirus antigens | • Seropositive children had more frequent lymphoproliferation 50/112 (45%) than seronegative 4/41, 10% children ($p < 0.0001$) and stronger lymphoproliferation against purified ($p = 0.010$), lysate ($p = 0.0031$) human rotavirus and bovine rotavirus ($p < 0.0001$)<br>• Seropositive children had higher IFN-γ producing PBMC compared to seronegative children ($p = 0.084$) | • Prior exposure to rotavirus induces both memory T-cell and B-cell immunity in children. |
| Makela et al., 2004 [26] | Healthy: rotavirus IgA and/or IgG seropositive or seronegative at 3 mos to 12 mos of age with primary ($n$ = 19) or secondary ($n$ = 5) rotavirus infections | Lymphoproliferation against purified and lysate human rotavirus | • Minimal or absent proliferation in children with low rotavirus antibody titers.<br>• Increase in antibody titers accompanied by stronger lymphoproliferation against lysate and purified human rotavirus ($p = 0.017$ and $p = 0.027$, respectively, Wilcoxon test) and more positive lymphoproliferation in 9/24 (37.5%) cases. In contrast, one child had lymphoproliferation without a simultaneous increase in rotavirus antibody titers.<br>• Lymphoproliferation more frequent in secondary infections than primary infections (NS)<br>• Rotavirus-specific antibody levels remained elevated throughout follow-up after rotavirus infection but lymphoproliferation declined shortly after infection and was detectable less than 12 months after primary infection (mean 5 months) | • T-cell immunity occurs in tight association with rotavirus antibody response.<br>• T-cell immunity can occur in absence of detectable increasing antibody response<br>• More persistent and stronger T-cell immunity develops after repeated rotavirus exposure<br>• Unlike antibodies, T-cell immunity to rotavirus is transient. |
| Offit et al., 1992 [28] | Healthy: age groups newborns ($n$ = 11), 16 days to <6 mos ($n$ = 11), 6 mos to <2 yrs ($n$ = 8), 2 yrs to 5 yrs ($n$ = 8), and 5 yrs to 18 yrs ($n$ = 10) with rotavirus neutralising antibody | Lymphoproliferation against human and simian rotavirus | • More newborns and children < 6 mos, had neutralizing antibodies against at least one rotavirus strain than lymphoproliferation<br>• In contrast, among older age groups between 6 mos and 18 yrs, most children had both lymphoproliferation and rotavirus neutralizing antibodies to at least one human or simian rotavirus strain. | • Development of T-cell immunity to rotavirus occurs in conjunction with the development of antibody responses in children<br>• In young infants aged <6 mos measurement of T-cell immunity is possibly more reliable in discriminating active from a passively acquired immune response<br>• Both T-cell and antibody immunity induced by rotavirus in children can be cross-reactive |
| Offit et al., 1993 [29] | RV-AGE: caused by P-type 1 and different G type strains followed up in convalescence and late convalescence with rotavirus IgA and neutralizing antibodies ($n$ = 8) | lymphoproliferation against human rotavirus | • Neutralizing antibodies were mounted against different P and G serotype infecting rotavirus strains and similarly, lymphoproliferation was also mounted against different infecting G serotypes strains | • Both rotavirus specific neutralizing antibody and T-cell immunity in children may not clearly distinguish P and G infecting serotypes |
| Parra et al., 2014 [31] | Rotavirus IgA seropositive vaccinated ($n$ = 35) and seronegative placebo ($n$ = 24) | Frequency of CD4 T-cells positive for rotavirus specific VP6-7 T-cell epitope | • Vaccinated seropositive children had a higher frequency of VP6-7 tetramer-positive activated CD4 T-cells (40–71%) than placebo seronegative children (0–8%) | • Rotavirus-specific antibody responses to vaccination are accompanied by rotavirus-specific CD4 T-cells in children. |
| Weinburg et al., 2018 [35] | PHEU and PHIV ($n$ = 42) vaccinated with pentavalent live rotavirus vaccine: IgA and neutralizing IgG | Frequency of several CD4 and CD8 T-cell phenotypes | • Higher CD4 T-cell frequency and counts marginally and significantly associated with higher IgG neutralizing antibodies to 3/5 viral strains tested<br>• Higher frequencies of $CD4^+FOXP3^+CD25^+$ and $CD8^+FOXP3^+CD25^+$ regulatory T-cells were marginally or significantly ($p < 0.1$) associated with higher rotavirus IgG neutralizing antibodies to 4/5 viral strains in the RV5 vaccinated and significantly associated with higher IgA antibodies. These associations remained at least marginally significant after adjustment for CD4 T-cell proportions.<br>• Significant negative correlations with antibody titers were observed for $CD4^+IL10^+$ regulatory T-cells | • Rotavirus CD4 T-cells are induced in positive association with the antibody response to vaccination<br>• $FOXP3^+CD25^+$ regulatory CD4 and CD8 T-cells may positively influence antibody responses by the protection of B cells against intense activation and apoptosis while $IL10^+$ regulatory CD4 T-cells may negatively influence this response by downregulation of immune responses via bystander mechanisms. |
| Wood et al., 1988 [36] | CHH ($n$ = 1) and CHARGE associated ($n$ = 1) T-cell deficiency and rotavirus IgG | Lymphoproliferation against mitogens, rotavirus antigen, and proportions of T-cells | • Poor lymphoproliferation and absent rotavirus specific IgG antibody response associated with persistent rotavirus diarrhea. | • Rotavirus-specific T-cell deficiency is associated with impaired antibody response and inability to clear rotavirus infection |

Abbreviation: CD4 = cluster of differentiation 4. CD8 = cluster of differentiation 8. CD25 = cluster of differentiation 25. CHH = cartilage hair hypoplasia. CHARGE = coloboma, heart defects, atresia choanae growth retardation, genital abnormalities, and ear abnormalities. FOXP3 = Forkhead box protein P3. IFN-γ = Interferon gamma. IgA = Immunoglobulin A. IgG = Immunoglobulin G. IL1–10 = Interleukin 10. mos. = months. NS = not significant. PBMC = peripheral blood mononuclear cells. PHEU = perinatally HIV exposed but uninfected. PHIV = perinatally HIV infected. $p$ = probability value. RV-AGE = rotavirus acute gastroenteritis. RV5 = pentavalent rotavirus vaccine. VP6-7 = viral protein 6-7. yrs = years.

### *3.6. CD4 and CD8 T-Cells Are of Low Circulating Frequency in Acute Rotavirus*

Five studies reported a lower circulating frequency of $CD4^+$ and $CD8^+$ T-cells in response to acute rotavirus infection. In one study, while healthy children had normal proportions of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell subsets, children with acute rotavirus diarrhea had selectively lowered $CD4^+$ T-cell proportion and a low $CD4^+$:$CD8^+$ T-cell ratio [22]. A case study of a single child with rotavirus diarrhea showed a depressed $CD4^+$ T-cell frequency and $CD4^+$:$CD8^+$ ratio in an acute phase that persisted up to one-month post-infection but normalized by convalescent period [23]. In another two studies close to half of the children with rotavirus diarrhea had absolute lymphopenia compared to children with or without previous rotavirus exposure but with non-rotavirus diarrhea and the

majority of children with acute (<7 days after the onset of illness) rotavirus diarrhea had total whole blood lymphocyte counts less than the lower limit of the normal count range in healthy children [27,34]. Additionally, among children with previous rotavirus exposure and those with rotavirus diarrhea, few had detectable cytokine-producing rotavirus-specific CD4 or CD8 T-cells [27]. Likewise, flow cytometry and gene expression T-cell analysis of children with rotavirus diarrhea revealed significantly lower mean frequencies of $CD4^+$ and $\alpha\beta^+CD4^+$ T-cells, $CD8^+$ and $\alpha\beta^+CD8^+$ T-cells and T-cell associated gene expression in children with rotavirus diarrhea in the acute phase than in healthy controls. In the convalescent phase, however, the frequencies of these T-cell populations significantly increased to similar levels observed in healthy children. Exceptionally, one child with rotavirus diarrhea was observed to have a minimal reduction in $CD4^+$ and $CD8^+$ T-cell frequencies in the acute stage but had a severe reduction in CD4 and CD8 T-cell subsets at convalescence [34]. Among vaccinated children, rotavirus antigen-experienced CD4 T-cells were detected in low frequencies two weeks post-vaccination [31]. Summary findings of these studies are outlined in Table 4.

**Table 4.** Proliferative, Helper, and cytotoxic T-cell frequency to rotavirus in children compared to adults and other stimulants.

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Elaraby et al., 1992 [22] | Healthy: ($n$ = 50); RV-AGE: ($n$ = 6) | CD3 (OKT3 pan), CD4 (OKT4 helper), CD8 (OKT8 frequency, CD4:CD8 T-cell ratio | • Depressed CD4 T-cell frequency (33.4%) and a lower CD4:CD8 ratio (1.36) in children with rotavirus diarrhea compared to normal CD4 (range 47.1% to 55.7%) and CD8 (23.8% to 25%) T-cell frequency and helper: suppressor ratio (1.9 to 2.23) in healthy children | • Lowered CD4 T-cells during acute infection may be a result of CD4 T-cell migration out of circulation to effector sites |
| Iwasa et al., 2008 [23] | RV-AGE: ($n$ = 1) | CD4 and CD8 T-cell frequency, CD4:CD8 T-cell ratio | • Depressed CD4 T-cells frequency (15.7%) and lowered CD4:CD8 ratio (0.41) but normal CD8 T-cell frequency (38.76%) in acute phase.<br>• Depressed CD4 T-cells frequency (14.55%) and lowered CD4:CD8 ratio (0.42) sustained in early convalescence but normalized in late convalescence | • Lowered CD4 T-cell during acute infection may be a result of CD4 T-cell migration out of circulation to effector sites<br>• CD4 T-cells may be more critical effectors than CD8 T-cells in mucosal tissue sites |
| Mesa et al., 2010 [27] | Non-RV-AGE seronegative ($n$ = 15) or seropositive ($n$ = 21) and RV-AGE ($n$ = 17) children. Healthy ($n$ = 21) and RV-AGE adults ($n$ = 5) | Lymphopenia and Th1, Th2, Th17 CD4 and cytotoxic CD8 T-cells | • Absolute lymphopenia in 5/12 (41.6%) children with RV-AGE compared to only 1/25 (4%) in children with non-RV-AGE<br>• Low (<0.06) or undetectable frequencies of IFN-$\gamma^+$, IL-$13^+$, IL-$2^+$, IL-$10^+$ and IL-$17^+$ CD4 T-cells in most children with non- and RV-AGE. The IFN-$\gamma^+$CD4 and CD8 T-cells were observed in a few 2/12 children with previous rotavirus exposure or rotavirus diarroea.<br>• In contrast, higher frequencies ($\leq$0.65%) of rotavirus-specific $CD4^+$IFN-$\gamma^+$ and $CD4^+$IL-$2^+$ T cells were detected in the majority 14/21 (66.7%) and 6/10 (60%) of healthy adults, respectively. Similarly, $CD8^+$IFN-$\gamma^+$ and $CD8^+$IL-$2^+$ T cells were observed in 8/20 (40%) and 1/9 healthy adults, respectively. | • Low circulating frequency of Th1, Th2, Th17, and cytotoxic T-cells in acute rotavirus that may result from effector T-cell functions at mucosal sites of infection<br>• Diminished rotavirus Th1 and cytotoxic responses in children compared to adults |
| Parra et al., 2014 [31] | Seropositive vaccinated: ($n$ = 35) and seronegative placebo: ($n$ = 24) | Rotavirus (VP6-7 tetramer) antigen experienced CD4 T-cells | • Low frequency (0.001–0.1%) rotavirus antigen experienced CD4 T cells in children two weeks post two-dose vaccination | • CD4 T-cells are expanded after rotavirus vaccination but low circulating frequency |
| Wang et al., 2007 [34] | RV-AGE: ($n$ = 10); Healthy ($n$ = 8) | Lymphopenia, frequencies of CD4, $\alpha\beta^+$CD4, CD8 and $\alpha\beta^+$CD8 T-cells | • Lymphopenia in majority 5/7 (71%) of children with RV-AGE and repressed T-cell proliferation, differentiation, activation, survival, and homeostasis mRNA gene expression<br>• Lower mean frequency of CD4 (20%, range 10.4% to 26.8%) and $\alpha\beta^+$CD4 (17% range 9% to 22.6%) T-cells in RV-AGE than in healthy children (50.9% range 38.6% to 60.5%) and (46.8% range. 36.7% to 53.7%) respectively ($p < 0.01$). CD4 T-cell frequencies significantly increased ($p < 0.01$) to that of healthy children at convalescence.<br>• Similarly, lower mean frequency of CD8 (2.8%, range 1.6% to 3.8%) and $\alpha\beta^+$CD8 (2.9%, range 1.7% to 3.7%) T-cells in RV-AGE than in the healthy children (10.9%, range 7.4% to 13.5%) and (8.6%, range 6.1% to 10.5%) respectively ($p < 0.05$). Both CD8 and $\alpha\beta^+$CD8 T-cell frequencies significantly increased ($p < 0.01$) to that of healthy children at convalescence. | • Altered T-cell homeostasis and low circulating frequency of CD4 and CD8 T-cells in acute rotavirus that may result from effector T-cell functions at mucosal sites of infection |
| Jaimes et al., 2002 [24] | RV-AGE children ($n$ = 12), rotavirus exposed asympomatic and symptomatic adults ($n$ = 19), healthy adults ($n$ = 7) | Th1 and Th2 CD4 and cytotoxic CD8 T-cell frequencies | • Lower mean rotavirus specific CD8 IFN-$\gamma$ T-cell frequency 0.02% (SEM 0.007% range −0.01 to 0.08%) in RV-AGE children than exposed adult 0.49% (SEM 0.17% range 0.2 to 1.13%); recently infected symptomatic adults 0.28% (SEM 0.11% range, 0.03 to 0.91%); and asymptomatic adults mean, 0.15% (SEM 0.06% range, 0.03 to 0.37%) ($p < 0.05$)<br>• Lower mean rotavirus-specific CD4 IFN-$\gamma$ T-cell frequency 0.02% (SEM 0.007% range −0.01 to 0.07%) in infected children than in exposed adults 0.1% (SEM 0.02% range, 0.02 to 0.19%); symptomatically infected adults mean 0.18% (SEM 0.10% range 0.02 to 0.94%) and asymptomatic rotavirus infected adults mean 0.05%; SEM 0.01%; range, 0.01 to 0.09%) ($p < 0.01$).<br>• CD4 IL-13 T-cell frequency mean 0.02%; SEM, 0.009%; range, 0 to 0.06% detected in children but not adults but no predominance in CD4 IFN-$\gamma$ or IL-13 T-cells in children. | • Lower circulating frequency of Th1 and cytotoxic T-cells in infected children than adults<br>• Mixed Th1 and Th2 responses in children contrasted to predominantly Th1 in adults. |

**Table 4.** *Cont.*

| Author, Year [Ref] | Population | T-Cell Response | Key Findings | Interpretation |
|---|---|---|---|---|
| Makela et al., 2004 [26] | Healthy (T1D at risk) children: ($n$ = 20); Healthy rotavirus exposed adults ($n$ = 16) | Lymphoproliferation | • Adults had stronger T-cell proliferation to bovine rotavirus (NCD) ($p$ = 0.0001–0.0067), human rotavirus lysate ($p$ = 0.0008–0.011) and purified human rotavirus ($p$ = 0.0044–0.083) than any age group of children.<br>• Similar T-cell proliferation to PPD in children and adults ($p$ = 0.53–0.91) | • Children have weaker T-cell responses to rotavirus compared to adults.<br>• Rotavirus is a poor inducer of T-cells in comparison to mycobacterial tuberculin |
| Makela et al., 2006 [25] | Healthy children (T1D at risk, $n$ = 183) | Lymphoproliferation | • Children had a higher median T-cell proliferative response to TT and PPD than to purified rotavirus, human rotavirus lysate, or bovine rotavirus (NCD) | • Rotavirus is a poor inducer of T-cells in comparison to mycobacterial tuberculin and tetanus toxoid |
| Parra et al., 2014 [30] | Healthy children ($n$ = 5) and healthy adults ($n$ = 25) | Cytokine secreting PBMC. Th1 CD4 and cytotoxic CD8 T-cells. CD4 and CD8 proliferation. | • IFN-γ, TNF-α, GM-CSF, RANTES, MCP-1 and IL-10 secreting PBMC in adults but not children<br>• Lower frequencies of IFN-γ, TNF-α, and IL-2 CD4 T-cells against rotavirus than against TT ($p$ = 0.0313) or Influenza ($p$ = 0.0313) in both children and adults.<br>• Monofunctional (single IFN-γ or TNF-α secreting) rotavirus specific CD4 T-cells predominant in both adults and children | • Diminished Th1 responses in children than adults.<br>• Rotavirus is a poor inducer of T-cells in comparison to tetanus toxoid and Influenza<br>• CD4 T-cell response to rotavirus involves predominantly Th1 subset |
| Rojas et al., 2003 [32] | RV-AGE children ($n$ = 9); Healthy adults ($n$ = 7) | Frequencies of Th1 and Th2 CD4 and cytotoxic CD8 T-cells | • Both IFN-γ CD4 ($p$ = 0.046) and IFN-γ CD8 ($p$ = 0.028) T-cells against rotavirus detected in adults but only IFN-γ CD8 $p$ = 0.018) and not CD4 T-cells ($p$ = *0.17*). detected in children with diarrhoea.<br>• Low but insignificant frequency of IL-4 CD4 T-cells against rotavirus detected in both adults and children ($p$ = 0.15). | • IFN-γ cytotoxic CD8 T-cells may be the main effector in acute rotavirus infected children<br>• Th2 CD4 T-cells may have a less significant role against rotavirus |

Abbreviations: CD3 = cluster of differentiation 3. CD4 = cluster of differentiation 4. αβCD4 = alpha beta cluster of differentiation 4. CD8 = cluster of differentiation 8. αβCD8 = alpha beta cluster of differentiation 8. GM-CSF = granulocyte-macrophage colony-stimulating factor. IFN-γ = interferon gamma. IL-2 = interleukin 2. IL-10 = interleukin 10. IL=13 = interleukin 13. IL-17 = interleukin 17. mRNA = messenger ribonucleic acid. MCP1 = monocyte chemoattractant protein 1. NCD = Nebraska Calf Diarrhoea. OKT3 = anti-CD3 monoclonal antibody. OKT4 = anti-CD4 monoclonal antibody. OKT8 = anti-CD8 monoclonal antibody. PBMC = peripheral blood mononuclear cells. $p$ = probability value. PPD = tuberculin purified protein derivative. RANTES = regulated on activation, normal T-cell expressed and secreted. RV-AGE = rotavirus acute gastroenteritis. SEB = staphylococcal enterotoxin B. SEM = standard error of measurement. T1D = type 1 diabetes. Th1 = T-helper type 1. Th2 = T-helper type 2. Th17 = T-helper type 17. TT = tetanus toxoid. TNF-α = tumor necrosis factor alpha. VP6-7 = viral protein 6-7.

### *3.7. Proliferative, Helper and Cytotoxic T-Cells Profiles to Rotavirus Differ in Children Compared to Adults and Other Stimulants*

Diminished responses and different profiles of proliferative, helper, and cytotoxic T-cell responses are elicited against rotavirus in children compared to adults or other stimulants as shown in Table 4. In a study by Jaimes et al., rotavirus-specific $CD4^+IFN\text{-}\gamma^+Th1$, $CD4^+IL\text{-}13^+Th2$, and $CD8^+IFN\text{-}\gamma^+$ cytotoxic T-cells, were investigated in children with rotavirus diarrhea in comparison to recently infected, exposed, and unexposed healthy adults. When compared, rotavirus-exposed adults had significantly higher mean proportions of rotavirus-specific Th1 and cytotoxic responses than children whose responses were similar to those observed in healthy adults. However, while the Th1 and cytotoxic T-cell responses were induced by rotavirus in both adults and children, the Th2 response was additionally observed in children with rotavirus diarrhea at a similar frequency to the Th1 response but not in adults [24]. In contrast, a study by Parra et al. showed a predominance of monofunctional $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ Th1 response in both adults and children [30]. Another study found T-cell proliferative responses to rotavirus were generally weaker in prospectively studied children compared to adults with the adults having significantly stronger T-cell proliferation to both bovine and human rotavirus strains than any age group of children [26]. A study looking at frequencies of $CD4^+IFN\text{-}\gamma^+$ or $IL\text{-}2^+Th1$, $CD4^+IL\text{-}13^+Th2$, $CD4^+IL\text{-}17^+Th17$ and $CD8^+IFN\text{-}\gamma^+$ cytotoxic T-cells in children with rotavirus and non-rotavirus diarrhea in comparison with healthy and acutely or convalescent rotavirus infected adults found similar observations. Little to no Th1, Th2, or Th17 rotavirus-specific T-cell responses were observed in children with diarrhea and few responses observed comprised Th1 and cytotoxic responses and were only observed among children with prior exposure to or existing acute rotavirus diarrhea. In contrast to children, a much larger proportion of adults, both healthy and acutely infected had detectable Th1 and cytotoxic T-cell responses [27]. These results are similar to another study that showed secretion of IFN-γ, TNF-α, GM-CSF, RANTES, MCP-1, and IL-10 from rotavirus stimulated cells in adults but not in children [30].

In comparison to other viral and bacterial stimulants, circulating rotavirus-specific T-cell responses are generally diminished. While significantly higher proliferation to rotavirus

was observed in adults than children, proliferation in response to mycobacterium purified protein derivative (PPD) in children was as high as that observed in adults [26]. Among healthy children, T-cell proliferation to rotavirus was observed to be generally lower in comparison to proliferation against tetanus toxoid (TT), mycobacterium PPD antigens, and Coxsackie B4 virus (CBV) antigen [25,30]. Significantly lower frequencies of IFN-γ, TNF-α and IL-2 producing CD4 T-cells were observed against rotavirus than in response to Influenza virus antigens in children [30]

### *3.8. Rotavirus Activates Proinflammatory, Regulatory and Gut Homing Effector T-Cell Phenotypes*

The T-cell immune response to rotavirus in children is characterized by an elevated activated and proinflammatory T-cell profile (Table 5). Children with rotavirus diarrhea show higher proportions of proinflammatory T-helper 17 cells complemented by higher levels of peripheral blood circulating pro-inflammatory IL-6 and IL-17 cytokines at the time of acute infection compared to healthy children [21]. Similarly, a case report of a child with rotavirus gastroenteritis reported elevated proportions of IFN-γ producing helper and cytotoxic T-cells in the acute phase of infection although these levels were reduced by convalescence [23]. Likewise, another study showed a positive correlation between T-cell proliferative responses to rotavirus and messenger ribonucleic acid (mRNA) expression of proinflammatory IFN-γ and IL-4 cytokines in healthy children [25]. Similar to these findings, a microarray analysis study of immune cell mRNA gene expression by Wang et al. revealed that children with rotavirus diarrhea had upregulation of genes encoding lymphocyte activation markers, proinflammatory cytokines, chemokines, and immune proteins in the acute stage compared to healthy children. Interestingly, although there was an elevated gene expression of lymphocyte activation markers CD69 and CD83 as well as genes encoding for the differentiation, maturation, activation, and survival of B lymphocytes, there was a reduced expression of genes involved in the proliferation, differentiation, activation, survival, and homeostasis of T lymphocytes in these rotavirus infected children [34].

The proinflammatory T-cell response to rotavirus may occur in association with either a lowered or elevated regulatory T-cell response (Table 5). Dong et al. found that rotavirus infected children had a significantly lower proportion of regulatory T-cells compared to healthy children. The lower regulatory cell profile corresponded to significantly lower levels of circulating immunosuppressive IL-10 and TGF-β cytokines [21]. In contrast, a study by Mesa et al. showed that a TGF-β dependent regulatory mechanism of rotavirus specific CD4 and CD8 IFN-γ T-cell response was absent in children with acute rotavirus gastroenteritis but present in adults, although only four and three adults were studied respectively, and showed that the lowered circulating frequency of rotavirus specific T-cells was not due to regulation by TGF-$\beta^{+}$ regulatory T-cells as both rotavirus-infected and healthy children had similar proportions of these circulating Treg profiles [27]. Furthermore, another study found a positive correlation between T-cell proliferative responses and immunosuppressive IL-10 but supporting the previous studies this was not observed for TGF-β [25]. One other study also found elevated expression of other inflammation-modulating proteins IL-1R antagonist, IFN α/β receptors and IFN-stimulated proteins in rotavirus infected children [34].

Two studies reported that a substantial proportion of rotavirus-experienced T-cells express gut homing markers. As shown in Table 5, one study by Rott et al. among children convalescing after acute rotavirus infection reported higher T-cell proliferative response to rotavirus in the $\alpha4\beta7^{hi}$ lymphocyte population than $\alpha4\beta7^{-}$ lymphocyte population although this was based on cellular data obtained from one child [33]. Likewise, another study among rotavirus vaccinated children found that most of the rotavirus antigen-experienced $CD4^{+}$ T cells expressed α4β7 gut homing marker with most cells expressing both, α4β7 and CCR9, gut homing markers [31].

**Table 5.** T-cell activation, proinflammatory, regulatory and homing phenotypes in response to rotavirus.

| Author, Year [Ref] | Child Population | T-Cell Response | Finding | Interpretation |
|---|---|---|---|---|
| Dong et al., 2015 [21] | RV-AGE ($n$ = 102); Healthy ($n$ = 30) | Th17 and Tregs frequency | • Frequencies of $CD4^+$ IL-$17^+$ Th17 cells and circulating IL-17 and IL-6 proinflammatory cytokines were increased ($p < 0.05$) in RV-AGE than healthy children. ($p < 0.05$)<br>• In contrast, the frequency of $CD4^+CD25^+$ Treg cells and levels of circulating IL-10 and TGF-β regulatory cytokines in children with rotavirus enteritis was significantly decreased when compared with the healthy children ($p < 0.05$). | • Th17 cells play a role in the protective immune response to rotavirus<br>• $CD4^+CD25^+$ T-cells and regulatory cytokines lowered in rotavirus infection |
| Iwasa et al., 2008 [23] | Infant with acute rotavirus gastroenteritis ($n$ = 1) | Th1 CD4 and cytotoxic CD8 T-cell frequencies | • Elevated IFN-γ$CD4^+$ (14.85%) and $CD8^+$ (77.58%) T-cell frequency during acute stage that decreased one month later to 3.46% and 0.19% respectively | • IFN-γ Th1 CD4 and cytotoxic CD8 T-cells are effectors against acute rotavirus |
| Makela et al., 2006 [25] | Healthy (T1D at risk), $n$ = 183) | IFN-γ, IL-4, IL-10 and TGF-β mRNA expression and T-cell proliferation | • Positive correlation between PBMC IFN-γ, IL-4 and IL-10 mRNA secretion and lymphoproliferation against rotavirus ($r$ = 0.48, $p$ = 0.003, r = 0.46, $p$ = 0.004, and $r$ = 0.36, $p$ = 0.026 respectively). No correlation with TGF-β | • Rotavirus T-cell responses includes Th1 and Th2 effectors<br>• IL-10 and not TGF-β regulatory T-cells may be important immune regulators of the proinflammatory response |
| Wang et al., 2007 [34] | RV-AGE ($n$ = 10); Healthy ($n$ = 8) | Gene expression of T-cell immune markers | • Elevated gene expression of inflammatory immune markers TNF- α, proIL-1 β, IL-1 β, IL-6, IL-8, GRO- β, IL-1R antagonist, IFN- α / β receptor and IFN α / β -stimulated proteins in rotavirus infected children than healthy children<br>• Elevated CD4 T-cell activation CD4/CD69 (from 2.7% to 10.5% [mean, 5.5%]), CD4/CD83 (from 10.5% to 25.8% [mean, 16.6%]), and CD8 T-cell activation CD8/CD69 (from 1.6% to 8.3% (mean, 3.5%), CD8/CD83 (from 4.4% to 16.1% [mean, 7.8%]) in RV-AGE than in healthy children range 0% to 0.5% (mean, 0.3%) for CD4/CD69, from 0.1% to 4.0% (mean, 1.2%) for CD4/CD83, from 0.1% to 0.7% (mean, 0.3%) for CD8/CD69, and from 0% to 0.4% (mean, 0.2%) for CD8/CD83 respectively. | • Rotavirus induces a pro-inflammatory immune response<br>• CD69 and CD83 activated CD4 and CD8 T-cells contribute to antiviral activity and recovery from disease in children |
| Mesa et al., 2010 [27] | RV-AGE ($n$ = 53) | $CD4^+CD25^+$, $CD4^+CD25^+CD127^{low}$, $CD4^+CD25^+CD127^{low}$ TGF-$\beta^+$ and $CD45RA^+$ regulatory T-cells (Tregs) and IFN-γ producing CD4 T-cells | • Rotavirus IFN-γ CD4 T-cells not affected by TGF-β regulation in children but in adults<br>• No difference in $CD4^+CD25^+$, $CD4^+CD25^+CD127^{low}$ and $CD4^+CD25^+CD127^{low}$ TGF-$\beta^+$ Tregs in RV-AGE and non-RV-AGE<br>• Most $CD4^+CD25^+CD127^{low}$ Treg cells and $CD4^+CD25^+CD127^{low+}$TGF-$\beta^+$ Treg cells in children are naïve phenotype (CD45RA) | • TGF-β does not regulate IFN-γ $^+$CD4-T cell to rotavirus in children.<br>• The naïve Treg profiles in children could result in their reduced immunomodulatory effects in response to rotavirus infection |
| Parra et al., 2014 [31] | Vaccine ($n$ = 3) | $CD62L^-$ $CD45RA^{+/-}$ and $CD26L^+CD45RA^-$ CD4 T-cells α4β7 and CCR9 | • Most of the rotavirus antigen VP6-7 $tetramer^+$ experienced CD4 T-cells expressed α4β7, or expressed both, α4β7 and | • Majority rotavirus CD4 T-cells are gut homing<br>• Generation of these T-cell gut homing phenotypes may be important for clearing rotavirus infection and protecting against re-infection |
| Rojas et al., 2003 [32] | RV-AGE ($n$ = 9) | Frequencies of CD4 and CD8 T-cells producing IL-4 and IFN-γ | • Detectable INF-γ CD8 ($p$ = 0.018) but not INF-γ CD4 ($p$ = 0.17) T-cells. IL-4 CD4 and IL-4 CD8 also not detected ($p$ = 0.15). | • INF-$\gamma^+$ cytotoxic CD8 T-cells may be more important for initial clearance of infection than the CD4 T-cell subset |
| Rott et al., 1997 [33] | convalescing RV-AGE ($n$ = 1) | T-cell proliferation | • α4β$7^{hi}$ blood lymphocytes showed a 2.6-fold greater proliferative response to rotavirus than α4β$7^-$ cells (SI 4.07 versus 1.54 respectively) | • Majority of rotavirus T-cell have α4β$7^{hi}$ phenotype |

Abbreviations: α4β7 = alpha 4 beta 7. CCR9 = C-C motif chemokine receptor 9. CD4 = cluster of differentiation 4. CD8 = cluster of differentiation 8. CD25 = cluster of differentiation 25. CD45RA = cluster of differentiation 45RA. CD62L = cluster of differentiation 62L. CD69 = cluster of differentiation 69. CD83 = cluster of differentiation 83. CD127 = cluster of differentiation 127. GRO-β = growth-regulated oncogene. IFN-α = interferon alpha. IFN- β = interferon beta. IFN-γ = interferon gamma. IL-1β = interleukin 1β. IL-1R = interleukin 1R. pro-IL-1β = precursor interleukin 1β. IL-4 = interleukin 4. IL-6 = interleukin 6. IL-8 = interleukin 8. IL-10 = interleukin 10. IL-17 = interleukin 17. mRNA = messenger ribonucleic acid. $p$ = probability value. PBMC = peripheral blood mononuclear cells. $r$ = Pearson's correlation coefficient. RV-AGE = rotavirus acute gastroenteritis. Th1 = T-helper type 1. Th2 = T-helper type 2. Th17 = T-helper type 17. T1D = type 1 diabetes. TNF-α = tumor necrosis factor alpha. Treg = regulatory T-cell. TGF-β = transforming growth factor beta.

## 4. Discussion

We provide an overview of the evidence and characteristics of T-cell immune responses to rotavirus in healthy, rotavirus infected, and vaccinated children. Although many research studies have been done, very few of them specifically address T-cell mediated immunity to rotavirus in children. We found only seventeen articles to include in this review.

### *4.1. Summary Findings and Implications*

The majority of studies identified were within the context of rotavirus infection and only two studies assessed T-cell responses in relation to rotavirus vaccination. This is particularly surprising considering the continued development and introduction of new rotavirus vaccines [6,38] and the fact that immune correlates of protection for rotavirus vaccines remain elusive to date [7]. Additionally, the least number of studies were conducted in African children which is of concern as this region bears the highest burden

of rotavirus diarrhea [3] and rotavirus vaccines within this region consistently exhibit diminished performance [17]. These findings highlight the gap in research elucidating the role of T-cell mediated immunity to rotavirus to explore their potential as immune correlates of vaccine protection and the need for a better understanding of rotavirus immune mechanisms. Such research would particularly help understand the reduced vaccine immunogenicity in African children.

T-cell immunity does play a role in the immune response to rotavirus in children. Lymphoproliferative assays provided evidence of circulating rotavirus-specific T-cells in children. The lack of proliferation observed in newborns, minimal proliferation in infants <1-year-old, and increasing proliferation with age are consistent with the exposure pattern to rotavirus in early life. However, the minimal rotavirus-specific T-cell proliferation in children aged below 1 year of age is of concern as rotavirus vaccines are administered within this period and vulnerability to rotavirus is highest in early infancy. While transplacental maternal antibody immunity is most probably important for protection in this age group, it may be necessary for new rotavirus vaccine formulations to incorporate designs allowing for enhanced T-cell activation such as the addition of adjuvants. Interestingly, evidence of rotavirus T-cell proliferation is also seen in some newborns that could be a result of in-utero or very early exposure to rotavirus antigens and is of significance for neonatal rotavirus vaccines strategies. Rotavirus vaccines administered at birth have been developed and found to be safe and highly efficacious in newborns. This birth dose vaccination could potentially impart rotavirus-specific memory T-cells thus providing an opportunity for cell-mediated protection very early on in life [39]. This early protection would have a considerable impact on further reduction of rotavirus burden in low-income countries where a sizeable proportion of children are infected with rotavirus before receipt of the first vaccine dose that has been associated with poor vaccine seroconversion [11,40].

Broadening of cross-reactive T-cells with increasing age is consistent with exposure to different rotavirus strains as children age. These results further implied that rotavirus-specific T-cells recognize epitopes shared by different infecting rotavirus serotypes indicating that T-cell immunity can provide cross-reactive protection. Rotavirus has a large strain diversity based on varying combinations of G- and P-serotypes and genotypes classified by antibody reactivity to VP7 and VP4 viral proteins respectively [8]. Rotavirus strains that cause infections in humans and commonly infect children aged <5 years are well known but evolutionary genetic mutation and reassortments eventually give rise to new strains [41]. This observed T-cell proliferation irrespective of infecting G-serotype suggests that rotavirus induced T-cell immunity in children is not G-serotype specific which is important for effective vaccine strategies. For instance, Rotarix, a monovalent G1P [8] rotavirus vaccine has shown protection against non-vaccine serotype rotavirus strains, however, vaccine strain breakthrough still occurs and the extent to which this cross-reactive immunity is mediated by T-cells or antibody responses is unclear and needs further investigation [42]. Total circulating antibody and homotypic and heterotypic neutralizing antibodies are associated but not entirely correlated with protection, which has suggested that other immune mechanisms like these cross-reactive T-cells are likely at play [7].

The available literature shows that both memory B and T-cell immunity are developed after rotavirus exposure with T-cell responses occurring in tight association with the antibody response. This review revealed more frequently detected T-cell responses in children that were seropositive than those seronegative for rotavirus-specific antibodies as well as in secondary versus primary infections. However, the antibody response is more persistent and due to the more transient nature of the T-cell response, T-cell immunity detected in children most likely reflects previous rather than active exposure. Therefore, in infants, T-cell immunity may be more useful as a measure of child-specific immune memory and in early infancy to discriminate from passively acquired maternal immune memory in response to infection. Additionally, in the context of vaccination, detection within shorter time periods post-vaccination would be required in the assessment of these

effector T-cell responses. Nevertheless, the detection of both T-cells and antibody responses is necessary to adequately describe the immune response to rotavirus in children infection.

Evidence of T-cell proliferation in the absence of increasing antibody titers in some children speaks towards the existence of anti-rotavirus protection mediated via a direct T-cell immune effector in children. The direct effector contribution of T-cells has been shown in murine model depletion and adoptive studies where depletion of CD8 T-cells resulted in the delayed rate of resolution of rotavirus infection, CD4 T-cell depletion was associated with chronic viral shedding and complete loss of protection [14], and adoptive transfer of rotavirus primed CD4 and CD8 T-cells resulted in shorter rotavirus shedding [43]. In such murine studies, a significant loss of protection against rotavirus has also been observed in T-cell deficient and T-cell receptor (TCR) knockout mice with the delayed resolution of rotavirus infection attributed to the depletion of the $CD4^+$ T-cell subset, while B-cell and TCR deficient mice remained protected [15]. In this review, direct effects of T-cell immunity were exemplified by the impaired rotavirus antibody response, chronic viral shedding, and inability to clear infection observed in T-cell immunodeficient children. In the context of vaccination, it is plausible that lowered antibody responses detected in non-seroconverting children based on fold change in antibody response may not entirely imply reduced protection as T-cell immunity may provide direct protective and immune memory functions. The contribution of T-cell immune memory in the measurement of vaccine immunogenicity may have implications for measures of vaccine efficacy.

The positive association between higher rotavirus CD4 T-helper cell response and rotavirus seropositivity or higher neutralizing IgG in children highlights the particular importance of indirect protection offered via the CD4 T-cell helper function in the production of the antibody response. In adoptive transfer murine models, rotavirus primed CD4 T-cells and not CD8 T-cells are associated with increased production and maintenance of secretory IgA that is important in mucosal immunity, and both serum IgA and IgG are currently recognized as valuable surrogate endpoints for protection [12]. Therefore, taking this into account, in regions of poor rotavirus vaccine performance, there is a need for elucidating detailed profiles of these CD4 T-cells in relation to the magnitude and neutralizing ability of the antibody responses among vaccinated children. Magnitude and maintenance of antibody response may be reliant on characteristics of the elicited CD4 T-cell response. Such T-cell studies may provide useful insights for the observed lower vaccine immunogenicity and effectiveness trends in these regions.

In children, these characteristics of CD4 and CD8 T-cell responses to rotavirus include predominantly Th1 but also Th17 responses. Activated CD4 and CD8 T-cells secreting proinflammatory cytokines particularly IFN-$\gamma$ and IL-17 appear important in this immune response. IFN-$\gamma$ cytokine has direct anti-viral effects and IL-17 is associated with the provision of protection via recruitment of other immune cells with both cytokines shown to be important in the clearance of rotavirus infection [44]. On the other hand, regulatory T-cells which may suppress the proinflammatory immune response in efforts to maintain homeostasis also occur in response to rotavirus. The regulatory T-cells can have a negative or positive influence on the immune response to rotavirus infection or vaccination. This review revealed $IL10^+$ and $FOXP3^+$ regulatory T-cells as distinct subpopulations with opposing effects on rotavirus antibody immunity. In this context, a distinct population of $CD4^+/CD8a^+$ $CCR6^+CXCR6^+$ Treg cells has been identified in the human colon, which responds to fecal bacterial species and produces IL-10 [45]. These cells could indeed drive distinct outcomes during rotavirus infection compared to their $FOXP3^+$ Treg counterparts. For live attenuated rotavirus vaccines, assessing these Th1 and Th17 inflammatory and $FOXP3^+$ and $IL\text{-}10^+$ regulatory T-cell profiles in children may provide insights into the observed vaccine immunogenicity.

In addition to these conventionally studied CD4 and CD8 T-cell subsets, recently identified innate-like T-cells such as the gamma delta T-cell ($\gamma\delta$T), mucosal-associated invariant T-cells (MAIT), and natural killer T-cells (NKT) are enriched in mucosal tissues and have been reported to provide protective effector activities against human intestinal

infections. Through direct cytokine action or indirectly via recruitment of other immune effector cells cytokine responses, these innate-like T-cells have been suggested to provide early antiviral immune protection in the interface between innate immunity and induction of adaptive immunity and have been associated with inhibited viral replication of important human viral pathogens [46,47]. There is an urgent need to also consider the characterization of these atypical T-cell profiles and how they relate to conventional CD4 and CD8 T-cell subsets in relation to observed rotavirus infection or vaccine immunogenicity.

Circulating rotavirus-specific T-cells in children are generally low in frequency during the acute than convalescent phase and much weaker than those generated in adults and against other pathogens. The lowered frequency of rotavirus-specific T-cells in the initial response may be a direct consequence of their migration from circulation to gut mucosal priming sites to carry out effector function. This is supported by literature documenting higher T-cell proliferation within $\alpha 4\beta 7^{hi}$ subset and a higher proportion of CD4 T-cells responding to rotavirus expressing $\alpha 4\beta 7$ or CCR9 gut homing markers. Current live attenuated oral rotavirus vaccines aim to mimic natural infection immune priming within the gut. The extent to which such vaccines elicit these gut homing effector T-cell phenotypes may relate to the protective effect of vaccination. With new parenterally administered rotavirus vaccines being introduced, their ability to elicit these gut homing phenotypes must also be studied. While murine models have documented the development of mucosal immunity from parenteral vaccination [48], the generation of gut homing rotavirus specific T-cells in children vaccinated with parenteral rotavirus vaccine remains to be determined although an observed reduction in viral shedding in clinical trials conducted thus far has implied generation of local mucosal effectors [49]. It will, therefore, be important to conduct studies assessing the homing phenotypes elicited by rotavirus vaccination which may influence effector abilities in the protection against rotavirus at the gut.

When compared to tuberculin, tetanus toxoid, and influenza-derived antigens for which childhood vaccines are also administered, the T-cell responses induced by rotavirus antigen were observed to be diminished. Reasons for such variations in antigen-specific responses in early life can include immune dysfunction in antigen-specific presentation and differences in antigen-specific T-cell activation, proliferation, and effector versus memory generating functions. A better understanding of these T-cell phenotypes responding to rotavirus in this context has the potential to be exploited for improved immunity [50]. Considering the role of T-cell phenotypes in the child's immune response to infection or vaccinations, it should be important when assessing immune responses in children to account for pathogens that have a strong modulatory effect on these T-cell populations. For instance, cytomegalovirus, a ubiquitous pathogen, and potent T-cell modulator have been shown to influence immune and vaccine-induced T-cell profiles in children [51,52] but data is unavailable on its modulatory effect on anti-rotavirus T-cell immunity in children.

### *4.2. Strengths and Limitations*

To the best of our knowledge, this is the first systematic review of the T-cell response to rotavirus in children using a clearly defined search and screening strategy to obtain existing literature. Our review gives an overview of research done prior to and post introduction of rotavirus vaccines and provides evidence supporting the need for more research on T-cell mediated immunity in children not only as it relates to infection but also vaccination. This review provides current knowledge in the literature on different subsets and characteristics of T-cells response to rotavirus encompassing general proliferation, specific phenotypes, functional cytokine secretion, and migratory profiles. The review also covered the relationship of T-cell responses to widely studied antibody responses.

Limitations in this review primarily arose from the nature of the studies identified. A substantial proportion of studies, particularly those conducted earlier, reported lymphoproliferative activity as an indication of T-cell immunity. However, caution must be taken in their interpretation as the detected proliferation potentially includes that of innate and B-cells. Lymphoproliferative-based measures, while giving insights to T-cell immu-

nity, do not provide specific T-cell immune data in comparison to current more advanced techniques such as multicolor flow cytometry. Additionally, aside from four studies, the majority were conducted within the last decade and as such did not utilize more recent immunological methods such as higher cell marker parameter flow cytometry to provide more comprehensive T-cell knowledge.

Another limitation is that the studies identified used a diverse range of immune stimulants to assess the rotavirus T-cell responses which included different rotavirus strains or mitogens and had variations in reporting format for the T-cell outputs. This introduced large methodological heterogeneity that presented a major challenge in the quantitative synthesis of the evidence that was provided. Additionally, there was a lack of sufficient reporting of statistical data in several studies and more so in studies conducted much earlier on, and for some studies, sample sizes were very small making generalization of findings difficult.

## 5. Conclusions

T-cells clearly have a role to play in the immune response to rotavirus in children. This review shows that these responses are heterotypic and although present at low circulating levels and less persistent than antibodies, can be detected in children and develop through repeated exposure. Both CD8 and CD4 T-cell subsets are involved in this response and are primarily of a Th1 and gut homing phenotype. However, there is a paucity of T-cells studies, wide methodological differences, and a lack of sufficient quantitative data sets directly associating T-cell immunity to protection from rotavirus infection or in relation to immunogenicity of rotavirus vaccines. Thus, it is imperative that further research be done investigating T-cell responses against rotavirus and the standardization of rotavirus-specific T-cells assays is needed in this population.

Africa bears a disproportionate burden of rotavirus diarrheal disease and has an urgent need for research in this area. Such studies may also establish whether the observed lower vaccine-induced anti-rotavirus antibodies in African children could be attributed to limited or impaired T-cell responses. There is also a need to address innate-like T-cell subsets and the inclusion of more phenotypic markers using more developed immunological assays to provide comprehensive T-cell immunology data. In rotavirus vaccinology, it will be important to assess T-cell immunity relationship to seroconversion rates and clinical protection against rotavirus infection. Such research could form a good basis for further exploration of T-cells as a potential immune correlate of protection and inform the development of next-generation vaccines.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/v14030459/s1, Table S1: PRISMA checklist, File S1: Search strategy example, Table S2: Quality assessment tool.

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# Evaluation of ROTARIX® Booster Dose Vaccination at 9 Months for Safety and Enhanced Anti-Rotavirus Immunity in Zambian Children: A Randomised Controlled Trial

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**Abstract:** Oral rotavirus vaccines show diminished immunogenicity in low-resource settings where rotavirus burden is highest. This study assessed the safety and immune boosting effect of a third dose of oral ROTARIX® (GlaxoSmithKline) vaccine administered at 9 months of age. A total of 214 infants aged 6 to 12 weeks were randomised to receive two doses of ROTARIX® as per standard schedule with other routine vaccinations or an additional third dose of ROTARIX® administered at 9 months old concomitantly with measles/rubella vaccination. Plasma collected pre-vaccination, 1 month after first- and second-dose vaccination, at 9 months old before receipt of third ROTARIX® dose and/or measles/rubella vaccination, and at 12 months old were assayed for rotavirus-specific IgA (RV-IgA). Geometric mean RV-IgA at 12 months of age and the incidence of clinical adverse events 1 month following administration of the third dose of ROTARIX® among infants in the intervention arm were compared between infants in the two arms. We found no significant difference in RV-IgA titres at 12 months between the two arms. Our findings showed that rotavirus vaccines are immunogenic in Zambian infants but with modest vaccine seroconversion rates in low-income settings. Importantly, however, a third dose of oral ROTARIX® vaccine was shown to be safe when administered concomitantly with measles/rubella vaccine at 9 months of age in Zambia. This speaks to opportunities for enhancing rotavirus vaccine immunity within feasible schedules in the national immunization program.

**Keywords:** rotavirus; ROTARIX®; vaccine; safety; booster dose; immunogenicity; Zambia; Africa

## 1. Introduction

Diarrhoeal disease is ranked third among the global leading causes of morbidity and mortality in young children, responsible for approximately 1.53 million deaths and contributing to over 80 million disability-adjusted life years, most of which occur within

Sub-Saharan African children aged below 5 years [1]. Among several infectious aetiologies of diarrhoeal disease, rotavirus is the most common cause of moderate to severe and less severe diarrhoea [2,3] and the leading cause of diarrhoeal disease-associated mortality that has been attributed to 128,515 deaths in a single year in this population [4].

The orally administered and widely introduced rotavirus vaccines ROTARIX® (GlaxoSmithKline) and RotaTeq® (Merck) have proved important early life interventions in mitigating the diarrhoeal disease burden in this population, with substantial reductions in rotavirus-associated and all-cause diarrhoea morbidity and mortality observed since their introduction [5]. However, vaccine immunogenicity and efficacy when administered in early infancy is consistently lower and variable in highly burdened and high mortality settings with several reasons postulated [6,7]. Improved vaccine performance is an important way in which rotavirus infections that occur even among vaccinated infants can be further prevented. For these oral rotavirus vaccines to provide maximal benefit in these settings, it is important to assess potential methods to enhance the immunogenicity of existing vaccines as their routine use continues.

Removal of the age restrictions for child vaccination and alternate schedules with booster doses of existing oral rotavirus vaccines have the potential to improve vaccine performance with benefits outweighing potential associated intussusception risks [8,9]. Modelling data predicts enhanced anti-rotavirus immunity from booster dose administration at 9 or 12 months of age and prevention of up to 19,600 additional rotavirus-associated deaths in the second year of life annually [10]. Administration of monovalent ROTARIX® and pentavalent RotaTeq® concomitantly with measles vaccine at 9 months has been demonstrated to induce significantly increased anti-rotavirus antibody titres without interference with measles seroresponses in Bangladeshi and Malian infants, respectively [11,12].

Zambia introduced the ROTARIX® vaccine in 2013 and recorded a seroconversion rate of 60.2% [13]. Although a significant decline in rotavirus-attributable childhood diarrhoea has been recorded especially in infants [14], it remains necessary to further reduce residual infection and disease burden. Newer oral rotavirus vaccines have been evaluated in our setting with similar low rates of vaccine seroconversion observed [15]. To date, no study has been conducted on safety and potential immunogenicity benefits of a booster ROTARIX® vaccine dose administered at 9 months of age in Zambian infants.

We aimed to assess a booster dose of ROTARIX® vaccine administered at 9 months of age as an alternative to the current two-dose schedule to enhance anti-rotavirus immunity in Zambian infants.

## 2. Materials and Methods

### *2.1. Study Design and Sample Size Calculation*

The study was a single-center, open-label, randomised, controlled trial assessing the safety and immunogenicity of a booster dose of the monovalent ROTARIX® vaccine at 9-month infant age. We anticipated a 15% or greater increase in log10 RV-IgA response after the booster ROTARIX® dose. Using a two-sample *t*-test and assuming equal SD at 5% level of significance, we therefore required a total of 196 infants (98 per arm) to detect an increase to 3.13 log10 RV-IgA due to the booster dose of ROTARIX® at an 80% power. We made an upward sample size adjustment of 9% to account for potential loss to follow-up to reach the total of 214 infants to be recruited in this study. The estimation was performed using Stata 14 MP "power" command (StataCorp™, College Station, TX, USA).

### *2.2. Study Participant Selection and Enrolment*

The study enrolled 214 infants aged 6 to 12 weeks from 13th September 2018 to 15th November 2018 at George Health Centre (GHC), a government-run peri-urban health facility serving a high-density, low-income population in Lusaka, Zambia. Mothers presenting with their infants for routine immunization visits were approached by study staff and sensitized about the study. Interested mothers were provided further study information at the clinical research site located within the GHC premises. Mothers that were willing

to participate were individually taken through an informed consent process and simple comprehension assessment test in private rooms. Eligibility criteria included that the infant was aged 6 weeks to 12 weeks old; the mother participated voluntarily, provided written informed consent (with a witness in the case of illiterate participant) and agreed to all study procedures; and the mother was resident in the study area and willing to come for scheduled visits for the duration of the study. Infants were not eligible if they had a contraindication to rotavirus vaccination; previously received rotavirus vaccination; had a recent history of immunosuppressive therapy; had a recent history of blood or blood product transfusion; existing congenital anomalies; or any condition deemed by the study investigator to pose potential harm to the participants or jeopardize the validity of study results.

### *2.3. Study Procedures and Randomization*

Enrolled mother-infant pairs were followed up until the infant was 36 months old. At baseline, eligible infants were randomised at a ratio of 1:1 using masked allocation into either the intervention arm to receive a booster dose of ROTARIX® concomitantly with measles/rubella (MR) vaccination or into the control arm to receive only MR vaccination at 9 months old. All children in the study received routinely administered first and second ROTARIX® vaccine doses (given from 6 weeks and ideally 4 weeks apart before the age of 2 years). ROTARIX® is an orally administered live, attenuated G1P [8] monovalent vaccine in routine use in Zambia. The batch number of ROTARIX® used in the study was AROLC044AA. Infants in both arms also received polio, Bacillus Calmette–Guérin (BCG), pentavalent diphtheria/pertussis/tetanus/Hepatitis B/*Haemophilus influenza*-type (DPT-HepB-Hib) and pneumococcal conjugate vaccines (PCV) according to the routine Zambian expanded immunization schedule.

Baseline sociodemographic and clinical data were collected from the participating mother/infant pairs. From all enrolled infants, whole blood samples (3–4 mL) were collected before receipt of the first ROTARIX® dose, 1 month after two-dose ROTARIX® vaccination, before receipt of MR vaccine (control arm) or MR and booster dose ROTARIX® at 9 months of age and when 12 months old. In a subset of infants, additional blood sampling was performed within 1 month after the first ROTARIX® dose. From baseline to the time the infant was 36 months old, anthropometric growth measurements were taken and data on incidence of clinical illness were recorded.

### *2.4. Immunogenicity Assessment*

Plasma from whole blood samples was tested for anti-rotavirus immunoglobulin A (RV-IgA) titres using an adaptation of a published and validated sandwich enzyme-linked immunosorbent assay (ELISA) based on the use of WC3 rotavirus antigen and mock infected African green monkey kidney (MA104) cell lysate [16]. All plasma testing for RV-IgA was performed at the Centre for Infectious Disease Research in Zambia Enteric Disease and Vaccine Research Laboratory in Lusaka, Zambia. In-house-generated pooled plasma from rotavirus-vaccinated adults was validated for use as the standard in the ELISA assay using pooled serum with known assigned RV-IgA units per millilitre (U/mL). The primary immunogenicity endpoint was the geometric mean titre of anti-rotavirus IgA at 12 months of age. The study also investigated RV-IgA seropositivity and vaccine seroconversion using published definitions. Seropositivity was defined as an RV-IgA titre $\geq$ 20 U/mL. Seroconversion was defined as a four-fold or greater change in RV-IgA titre 1 month after dose two if pre-vaccination titre was <20 U/mL [13].

### *2.5. Safety Assessment*

All enrolled infants received ROTARIX® vaccination together with other routine vaccines as per the Zambian immunization schedule. Prior to vaccination, all participants were screened for any medical condition. Following vaccination, all infants were reviewed by the study staff to identify any immediate adverse events (AE). Participant mothers or

guardians were provided with and trained in completing a post-vaccination diary card to record presence or absence of solicited AE including fever, diarrhoea, vomiting, loss of appetite and irritability over the next 5 days following immunization, which was returned to the study clinic at the next study visit. Mothers were also encouraged to bring the infant to the study clinic whenever the child was unwell, at which point standard of care was given and the presenting AE was recorded using structured case report forms. For the AE, information collected included but was not limited to the presenting symptoms, evolution of the presentation of symptoms, examination findings, investigations and drugs given (dosage, route and duration). In the case of serious adverse events (SAE), every effort was made to make physical contact and access the medical records in the admitting health facility. For both AE and SAE, the infants were followed up until resolution whilst offering the necessary standard medical care. Once resolved, the study participant documents were updated accordingly and where required, the local authorities were updated accordingly as per regulatory guidelines. All SAE were also reviewed at regular intervals by the study Data Safety and Monitoring Board (DSMB) comprised of clinicians from the study, those independent of the study and reported to the relevant national regulatory authorities. During routine scheduled study visits to the clinic, mothers were also specifically asked about diarrhoea occurrence and any other illnesses that the infant may have had in the period preceding the visit. All stool samples passively collected from children presenting with diarrhoeal disease during clinic visits were tested for rotavirus. Genotyping was performed on all rotavirus-positive stool samples to determine infecting strains. We documented and described the incidence of clinical AE and SAE within a month following administration of the third dose ROTARIX® + MR and MR alone in infants in the intervention and control arm respectively as the primary measure on safety.

### *2.6. Statistical Analysis*

For the immunogenicity analysis, the characteristics of participating infants at 9-month follow-up were tabulated for each arm. Analysis was based on the intention-to-treat population. In the primary analysis, we used two-sample t-test to test the difference in RV-IgA titre at 12-month infant age between the two arms. Linear regression model on log-transformed RV-IgA titre at 12-month infant age was used to estimate the geometric mean ratio and 95% confidence interval (CI), adjusted for potential confounders. *p*-values were considered significant at 5%. For the safety analysis, AE, and SAE incidence within 1 month after receipt of booster ROTARIX® dose and MR vaccine or MR vaccine alone were tabulated for each arm and 95% CI was calculated for the proportion of infants with any AE or SAE in each arm. All analyses were performed in Stata 17 MP (StataCorp, College Station, TX, USA) and R-Software.

## 3. Results

### *3.1. Participant Enrolments and Baseline Characteristics*

As summarised in Figure 1, the study enrolled and randomised 214 infants between 13th September 2018 and 15th November 2018. Pre-vaccination whole blood was obtained from 211/214 (98.6%) enrolled infants at baseline. 170/214 (88/170 in intervention and 82/170 in control arm) infants had a clinic visit 28 days after their second dose of ROTARIX®. A total of 168 out of 214 (78.5%) infants attended and gave a whole blood sample at their 9-month-age study visit of which 88/168 (52.4%) infants were in the intervention (ROTARIX® + MR vaccination) arm and 80/168 (47.6%) infants in the control (MR vaccination) arm. Of these, 159/168 (94.6.2%), of which 85/159 (53.5%) and 74/159 (46.5%) were in the intervention and control arm, respectively, also attended and gave a whole blood sample at their 12-months-of-age study visit. Infants that had 9- and 12-months-of-age whole blood samples collected were included in the final analysis, whereas others were not included due to dropouts caused by mother's relocation from study site, withdrawal of consent, non-study related infant deaths, and losses to follow-up of participating mothers during follow-up period.

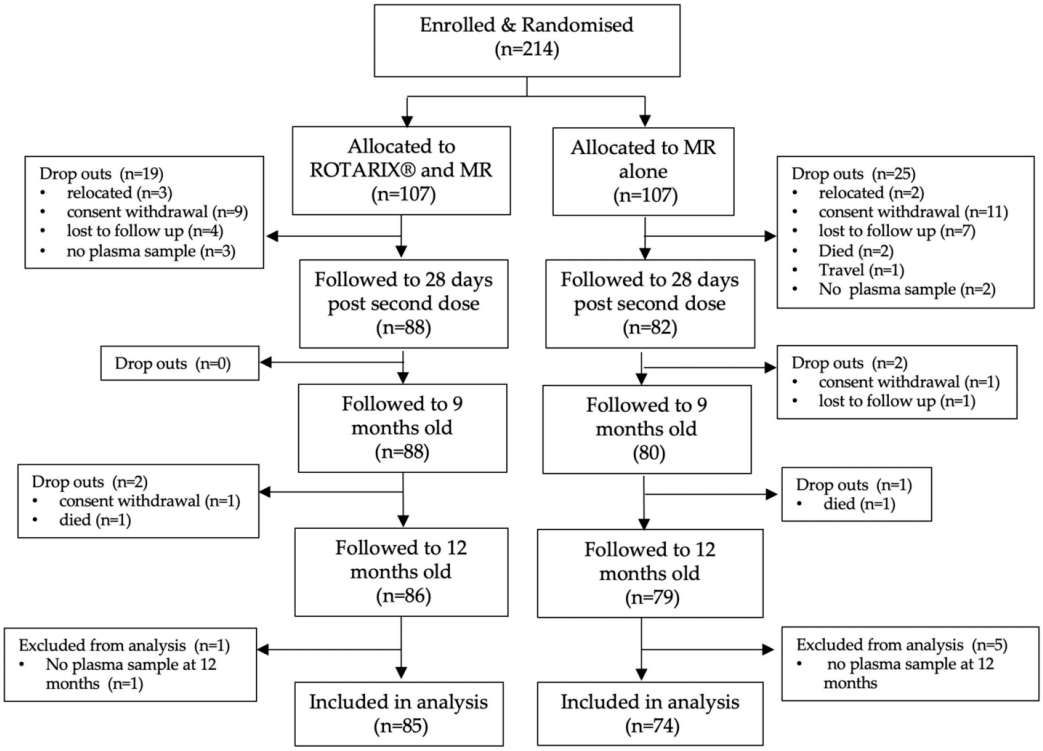


**Figure 1.** Study participant flow chart. Abbreviations: MR (measles/rubella vaccine).

As outlined in Table 1, infants were from low-income households with poor water sanitation and hygiene (WASH). The majority of the infants were from households with shared toilet facilities and using public water sources. Infants at enrollment had a median age of 6 weeks, the majority were HIV unexposed, full-term with normal weight at birth, generally healthy and mostly breastfed. The RV-IgA seropositivity (RV-IgA titre $\geq$ 20 U/mL) rate was low at baseline at 4.8% overall and 3.5% and 6.3% in the intervention and control arms, respectively. There was no statistically significant difference in these baseline characteristics between the two study arms.

**Table 1.** Baseline characteristics of mother/infant pairs by trial arm.

| | **Total Population (N = 168 [a])** | **ROTARIX® + MR (n = 88)** | **MR (n = 80)** |
|---|---|---|---|
| | **n (%)** | **n (%)** | **n (%)** |
| **Infant Characteristic** | | | |
| **Age, weeks** | | | |
| Median (IQR) | 6 (6–6) | 6 (6–6) | 6 (6–6) |
| **Sex** | | | |
| Male | 89 (53.0) | 38 (43.2) | 41 (51.3) |
| Female | 79 (47.0) | 50 (56.8) | 39 (48.8) |
| **Gestation** | | | |
| Pre-term | 11 (6.6) | 5 (5.7) | 6 (7.5) |
| Full-term | 157 (93.5) | 83 (94.3) | 74 (92.5) |

**Table 1.** *Cont.*

| | Total Population (N = 168 [a]) | ROTARIX® + MR (n = 88) | MR (n = 80) |
|---|---|---|---|
| | n (%) | n (%) | n (%) |
| **Mode of Delivery** | | | |
| Vaginal | 160 (95.2) | 84 (95.5) | 76 (95.0) |
| Caesarean | 8 (4.8) | 4 (4.6) | 4 (5.0) |
| **Feeding** | | | |
| Expressed/direct Breastmilk | 158 (94.1) | 83 (94.3) | 75 (93.8) |
| Mixed breast and formula | 10 (6.0) | 5 (5.7) | 5 (6.3) |
| **Birth weight, kg (N = 167)** | | | |
| <2.5 | 11 (6.6) | 3 (3.5) | 8 (10) |
| ≥2.5 | 156 (93.4) | 84 (96.6) | 72 (90.0) |
| **Weight at enrolment, kg** | | | |
| Mean (SD) | 4.6 (0.6) | 4.6 (0.6) | 4.7 (0.7) |
| **Length at enrolment, cm** | | | |
| Median mean (SD) | 54 (2.6) | 54 (2.7) | 54 (2.6) |
| **Malnourished (WLZ < −2) (N = 167)** | | | |
| No | 164 (98.2) | 85 (97.7) | 79 (98.8) |
| Yes | 3 (1.8) | 2 (2.3) | 1 (1.3) |
| **Stunting (LAZ < −2)** | | | |
| No | 138 (82.1) | 70 (79.6) | 68 (85.0) |
| Yes | 30 (17.9) | 18 (20.5) | 12 (15.0) |
| **Wasting (WAZ < −2)** | | | |
| No | 153 (91.1) | 79 (89.8) | 74 (92.5) |
| Yes | 15 (8.9) | 9 (10.2) | 6 (7.5) |
| **HIV exposure** | | | |
| Unexposed | 119 (70.8) | 60 (68.2) | 59 (73.8) |
| Exposed | 49 (29.2) | 28 (30.8) | 21 (26.3) |
| **RV-IgA seropositive (N = 166)** | | | |
| No | 158 (95.2) | 84 (96.6) | 74 (93.7) |
| Yes | 8 (4.8) | 3 (3.5) | 5 (6.3) |
| **Maternal characteristics** | | | |
| **Age, years** | | | |
| <20 | 23 (13.7) | 10 (11.4) | 13 (16.3) |
| 20–24 | 53 (31.6) | 29 (33.0) | 24 (30.0) |
| 25–29 | 51 (30.4) | 27 (20.7) | 24 (30.0) |
| ≥30 | 41 (24.4) | 22 (25.0) | 19 (23.8) |
| **Parity** | | | |
| Low parity (1–2) | 98 (58.3) | 50 (56.8) | 48 (60.0) |
| Multiparity (3–4) | 54 (32.1) | 27 (30.7) | 27 (33.8) |
| Grand multiparity (5+) | 16 (9.5) | 11 (12.5) | 5 (6.3) |
| **Education level** | | | |
| No education | 6 (3.6) | 5 (5.7) | 1 (1.3) |
| Some/complete primary | 55 (32.7) | 29 (33.0) | 26 (32.5) |
| Some/complete secondary | 102 (60.7) | 52 (59.1) | 50 (62.5) |
| Attended/completed university | 5 (3.0) | 2 (2.3) | 3 (3.8) |
| **Monthly household income, ZMW** | | | |
| <500 | 64 (38.3) | 35 (39.8) | 29 (36.7) |
| 500–1000 | 49 (29.3) | 25 (28.4) | 24 (30.4) |
| >1000 | 54 (32.3) | 28 (31.8) | 26 (32.9) |
| **Share toilet facilities** | | | |
| No | 33 (19.6) | 23 (26.1) | 10 (12.5) |
| Yes | 135 (80.4) | 65 (73.9) | 70 (87.5) |

**Table 1.** *Cont.*

| | Total Population (N = 168 [a]) | ROTARIX® + MR (n = 88) | MR (n = 80) |
|---|---|---|---|
| | n (%) | n (%) | n (%) |
| **Source of water** | | | |
| Public tap/pipe | 93 (55.4) | 45 (51.1) | 48 (60.0) |
| Piped into house/yard | 33 (37.5) | 33 (37.5) | 26 (32.5) |
| Yard/public borehole | 8 (4.8) | 3 (3.4) | 5 (6.3) |
| Protected/unprotected well | 8 (4.8) | 7 (8.0) | 1 (1.3) |

[a] Infants that attended the 9-month visit. Abbreviations: cm (centimeter); HAZ (height-for-age Z-score); HIV (human immunodeficiency virus); IQR (interquartile range); kg (kilogram); MR (measles/rubella vaccine); RV-IgA (rotavirus-specific immunoglobulin A); WAZ (weight-for-age Z-score); WLZ (weight-for-length Z-score); ZMW (Zambian Kwacha).

### *3.2. Seroconversion Rates and Anti-Rotavirus IgA Titres in Two-Dose and Booster Dose ROTARIX® Vaccinated Infants*

As shown in Figure 2, pre-vaccination mean RV-IgA antibody titres were low in the infants but increased after each ROTARIX® vaccine dose. Statistically significant increases in mean RV-IgA titres were observed between baseline and 1 month after the first dose of ROTARIX® in both the control arm ($p = 0.046$) and intervention arm (0.012). However, this increase was less apparent between the first and second doses for both control ($p = 0.447$) and intervention arms ($p = 0.068$). Interestingly, after two-dose vaccination, significant increases in RV-IgA titres in the control ($p = 0.001$) and intervention arms ($p < 0.001$) were observed by 9 months of age. Similarly, a significant increase ($p < 0.001$) in RV-IgA titres was seen in both arms by 12 months of age.

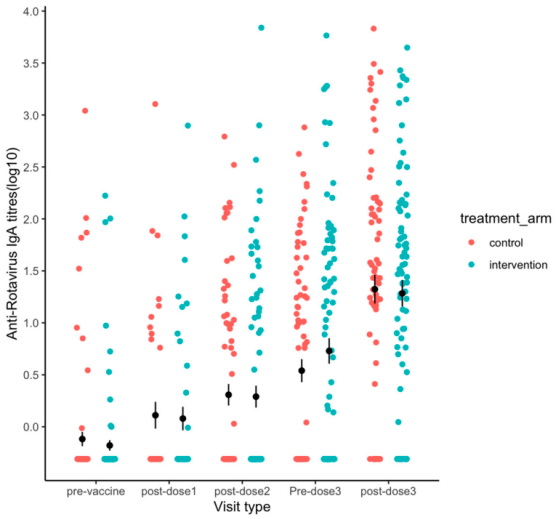


**Figure 2.** Trends in rotavirus-specific immunoglobulin A (RV-IgA) titres pre and post rotavirus vaccination compared between the control (red circle) and intervention (blue circle) arms. Each circle represents an infant's log10 RV-IgA titre. Black circle represents mean and standard error of log RV-IgA titre.

In general, mean RV-IgA antibody titres were similar in the intervention and control arms at baseline ($p = 0.06$), 1 month after the first dose ($p = 0.944$) and 1 month after the second dose ($p = 0.644$). Similarly, mean RV-IgA titres in the two arms at 9 months old were

not significantly different ($p = 0.207$), but the mean RV-IgA titres at 9 months old showed a higher trend among infants in the intervention arm. At 12 months old, the difference in mean RV-IgA titres between the control and intervention arms did not reach statistical significance ($p = 0.688$).

Vaccine seroconversion approximately 1 month after two-dose ROTARIX® was low in this study population with 47/169 (27.8%) infants seroconverting, of which 25/47 (53.2%) were from the intervention arm and 22/47 (46.8%) from the control arm.

### *3.3. Effect of Booster Dose ROTARIX® at 9 Months on Anti-Rotavirus IgA Geometric Mean Titres at 12 Months of Age*

We observed no statistically significant differences in RV-IgA GMT ratios at 12 months of age between infants that received the third ROTARIX® vaccine dose and those that did not (Table 2).

**Table 2.** Rotavirus IgA geometric titre mean ratio at 12 months by study arm.

| Arm | N (% of Total) | GMT (95% CI) | Two-Sample *t*-Test, *p*-Value | GMT Ratio (95% CI) | *p*-Value | Adjusted GMT Ratio * (95% CI) | *p*-Value |
|---|---|---|---|---|---|---|---|
| MR | 74 (46.5) | 3.98 (3.50–4.51) | | 1 | 0.689 | 1 | 0.223 |
| ROTARIX + MR | 85 (53.5) | 3.85 (3.41–4.35) | 0.688 | 0.84 (0.35–2.00) | | 0.61 (0.27–1.35) | |

* Adjusted for malnutrition, sex, water source, income, pre-dose three RV-IgA titres using linear regression on log-transformed titres. Abbreviations: MR (measles/rubella vaccine); GMT (geometric mean titre).

### *3.4. Safety: Incidence of Adverse Events and Serious Adverse Events by Trial Arm*

Primary safety assessment was conducted on infants who successfully attended the 9-months-of-age study visit and remained in follow-up 1 month thereafter. In these infants, respiratory tract illness (RTI) was the most common AE, followed by diarrhoeal disease with comparable incidence between the intervention and control arms (Table 3). Other AEs observed included conjunctivitis, dermatitis, candidiasis, febrile illness, emesis and otitis with comparable incidences between the two arms (Table 3). Out of 76 stool samples that were passively collected from infants presenting with diarrhoea during unscheduled visits, 4 (5.3%) tested positive for rotavirus. Genotyping of 3 out of the 4 stool samples that had sufficient volumes revealed two G3 and one G4 genotype. Of the G3 genotype infections, one was in an infant in the intervention arm and the other was an infant in the control arm. The G4 genotype was observed in an infant from the control arm.

**Table 3.** Incidence of adverse events within 1 month after third dose ROTARIX® (+MR) compared to MR vaccination.

| Arm | Diarrhoea (n), Incidence * (95% CI) | RTI (n), Incidence (95% CI) | Conjunctivitis (n), Incidence (95% CI) | Dermatitis (n), Incidence (95% CI) | Candidiasis (n), Incidence (95% CI) | Febrile Illness (n), Incidence (95% CI) | Emesis (n), Incidence (95% CI) | Otitis (n), Incidence (95% CI) |
|---|---|---|---|---|---|---|---|---|
| MR | 8 3.33 (1.7–6.7) | 12 5.0 (2.8–8.8) | 1 0.4 (0.05–3.0) | 0 | 1 0.4 (0.06–3.0) | 1 0.4(0.05–3.0) | 3 0.8 (0.2–3.3) | 1 0.4 (0.06–3.0) |
| ROTARIX + MR | 4 2.4 (0.6–4.0) | 8 3.0 (1.5–6.1) | 2 0.8 (0.2–3.0) | 3 1.1(0.2–1.8) | 1 0.4 (0.1–2.7) | 1 0.4 (0.05–2.7) | 1 0.4 (0.05–2.7) | 0 |
| Rate ratio (95% CI), *p*-value | 1.75 (0.14–1.51), 0.186 | 1.23 (0.25–1.48), 0.268 | 1.82 (0.17–20.05), 0.620 | - | 0.91 (0.06–14.53), 0.946 | 0.91 (0.06–14.5) 0.946 | 0.46 (0.04–5.01), 0.509 | - |

* Incidence per 1000 infant days. Abbreviations: CI (confidence interval); MR (measles/rubella vaccine); RTI (respiratory tract illness).

Throughout the three year study follow-up period, a total of 30 SAEs were recorded. Among these SAEs, 7/30 (23%) had acute gastroenteritis among the presenting symptoms. The study recorded four deaths among these SAE, of which three were in the control arm and one was in the intervention arm.

Only two SAEs, one within each arm, occurred within 1 month after the intervention at 9 months. The SAE recorded in the control arm was acute gastroenteritis with severe dehydration in severe anemia and failure to thrive. The SAE recorded in the intervention arm was acute gastroenteritis with severe dehydration. None of these SAEs recorded were related to the study (Table 4).

**Table 4.** Occurrence of serious adverse events in intervention (ROTARIX® +MR) compared to control (MR) arm.

| Arm | At Least One SAE, Incidence * (95% CI) | At Least One Related SAE, Incidence (95% CI) | Deaths |
|---|---|---|---|
| MR | 1<br>0.4 (0.06–3.0) | 0 | 3 |
| ROTARIX + MR | 1<br>0.4 (0.06–2.8) | 0 | 1 |
| Rate ratio, *p*-value | 0.94 (0.06–15.0),<br>0.9633 | | |

Abbreviations: CI (confidence interval); MR (measles/rubella vaccine); SAE (serious adverse event). * Incidence per 1000 infant days within 1 month after third dose ROTARIX®.

## 4. Discussion

In this clinical trial, we assessed the safety and immune boosting effects of a third dose of ROTARIX® vaccine administered at 9 months of age. This is the first clinical trial assessing administration of this oral rotavirus vaccine in Zambia outside of the recommended age range and our data show that a third dose of ROTARIX® given at 9 months of age in Zambian infants is well tolerated. Our results are consistent with studies conducted elsewhere, where no difference in AE and/or SAE frequency was observed between intervention and control arms [11,12].

We found no difference in geometric mean titres and ratios of anti-rotavirus IgA at 12 months of infant age in the intervention arm from a booster third dose of ROTARIX® vaccine given at 9 months compared to the control arm. This contrasts with findings from a study conducted in Mali where a three-fold or greater rise in RV-IgA and greater seropositivity rate 28 days after vaccination was seen among infants who received the booster dose of pentavalent ROTATEQ at 9 to 11 months of age (in addition to doses given at 6, 10 and 14 weeks of age) compared to those who did not. [12]. Another study in Bangladesh also observed an increase in RV-IgA seropositivity and geometric mean titres in infants given a booster dose of ROTARIX® at 9 to 10 months when immunogenicity outcome was assessed 2 months later. This was in comparison to infants that received measles/rubella vaccine alone in which no apparent changes were observed [11]. Both these studies made use of the same WC3 based ELISA methods as used in our current study.

A notable difference of these two studies with our study was that immunogenicity assessment was performed earlier at 1 month and 2 months after rotavirus booster vaccination, whilst our study measured the immunogenicity effect 3 months later. The peaking of RV-IgA tends to occur within 1 month after vaccination, and it is possible that the 3 month period in our study saw a waning of vaccine induced immune responses in the intervention arm such that by our outcome sampling timepoint RV-IgA levels became comparable to the control arm. We chose to assess boosting at 12 months of age as we believed the timepoint was close enough to detect a boosting effect and gave a window between blood sampling timepoints that reduced the frequency of blood draws.

Additionally, of note is the influence that natural rotavirus immunity may have on observed booster dose immunogenicity. The Malian study observed rise in RV-IgA seroresponses among infants who did not receive the additional ROTATEQ dose, suggesting

natural rotavirus exposure may have contributed to a rise in titres [12]. We observed similar increase in RV-IgA among infants who did not receive the third dose within the 3 months after intervention. This may indicate that infants in our study had exposure to wild-type infection and the exposure during the three-month period after intervention in our study may have factored into results observed between arms. In Mali, about half of the infants had RV-IgA titres below <20 U/mL (seronegative) prior to receiving the booster dose [12]. In Bangladesh, pre-boost RV-IgA seropositivity was ~52.7%; however, an improvement in boosting effect was observed among infants that were seronegative pre-boost. In our study, higher levels of RV-IgA titres relative to post-two-dose vaccination were apparent in infants at 9 months of age with slightly higher levels in the intervention arm though difference did not reach significance. These higher pre-boost titres in the intervention arm could perhaps have influenced responses observed in diminishing immunogenicity of the booster dose. Nevertheless, differences in population ages, time post-boost and vaccines assessed (monovalent versus pentavalent) could also play roles in these contrasting findings.

This study had the opportunity to investigate pre-vaccination seropositivity and vaccine seroconversion as secondary immune measures. We found minimal baseline rotavirus seropositivity and low post-ROTARIX®-vaccination seroconversion rate comparable to estimates reported in a study performed within a similar population in the same setting [15]. These findings show that while ROTARIX® vaccine is immunogenic among infants in our setting, the phenomenon of modest immunogenicity persists. Although our study was not designed to assess the protective effect of vaccination, rotavirus infections were present, and incidence of diarrhoea was among the commonly reported illnesses among vaccinated infants. Detected rotavirus infections were G3 and G4 non-vaccine strains. Whilst ROTARIX® is a monovalent vaccine containing G1P [8] strain protection against non-vaccine infecting strains has been shown [17]. Nevertheless, detection of non-vaccine strains of rotavirus infections among ROTARIX® vaccinated infants may reduce the effectiveness of these vaccines within our settings and speaks towards the need for vaccines covering multiple strains. Such findings in this study emphasize need for continued surveillance of circulating rotavirus strains including other viral, bacterial and parasitic enteric pathogens that may become important in the post-vaccine era.

Among the strengths of the study was the that it was a randomised control design and was conducted in a population in which rotavirus vaccines would be of most benefit. The local implementation of an ELISA method that is widely employed in other rotavirus vaccine trials elsewhere was another strength that enabled comparison of findings to other similar studies. Generally, there are limited studies assessing booster rotavirus vaccine doses at later ages in Africa and this study was the first to be performed in Zambia. Another strength was the ability in our study to demonstrate rotavirus immunity status of the children from pre-vaccination. Our study design enabled determination of pre-vaccination immune status and seroconversion rates after routine two-dose vaccination and accounting for this in our interpretations which was not done in the two studies conducted in Bangladesh and Mali [11,12]. This study design also allowed determination of seroresponses of the vaccine in different localities and sub-population but within the same setting of Zambia by comparison to that performed previously when vaccination was introduced [13].

Notable study limitations included the high losses to follow-up encountered early during the trial which may have reduced the power to detect the boosting effect of the third dose. We measured RV-IgA as an immunogenicity outcome, and, while being the most widely utilised measure for rotavirus vaccine immunogenicity, it is a sub-optimal correlate [18]. Measurement of other rotavirus-specific humoral and cellular immune responses to vaccination is necessary to further inform immunogenicity and potentially correlates of protection. We did not assess the potential impact of the third rotavirus vaccine dose on immunogenicity of the measles/rubella vaccine in our setting; however, studies conducted elsewhere have observed no influence of booster oral rotavirus vaccine given at this age on measles vaccine responses and attainment of sero-protection [11,12].

## 5. Conclusions

Despite showing evidence that ROTARIX® vaccine is well tolerated at 9 months of age, our study findings do not support improved immunogenicity by 12 months of age from a booster dose vaccination at this age in our study setting. However further research is needed to generate stronger clinical evidence for policymakers. Evaluation of alternative vaccine formulations for improved immunogenicity may be important in our setting to increased effectiveness and further reduce the burden of rotavirus.

**Author Contributions:** Conceptualization R.C., S.B. and M.S.; methodology, R.C., S.B. and M.S.; formal analysis, S.B. and M.C.; investigation, N.M.L., A.C., M.C.-C., N.S., C.C., R.V., K.N., C.M. and I.M.; writing—original draft preparation, N.M.L.; writing—review and editing, R.C., S.B., M.S., M.C., A.C., M.C.-C., N.S., C.C., R.V., K.N., C.M., I.M. and M.R.G.; visualization, S.B. and M.C.; funding acquisition R.C. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the University of Zambia Biomedical Research Ethics Committee (UNZ-ABREC Ref: 003-02-18). The study also received approval from the Zambia Medicines Regulatory Authority (ZAMRA Ref: CT 078) and the National Health Research Authority (NHRA) prior to study initiation. The study was registered in the Pan African Clinical Trial Registry (PACTR) (Ref: PACTR201804003096919).

**Informed Consent Statement:** Written informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to institutional data policy restrictions.

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**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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**Research Article**

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Research Article

# Human cytomegalovirus seropositivity and its influence on oral rotavirus vaccine immunogenicity: a specific concern for HIV-exposed-uninfected infants

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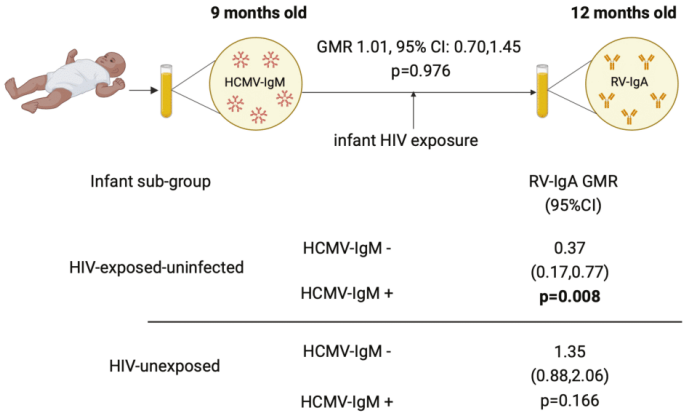
## Abstract

Oral rotavirus vaccines demonstrate diminished immunogenicity in low-income settings where human cytomegalovirus infection is acquired early in childhood and modulates immunity. We hypothesized that human cytomegalovirus infection around the time of vaccination may influence immunogenicity. We measured plasma human cytomegalovirus-specific immunoglobulin M antibodies in rotavirus vaccinated infants from 6 weeks to 12 months old and compared rotavirus immunoglobulin A antibody titers between human cytomegalovirus seropositive and seronegative infants. There was no evidence of an association between human cytomegalovirus serostatus at 9 months and rotavirus-specific antibody titers at 12 months (geometric mean ratio 1.01, 95% CI: 0.70, 1.45; $P = 0.976$) or fold-increase in RV-IgA titer between 9 and 12 months (risk ratio 0.999, 95%CI: 0.66, 1.52; $P = 0.995$) overall. However, HIV-exposed-uninfected infants who were seropositive for human cytomegalovirus at 9 months old had a 63% reduction in rotavirus antibody geometric mean titers at 12 months compared to HIV-exposed-uninfected infants who were seronegative for human cytomegalovirus (geometric mean ratio 0.37, 95% CI: 0.17, 0.77; $P = 0.008$). While the broader implications of human cytomegalovirus infections on oral rotavirus vaccine response might be limited in the general infant population, the potential impact in the HIV-exposed-uninfected infants cannot be overlooked. This study highlights the complexity of immunological responses and the need for targeted interventions to ensure oral rotavirus vaccine efficacy, especially in vulnerable subpopulations.

## Graphical Abstract




**Keywords:** rotavirus, vaccine, antibody, human cytomegalovirus, Zambia

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## Introduction

Rotavirus, a leading cause of diarrheal disease in children [1], remains a public health concern particularly in low- and middle-income countries (LMICs). The use of oral rotavirus vaccines (ORV) [2] has decreased the degree of diarrheal disease caused by rotavirus in children residing in LMICs especially in Africa [3, 4]. The impact has been to bring down hospitalizations for rotavirus diarrhea in those children aged 5 years and below [4]. However, these vaccines demonstrate diminished seroconversion rates in LMICs, a phenomenon not yet fully understood [5]. Zambia has seen a decrease in rotavirus diarrhea since ORV introduction [6, 7], but low seroresponse rates persist, estimated between 27% and 60% [8, 9]. Researchers have pinpointed numerous factors that could play a role [5], but the impact of persistent viral infections when receiving the vaccine is yet to be examined.

Human cytomegalovirus (HCMV), a β-herpesvirus [10], is common and can be transmitted congenitally and during nursing across different regions including Africa [11]. HCMV infection occurs early in childhood in Africa, with over 80% of infants infected by their first birthday [12]. In Zambia, about 83% of infants acquire HCMV infection by 18 months of age [13]. The high HCMV prevalence, its effects on host immunity and the observed poor ORV immunogenicity in these settings necessitate longitudinal studies to investigate temporal associations with childhood vaccine responses as argued by others [14].

Studies regarding HCMV's influence on immunogenicity of childhood vaccines in Africa are scarce and show inconsistent findings. For some vaccines such as measles, HCMV has been found to have no effect [15], beneficial effects [16, 17] but also associated with reduced immune responses [17]. The effect of HCMV on other vaccinations such as meningococcal [16], Hepatitis B (HepB) [15, 18], diphtheria-pertussis-tetanus (DPT) [15, 17–19], and Bacille Calmette-Guérin (BCG) [15, 19] has been conflicting, with studies showing varying associations with vaccine-induced cellular and humoral responses. In Zambia, no significant associations between HCMV and oral polio vaccine antibody responses have been observed [20].

The current ambiguity in the direction of HCMV's influence on infant vaccine responses and the absence of data for ORV signal the need for additional research. This study explores HCMV-IgM seroconversion in the first year of life in Zambia and its effect on rotavirus specific antibody responses among rotavirus vaccinated infants. It addresses the complex relationship between HCMV and vaccine immunogenicity in the context of low-income settings, infant health, and current vaccination strategies, shedding light on an understudied yet vital area of pediatric infectious disease management.

## Materials and methods

### Study design and participants

We conducted a longitudinal study nested within an open label, two-arm parallel group, randomized controlled trial (RCT). The RCT compared a two-dose (control arm) and three-dose (intervention arm) Rotarix™ vaccination schedule among Zambian infants. The details of the study design have been published elsewhere [21]. Briefly, 214 infants aged 6–12 weeks were enrolled in the parent RCT and followed up until they were 3 years of age between 2018 and 2021. During the first year of follow-up, all infants were given two doses of an ORV (Rotarix, GlaxoSmithKline) with the first dose administered from 6 weeks old and the second dose administered from 10 weeks old, along with polio, BCG, DPT-HepB-Hib and pneumococcal conjugate vaccines as part of the regular Zambia national immunization schedule. When the infants reached 9 months of age, they were randomly assigned to either a control arm (receiving only a measles-rubella (MR) vaccination) or an intervention arm (receiving MR vaccine and a third dose of Rotarix).

Plasma samples were collected at specific intervals: at enrollment (baseline, aged 6-12 weeks) before the first Rotarix dose, 1 month after the second Rotarix dose (aged 14-20 weeks, when vaccine seroconversion was determined), at 9 months (before receipt of the third Rotarix dose and/or MR vaccine), and at 12 months (when the immune-boosting effect of third Rotarix dose was assessed; Fig. 1). These plasma samples were tested for rotavirus-specific immunoglobulin A (RV-IgA) antibodies. RV-IgA seropositivity was defined as an RV-IgA titer ≥ 20 units/ml. Vaccine seroconversion was defined as a 4-fold or greater change in RV-IgA antibody titer 1 month after dose two of Rotarix if pre-vaccination titer was less than 20 U/ml [21].

For this nested study, infants with available RV-IgA results and sufficient plasma sample to test for HCMV-IgM antibodies at baseline and at least one of the three subsequent time points up to 12 months of age were included. The design thus facilitated the examination of the association between HCMV infection and ORV immunogenicity by focusing on multiple factors and time points, enabling a comprehensive analysis of the relationship.

### Laboratory procedures

**Determination of HCMV serostatus:**

HCMV-IgM antibodies were measured using enzyme-linked immunosorbent assay (ELISA) kits from Demeditec Diagnostics GmbH (Germany) and Alpha Diagnostic

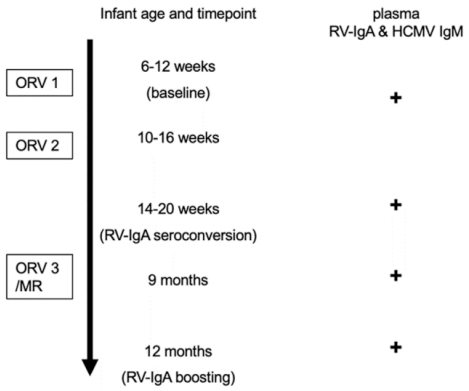


**Figure 1.** Study design. An illustration of the parent RCT study design and plasma collection timepoints.

International (USA). The procedure followed the manufacturer's guidelines. Infant plasma samples were diluted and added to a 96-well microtiter plate that had been precoated with a purified HCMV antigen. To detect any HCMV-IgM in these samples, they were treated with horse-radish peroxidase (HRP)-conjugated anti-human IgM. Subsequently, the tetramethylbenzidine (TMB) substrate was added, initiating an enzyme-substrate hydrolysis reaction, which resulted in color development. The color's absorbance was immediately measured at a 450-nm wavelength using an Epoch 2 microplate reader by Agilent (South Africa). Proper quality control of each experiment was ensured using the calibrators and controls provided in the kit. To check consistency in results, selected HCMV seropositive samples were tested with both kits. The test outcomes for HCMV-IgM serostatus in the plasma samples were categorized as either positive or negative based on specific cut-off control values for each experiment.

**Quantification of rotavirus-specific immunoglobulin A:**

The measurement of rotavirus-specific immunoglobulin A (RV-IgA) was carried out using a sandwich ELISA method, as outlined in the parent rotavirus vaccine trial [21]. In the procedure, infant plasma samples were placed on a 96-well microtitre plate, which was coated with alternating columns of rotavirus infected and uninfected cell lysate. To detect the RV-IgA, the samples underwent a subsequent treatment with biotinylated anti-human IgA and an avidin-biotin-peroxidase complex. The addition of the o-phenylenediamine dihydrochloride substrate initiated a color change, whose intensity was measured at a 492-nm wavelength with a microplate reader. The concentration of RV-IgA was determined based on these readings, compared against a standard curve created from a known rotavirus IgA plasma standard.

### Statistical analysis

Background characteristics were summarized with mean and standard deviation (SD) or median and interquartile range (IQR) for continuous variables. Categorical variables were summarized using frequency and proportion. Pearson's chi-square or Fisher's exact test was used to compare the distribution of categorical background characteristics by HCMV-IgM serostatus at 9 months and 4-fold change in RV-IgA titers between 9 and 12 months. For RV-IgA titers at 12 months, we used Student *t*-test on log-transformed values. For anthropometric indices, we calculated *z*-scores using the 2006 World Health Organization child growth standards. The exposure of interest was HCMV-IgM serostatus at 9 months and the primary outcome was RV-IgA titers at 12 months. Secondary outcome was proportion with 4-fold-change in RV-IgA titers between 9 and 12 months. The primary analysis was conducted among infants that had HCMV serostatus result at the 9-month time point and RV-IgA titer result at both the 9- and 12-month timepoints.

We used linear regression model of log-transformed RV-IgA titers to estimate the effect of HCMV-IgM serostatus at 9 months or cumulative HCMV-IgM seroconversion by 9 months on RV-IgA GMT at 12 months, adjusting for potential confounders. *P*-value of <0.05 was considered statistically significant. The RV-IgA titer below the range of the standard curve were imputed as "1" prior to log-transformation. We used generalized linear model, adjusted for potential confounders, to estimate the effect of HCMV-IgM serostatus at 9 months or cumulative HCMV-IgM seroconversion by 9 months on the proportion with a 4-fold or greater change in RV-IgA titers between 9 and 12 months. In a subgroup analysis, we used likelihood ratio test of interaction to investigate whether the effect of HCMV-IgM serostatus at 9 months on RV-IgA titer at 12 months varied by two-dose versus three-dose vaccination or by infant human immunodeficiency virus (HIV) exposure. In exploratory analyses, we examined the proportion of infants testing seropositive for HCMV-IgM for each time point and the relationship between HCMV-IgM point seropositivity or cumulative HCMV-IgM seroconversion and vaccine seroconversion after two dose vaccination. All analyses were performed in Stata 17 (StataCorp, College Station, TX, USA) and GraphPad Prism v9 (GraphPad Software, LLC).

## Results

The parent Rotarix RCT enrolled and quantified RV-IgA titers for 214 infants of which 177 had sufficient plasma available at baseline and for at least one other timepoint; these were also tested for HCMV-IgM. Of these a total of 155/177 (88%) infants met the criteria for inclusion in our primary analysis and included HIV-exposed-infected, HIV-exposed-uninfected and HIV unexposed infants (Fig. 2).

### Infant baseline characteristics

As shown in Table 1, among the infants included in the primary analysis ($n = 155$), median age at baseline was 6 weeks, majority were vaginally delivered (95%) at full term (94%), with normal birthweight (90%), and were predominantly breastfed exclusively (94%). Most infants came from homes with less than ideal water and sanitation, often sharing toilets with other households (79%) and getting water from public sources (65%) and approximately one-third of infants had HIV-positive mothers. There were 60 HCMV-IgM seropositive and 95 HCMV-IgM seronegative infants at 9 months of age. There was no statistically significant relationship between baseline characteristics and HCMV-IgM serostatus at 9 months of age (Table 1).

We additionally assessed for associations between infant baseline characteristics and the primary outcomes of RV-IgA at 12 months old and secondary outcome of 4-fold increase in RV-IgA titer between 9 and 12 months old. We found that infants residing in households that did not share toilet facility had higher RV-IgA GMT at 12 months compared to infants from households with shared toilet facility ($P = 0.027$) but for all other baseline characteristics, no statistically significant relationship was observed (Supplementary Table S1). There was no statistically significant relationship observed between infant baseline characteristics and 4-fold or greater increase in RV-IgA titer between 9 and 12 months (Supplementary Table S2).

### HCMV-IgM serostatus by age

To assess HCMV-IgM seropositivity by age, we included infants that had an HCMV-IgM result at all the four age timepoints 6-12 weeks, 14-20 weeks, 9 months, and 12 months ($n = 148$) out of the 177 that had a baseline and at least one follow-up sample collected. The proportion of infants

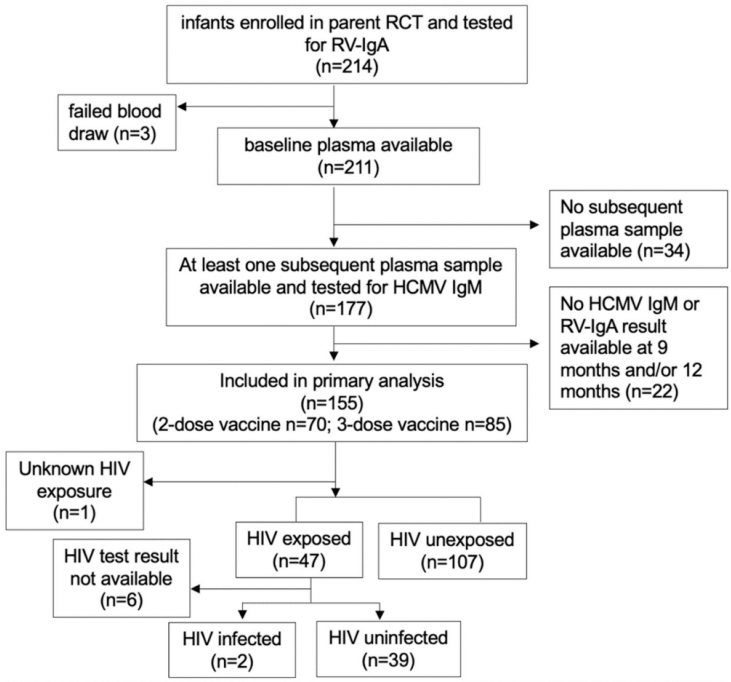


**Figure 2.** Flow diagram of infant samples included in the study. A schema of the participant flow and criteria used in the selection of plasma samples and infant subgroups included in analysis.

that were HCMV-IgM seropositive at each age timepoint increased from 9.5% (14/148) at ages 6-12 weeks, to 27.0% (40/148) at 14-20 weeks, 37.2% (55/148) at 9 months and 59.5% (88/148) at 12 months (Fig. 3A). We also assessed cumulative HCMV-IgM seroconversion with infants defined as HCMV-IgM seroconverters when they became HCMV-IgM seropositive after having HCMV-IgM seronegative results for all preceding timepoints. By 12 months old, the cumulative HCMV-IgM seroconversion was 79.1% (117/148), and 20.9% (31/148) infants were HCMV-IgM seronegative throughout (Fig. 3B).

## Effect of HCMV-IgM serostatus on rotavirus antibody response

In the overall study population ($n$ = 155), the RV-IgA GMT were 1.3 units/ml (95%CI: 1.1, 1.6) at 6–12 weeks ($n$ = 154), 3.2 units/ml (95%CI: 2.3, 4.4) at 14–20 weeks ($n$ = 149), 6.8 units/ml (95%CI: 4.7, 9.8) at 9 months ($n$ = 155) and 24.8 units/ml (95%CI:16.6, 36.9) at 12 months ($n$ = 155). There were 7/154 (4.6%), 27/149 (18%), 49/155 (31.6%), and 84/155 (54.2%) infants that were RV-IgA seropositive at 6–12 weeks, 14–20 weeks, 9 months, and 12 months, respectively. A total of 148 infants had RV-IgA results at both 6–12 and 14–20 weeks and among these, 40/148 (27.0%) were vaccine seroconverters and 108/148 (73%) were vaccine non-seroconverters.

At 12 months, the RV-IgA geometric mean titers (GMT) was 23.2 units/ml (95%CI: 12.32, 43.5) among infants seropositive for HCMV-IgM at 9 months and 25.8 units/ml (95%CI: 15.3, 43.7) among those that were HCMV-IgM seronegative. As shown in Table 2, irrespective of the number of vaccine doses and after adjusting for the potential confounding effect of sex, breastfeeding, stunting, wasting, and toilet facility, there was no statistically significant difference in RV-IgA GMT at 12 months between HCMV-IgM seropositive and HCMV-IgM seronegative infants at 9 months (geometric mean ratio (GMR) 1.01, 95%CI: 0.70,1.45; $P$ = 0.976). A 4-fold or greater increase in RV-IgA titer between 9 and 12 months of age was observed in 61/155 infants (39.4%) and 23/60 (37.3%) were from HCMV-IgM seropositive and 38/95 (40.0%) were from HCMV-IgM seronegative infants. Irrespective of the number of vaccine doses and after adjusting for the potential confounding effect of sex, breastfeeding, stunting, wasting, and toilet facility, there was no evidence of an association between HCMV-IgM serostatus at 9 months and the 4-fold or greater increase in RV-IgA titer (risk ratio (RR) 0.99, 95% CI: 0.66,1.52; $P$ = 0.995) (Table 2). Similarly, we found no statistically significant relationship between cumulative HCMV-IgM seroconversion status by 9 months and RV-IgA GMT at 12 months (GMR 1.24, 95%CI: 0.86, 1.78; $P$ = 0.239) or a 4-fold or greater increase in RV-IgA titer between 9 and 12 months (RR 0.88, 95% CI: 0.59,1.32; $P$ = 0.539) (Table 2).

**Table 1.** Baseline characteristics by HCMV-IgM serostatus at 9 months

| | HCMV-IgM serostatus at 9 months old | | | |
|---|---|---|---|---|
| | | Seronegative | Seropositive | |
| Characteristic | *n* (% of total) | *n* (% of total) | *n* (% of total) | *P* value |
| Age | | | | |
| Median(IQR) | 6 (6.6) | 6 (6.6) | 6 (6.6) | 0.927 |
| Sex | | | | |
| Female | 73 (47.1) | 41 (43.2) | 32 (53.3) | 0.249 |
| Male | 82 (52.9) | 54 (56.8) | 28 (46.7) | |
| Gestation | | | | |
| Full-term | 146 (94.2) | 89 (93.7) | 57 (95.0) | 1.000 |
| Pre-term | 9 (5.8) | 6 (6.3) | 3 (5.0) | |
| Mode of delivery | | | | |
| Caesarean | 8 (5.2) | 4 (4.2) | 4 (6.7) | 0.712 |
| Vaginal | 147 (94.8) | 91 (95.8) | 56 (93.3) | |
| Feeding | | | | |
| Breastmilk | 145 (93.5) | 91 (95.8) | 54 (90.0) | 0.187 |
| Breastmilk + formula | 10 (6.5) | 4 (4.2) | 6 (10.0) | |
| Birth weight, kg (*n* = 154) | | | | |
| < 2.5 | 16 (10.4) | 10 (10.5) | 6 (10.2) | 1.000 |
| ≥ 2.5 | 138 (89.6) | 85 (89.5) | 53 (89.8) | |
| Stunting (LAZ < –2) | | | | |
| No | 129 (83.2) | 75 (79.0) | 54 (90.0) | 0.081 |
| Yes | 26 (16.8) | 20 (21) | 6 (10.0) | |
| Malnourished (WLZ < -2) | | | | |
| No | 152 (98.1) | 92 (96.8) | 60 (100.0) | 0.284 |
| Yes | 3 (1.9) | 3 (3.2) | 0 (0.0) | |
| Maternal HIV (*n* = 154) | | | | |
| Negative | 107 (69.5) | 65 (68.4) | 42 (71.2) | 0.857 |
| Positive | 47 (30.5) | 30 (31.6) | 17 (28.8) | |
| Toilet facility sharing across households | | | | |
| Not shared | 32 (20.6) | 22 (23.2.7) | 10 (16.7) | 0.416 |
| Shared | 123 (79.4) | 73 (76.8) | 50 (83.3) | |
| Water source | | | | |
| Piped into household | 55 (35.5) | 35 (36.8) | 20 (33.3) | 0.731 |
| Wells/public taps and boreholes | 100 (64.5) | 60 (63.2) | 40 (66.7) | |
| Number of children in household | | | | |
| 1–3 | 122 (78.7) | 76 (80.0) | 46 (76.7) | 0.872 |
| 4–6 | 29 (18.7) | 17 (17.9) | 12 (20.0) | |
| 7–9 | 4 (2.6) | 2 (2.1) | 2 (3.3) | |
| Total | 155 (100) | 95 (61.3) | 60 (38.7) | |

## Subgroup analysis by infant HIV exposure and vaccine dose schedule

Of the 47/154 infants maternally exposed to HIV, 41/47 (87.2%) had an HIV result available. Of these, 39/41 (95.1%) were uninfected (HIV-exposed-uninfected, HEU), and 2/41 (4.9%) were infected (HIV-exposed-infected). We excluded the HIV-exposed-infected ($n = 2$) from subsequent analysis. As shown in Fig. 4A, analysis of HEU and HIV-unexposed infants (HU, $n = 146$), demonstrated an effect of point HCMV-IgM serostatus at 9 months on RV-IgA titers at 12 months according to infant HIV exposure status (likelihood ratio test of interaction $P = 0.002$). In contrast, there was no evidence of an interaction between infant HIV status and the effect of cumulative HCMV-IgM seroconversion by 9 months on RV-IgA titers at 12 months (likelihood ratio test of interaction $P = 0.138$) in this grouping (Fig. 4B).

As shown in Fig. 5, we found no evidence that the effect of HCMV-IgM seropositivity at the 9 months timepoint (Fig. 5A) or cumulative HCMV-IgM seroconversion by 9 months (Fig. 5B) on RV-IgA titer at 12 months of age varied by the vaccine dose schedule for the entire cohort (two versus three doses of Rotarix, $n = 155$) (likelihood ratio test of interaction $P = 0.318$ and $P = 0.737$, respectively).

Among the HU infants ($n = 107$), there was no statistically significant difference in RV-IgA GMT at 12 months between infants that were HCMV-IgM seropositive (GMT 29.4, 95%CI: 13.9, 61.9) and HCMV-IgM seronegative (GMT 16.4, 95%CI: 9.1, 29.4) at 9 months (GMR 1.35, 95%CI: 0.88,

2.06; $P = 0.166$) (Table 3). In the HEU group, there was evidence that the RV-IgA GMT at 12 months was decreased by 63% in infants that were HCMV-IgM seropositive compared to those that were HCMV-IgM seronegative at 9 months (GMR 0.37, 95%CI: 0.17,0.77; $P = 0.008$) (Table 3).

As shown in Fig. 6, there was no statistically significant difference in the frequency of vaccine seroconversion one month after two dose vaccination either by HCMV-IgM serostatus at 6-12 weeks ($n = 148$, $P = 0.528$) and 14–20 weeks timepoints ($n = 148$, $P = 0.407$) or by cumulative

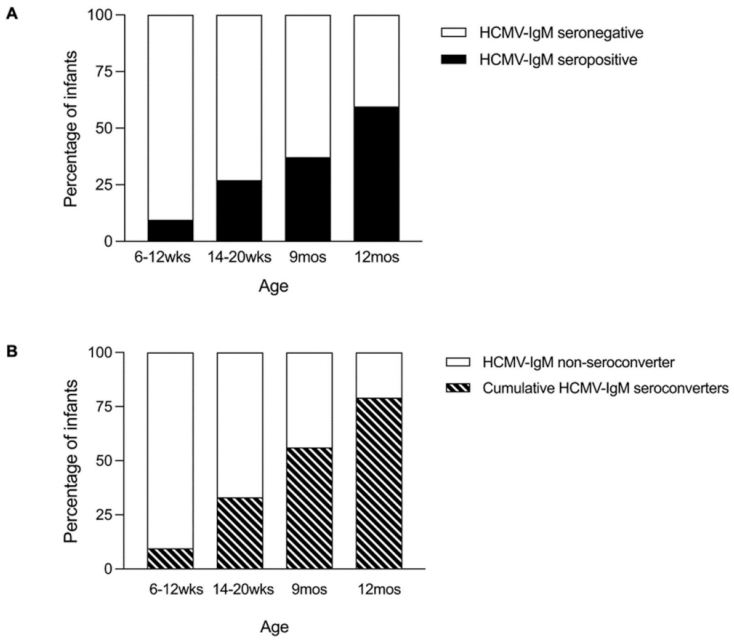


**Figure 3.** Infant HCMV-IgM serostatus by age. The percentage of HCMV-IgM seropositive and seronegative infants (panel A) and cumulative HCMV-IgM seroconverters and non-seroconverters (panel B) at each age timepoint is shown as bars ($n = 148$).

**Table 2.** Effect of HCMV-IgM serostatus on RV-IgA titers and 4-fold increase

| HCMV at 9 months | Number infants *N* (% of total) | RV-IgA GMT at 12 months (95%CI) | RV-IgA *GMR at 12 months (95% CI) | *P*-value | Mounted ≥ 4-fold rise in RV-IgA titer between 9 and 12 months *n* (%) | *RR (95% CI) | *P*-value |
|---|---|---|---|---|---|---|---|
| HCMV-IgM point serostatus | | | | | | | |
| HCMV IgM - | 95 (61.3) | 25.8 (15.3,43.7) | 1 | 0.976 | 38 (40.0) | 1 | 0.995 |
| HCMV IgM + | 60 (38.7) | 23.2 (12.3,43.5) | 1.01 (0.70,1.45) | | 23 (38.3) | 0.99 (0.66,1.52) | |
| HCMV-IgM cumulative seroconversion | | | | | | | |
| HCMV-IgM ns | 67 (43.2) | 20.67 (11.7,36.6) | 1 | 0.239 | 28 (41.8) | 1 | 0.539 |
| HCMV-IgM s | 88 (56.8) | 28.4 (16.2,49.8) | 1.24 (0.86,1.78) | | 33 (37.5) | 0.88 (0.59,1.32) | |
| Total | 155 (100) | 24.76 (16.6,36.9) | — | | 61(39.4) | | — |

Abbreviations: CI (confidence interval), ns (non-seroconverter), RR (risk ratio), s (seroconverter).
*Estimates were adjusted for sex, breastfeeding, stunting, wasting, and toilet facility.

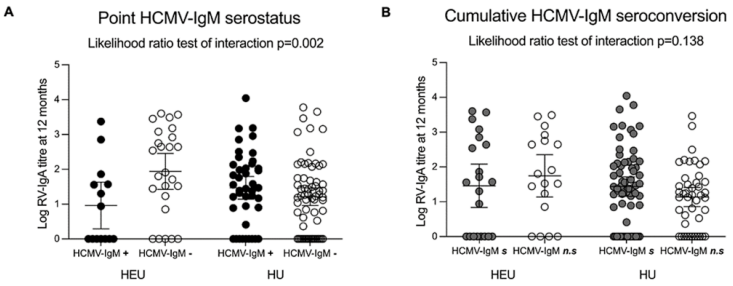


**Figure 4.** RV-IgA titres at 12 months infant age by point and cumulative HCMV-IgM serostatus at 9 months old stratified by infant HIV exposure. Each circle represents the log-transformed RV-IgA titer for a single infant ($n$ = 146) among HIV-exposed-uninfected (HEU, $n$ = 39) and HIV-unexposed (HU, $n$ = 107) infants. Black and white circles indicate HCMV-IgM seropositive (+) and HCMV-IgM seronegative (–) infants at 9 months, respectively (panel A). Grey and white circle indicate cumulative HCMV-IgM seroconverting (*s*) and non-seroconverting (*n.s.*) infants at 9 months respectively (panel B). Solid horizontal bar and error bars indicates the mean value with 95% confidence intervals.

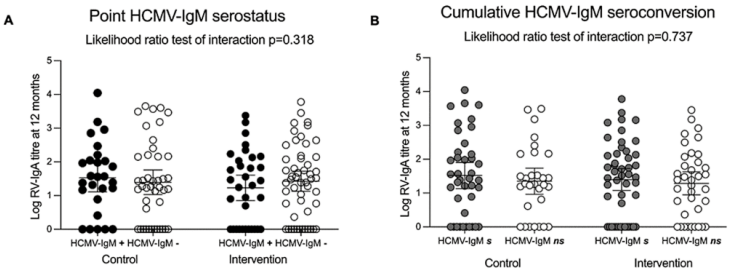


**Figure 5.** Mean rotavirus antibody titers at 12 months infant age by point and cumulative HCMV-IgM serostatus at 9 months old stratified by vaccine dose schedule. Each circle represents the log-transformed RV-IgA titer for a single infant ($n$ = 155) randomized to the intervention arm ($n$ = 85) and control arm ($n$ = 70). Black and white circle indicates HCMV-IgM seropositive (+) and seronegative (–) infants at 9 months, respectively (panel A). Grey and white circle indicates cumulative HCMV-IgM seroconverting (*s*) and non-seroconverting (*ns*) infants at 9 months, respectively (panel B). Solid horizontal bar and error bars indicates the mean value with 95% confidence intervals.

**Table 3.** Effect of HCMV-IgM serostatus on RV-IgA titers by infant HIV status

| Subgroups[a]) | Number of infants *N* (% of total) | RV-IgA GMT at 12 months (95%CI) | RV-IgA *GMR (95% CI) | *P*-value |
|---|---|---|---|---|
| HIV-unexposed | 107 (73.3) | 20.6 (13.0,32.5) | 1.35 (0.88,2.06) | 0.166 |
| HCMV-IgM- | 65 (60.8) | 16.4 (9.1, 29.4) | | |
| HCMV-IgM+ | 42 (39.2) | 29.4 (13.9, 61.9) | | |
| HIV-exposed-uninfected | 39 (26.7) | 38.8 (14.8, 102.0) | 0.37 (0.17,0.77) | **0.008** |
| HCMV-IgM- | 25 (64.1) | 87.2 (26.5, 286.4) | | |
| HCMV-IgM+ | 14 (35.9) | 9.1 (2.0, 42.8) | | |
| Total | 146 | 24.4 (16.0, 37.1) | | |

[a]Likelihood ratio test of interaction *P*-value = 0.002. *Estimates were adjusted for sex, breastfeeding, stunting, waisting, and toilet facility.

HCMV-IgM seroconversion at 14–20 weeks timepoint ($n$ = 147, $P$ = 0.166).

## Discussion

Our study aimed to investigate the influence of HCMV infection around the time of oral rotavirus vaccination on the vaccine immunogenicity in a low-income setting where early childhood HCMV infection is prevalent and may modulate immune responses. We measured HCMV-IgM in vaccinated infants when they were 9 months of age, which coincided with the time of a third dose of ORV. We specifically examined any association between the presence of HCMV-IgM (indicative of recent HCMV infection or reactivation) and the antibody response to rotavirus vaccine (measured as RV-IgA titers). Overall, there was no evidence of association, at 5%

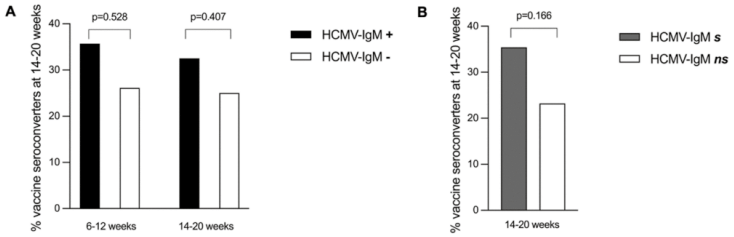


**Figure 6.** Vaccine seroconversion by HCMV-IgM serostatus. Each bar represents the percent vaccine seroconverters at 14–20 weeks (1 month after two dose vaccination) among infants that were HCMV-IgM seropositive ($n$ = 14) and HCMV IgM seronegative ($n$ = 134) before vaccination at 6–12 weeks old ($n$ = 148); HCMV IgM seropositive ($n$ = 40) and HCMV IgM seronegative ($n$ = 108) at 14–20 weeks old ($n$ = 148); and cumulative HCMV-IgM seroconverting ($n$ = 48) and non-seroconverting ($n$ = 99) infants at 14–20 weeks old ($n$ = 147). Black and white bars indicate HCMV-IgM seropositive (+) and seronegative (–) infants, respectively (panel A). Grey and white bars indicate cumulative HCMV-IgM seroconverting (s) and non-seroconverting (ns) infants respectively (panel B).

level of significance, between the presence of HCMV-IgM at 9 months of age and RV-IgA titers at 12 months. This suggests that for the majority of infants, HCMV infection does not seem to notably affect the vaccine's immunogenicity. However, among HEU infants who were HCMV-IgM seropositive at 9 months, a 63% reduction in RV-IgA titer at 12 months was observed compared to their HEU-HCMV-IgM seronegative counterparts. This points to a possible-specific immune modulation effect of HCMV in HEU infants in our setting.

Our findings are similar to what has been reported for another orally administered pediatric vaccine, oral polio vaccine (OPV), where a study in Zambia showed no effect of HCMV DNAemia or HCMV serostatus in 18-month-old infants on poliovirus antibody titers or proportion of infants with seroprotective levels in the overall study population [20]. In contrast to our findings on oral rotavirus vaccine, while significantly reduced poliovirus antibody responses were observed in maternally HIV exposed infants and HIV seropositive infants, the OPV study did not find any difference in poliovirus antibody responses by HCMV DNAemia or HCMV serostatus among the HEU infants [20]. Notably, trends of reduced OPV antibody responses among HIV-positive infants that had HCMV DNAemia compared to those without HCMV DNAemia were observed [20] although differences in determination of HCMV serostatus with our study (HCMV-IgG versus IgM) may limit comparisons. In our rotavirus vaccine study and the OPV study, while vaccine immunogenicity in the broader population was not impacted by HCMV infection, reduction of vaccine responses was seen within specific subgroups (HEU- and HIV-positive infants). This nuanced finding underscores the complexity of immunologic responses, especially in populations with various health challenges. Interestingly, in studies elsewhere with reported HIV prevalence of below 5%, HCMV infected infants are observed to have reduced antibody responses to tetanus toxoid after DPT vaccination and lowered T-cell effector responses post measles vaccination compared to HCMV uninfected infants despite no impact on infants' attainment of vaccine-specific seroprotective levels; however, the infant HIV status in these studies was not ascertained [15, 17]. We could not speculate on the clinical significance of the reduced RV-IgA titer in HEU HCMV-IgM seropositive infants as there is currently no defined seroprotective threshold level of RV-IgA; however, higher RV-IgA titers are associated with reduced risk of rotavirus infection and diarrhea [22]. Thus, factors like HCMV associated with reductions in these RV-IgA responses among HEU infants have the potential to negatively impact overall vaccine efficacy and effectiveness.

HCMV affects the immune system by committing a substantial proportion of T cells toward its immune response [23] and causing accumulation of differentiated immune cells [23–25] and restricted T-cell repertoires [26] characteristic of immune senescence. HCMV can also disrupt antigen presentation to T cells, suppress immune effector function, and limit immune cell proliferation [27–29]. Expansion of differentiated T-cell immunity by HCMV is consistent with immunological profiles observed in HEU infants compared to HIV unexposed infants [30]. In the HEU population, HCMV may therefore attenuate vaccine immune responses via this T-cell immune perturbation [31]. We found evidence of an impact of HCMV on rotavirus-specific antibody response in HEU infants thus for rotavirus vaccines, further studies investigating the effect of HCMV infection on T-cell immune responses to vaccination in HEU infants are merited to elucidate such possible effects. HCMV also alters intestinal microbiome [32] favoring increased composition of *Bacteroidetes* [33]. Abundance of specific *Bacteroidetes* genera, *Bacteroides* and *Prevotella*, has been significantly correlated with a lack of seroresponse to rotavirus vaccination in infants [34] and is reported to be significantly increased in HEU compared to HIV unexposed infants [35]. Human microbiome colonization and immune development are intimately related and influence infant immune responses to vaccines [36]. Early life HCMV may therefore be an important determinant of oral vaccine responses in HEU via its effect on the intestinal microbiome composition but additional studies are needed to confirm this.

A major strength of this study is its focus on a vulnerable population (infants) within a low-income setting. We addressed an important prevailing question as to why oral rotavirus vaccines perform sub-optimally in such regions. Measuring plasma HCMV-IgM prior to vaccination provided a clear temporal relationship between HCMV infection and vaccine immunogenicity. However, some limitations exist. The sample size might not have been sufficiently large to detect more subtle differences across the infant groups. Also, the study does not delve into potential biological mechanisms underlying the observed association in HEU infants, which might provide clearer insights. We determined HCMV

infection based on HCMV-IgM without confirmation of DNAemia by molecular methods, which could have introduced classification bias. HCMV-IgM serology can identify infant HCMV-specific responses in early life, as opposed to passively acquired transplacental maternal HCMV-IgG antibodies, but may show false positives due to cross reactivity with other herpesviruses such as EBV [37]. HCMV-IgM can also be transient and cleared rapidly in some individuals, thus contributing to misclassification of HCMV status [38]. We also acknowledge that the phenomena of reduced oral rotavirus vaccine immunogenicity may occur in areas with lower HIV seroprevalence and thus limit the generalizability of our findings. Furthermore, genetic, environmental and maternally derived factors known to influence oral rotavirus vaccine immunogenicity [5] were not included in this analysis but which may be involved in the immune responses observed.

Our findings shed light on a potentially critical area of vaccine research, especially in Zambia where HIV prevalence is high. More robustly designed studies may be needed to verify the observed association between HCMV infection and reduced oral rotavirus vaccine response in HEU infants. It would be pertinent to investigate the underlying immunological mechanisms that may be driving this impaired response. Also, it would be useful to study other vaccines' immunogenicity in relation to HCMV within the HEU infant population to understand if this observation is rotavirus vaccine-specific or a broader immunological phenomenon. If future studies corroborate these findings, it could have profound implications for vaccine policy in regions with high HIV prevalence. There might be a need to revisit vaccine schedules, dosages, or even the development of specific vaccine formulations tailored to the needs of HEU infants. Additionally, understanding such interactions can guide health campaigns and interventions, especially in low-income settings where both HCMV and HIV are prevalent. Public health officials might need to consider additional interventions or strategies to enhance vaccine efficacy in vulnerable subpopulations.

## Conclusion

While the broader implications of HCMV infections on oral rotavirus vaccine response might be limited in the general infant population, the potential impact on HEU infants cannot be overlooked. This study highlights the complexity of immunological responses and the need for targeted interventions to ensure vaccine efficacy, especially in vulnerable subpopulations.

## Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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## Ethical approval

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) (reference number 003-02-18) and the London School of Hygiene and Tropical Medicine (LSHTM) Research Ethics Committee (reference number 16168). Informed consent was obtained from all study participants.

## Conflict of interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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## Data availability

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to institutional data policy restrictions.

## Author contributions

N.M.L.: conceptualization, investigation, formal analysis, visualization, writing—original draft preparation, writing—review and editing, funding acquisition; S.B.: formal analysis, visualization writing—review and editing; R.C.: funding acquisition, writing—review and editing; M.S.: writing—review and editing; C.C.: writing—review and editing; H.N.: investigation, writing—review and editing; K.M.C.: investigation, writing—review and editing; M.G.: conceptualization, writing—review and editing. All authors have read and agreed to the published version of the manuscript

## Clinical trial registration

The parent trial is registered under the Pan African Clinical Trial Registry as PACTR201804003096919.

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