Epstein-Barr virus infection and its lifelong autoimmune ramifications in developed and developing nations

by

Rana Jafari-Minab

B.Sc., Simon Fraser University, 2017

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2021

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

Epstein-Barr virus infection and its lifelong autoimmune ramifications in developed and developing nations

submitted by Rana Jafari-Minab in partial fulfillment of the requirements for

the degree of Master of Science

in Pathology and Laboratory Medicine

Examining Committee:

Bruce Verchere, Pathology and Laboratory Medicine

Co-supervisor

Peter van den Elzen, Pathology and Laboratory Medicine

Co-supervisor

Marc Horwitz, Microbiology and Immunology

Supervisory Committee Member

Manish Sadarangani, Pediatrics

Additional Examiner

Conelia Laule, Pathology and Laboratory Medicine

Additional Examiner

Additional Supervisory Committee Members:

Soren Gantt, Experimental Medicine

Supervisory Committee Member

Abstract

Background.
Epstein-Barr virus (EBV) infection is a major cause of malignancy worldwide. Maternal antibodies are thought to prevent EBV infection because infection is uncommon in early infancy. Additionally, maternal HIV infection is associated with an increased incidence of EBV infection in exposed infants possibly resulting from impaired transfer of EBV maternal antibodies. EBV, possibly in its acute form manifesting as infectious mononucleosis (IM), is also the highest environmental risk factor for the development of multiple sclerosis (MS), a devastating neurologic disease caused by autoimmune destruction of myelin. IM can be diagnosed by the presence of heterophile antibodies which target an antigen that is akin to certain myelin glycans in the brain.

We hypothesized that maternal antibodies can protect against EBV acquisition by studying infants with and without maternal HIV exposure. Furthermore, , since the degeneration of myelin is a hallmark of MS, we hypothesized that EBV infection, specifically IM, primes the immune system to cross-react and attack myelin.

Methods.
Ugandan infants were followed for EBV acquisition from birth and measured antibody binding to EBV glycoproteins involved in B cell and epithelial cell entry, as well as viral neutralization and antibody-dependent cellular cytotoxicity (ADCC). Serum antibodies against major brain glycans were measured using a Luminex based glycan array for 6 infant samples (pre-post EBV infection) and 9 adults with IM.

Results.
HIV-exposed uninfected infants had significantly higher titers than HIV-unexposed uninfected infants for all EBV-binding and neutralizing antibodies measured ($p<0.01$), but not ADCC activity, which was similar between groups. No antibody measure was associated with a decreased risk of EBV acquisition in the cohort. IM-positive sera had 10 times the anti-glycan antibodies to major brain glycans compared to pre and post-EBV infected infants, while there was no significant difference between pre-post EBV infected infant samples.

Conclusions.
Our findings indicate that in this cohort maternal antibody did not protect infants against EBV infection through viral neutralization. The increase in anti-myelin antibodies in individuals with IM suggests that the development of cross-reactivity towards myelin may be a mechanistic link between IM and MS.

Lay Summary

Epstein-Barr virus (EBV) infects over 90% of all humans and although it is usually harmless, it is strongly associated with some cancers and autoimmune diseases, particularly multiple sclerosis (MS), a degenerative autoimmne disease of the nervous system. In developing nations, almost all children are infected with EBV by the age of 3, and this is also when some EBV associated cancers are common. In developed nations, EBV infection is late and often manifests as infectious mononucleosis (mono) which increases the risk of MS by 3-fold. To study the immune response to EBV infection, samples from infants in Uganda and adults in Canada were studied. We found that maternal antibodies are not as protective against EBV as initially thought. In addition, during mono, antibodies are produced that recognize fat molecules in the brain that are usually degraded in MS, suggesting that mono is a possible risk factor for MS.

Preface

This thesis is based on the analysis of specimens and clinical data collected by the Primary Herpesvirus Infection in Children Study (PHICS) prospective cohort under the supervision of Dr. Soren Gantt. Infectious mononucleosis samples were collected for a pilot project at St. Paul's Hospital and BC Children's Hospital. All experiments for the PHICS study were studied at our collaborators' laboratories at the National Institute of Health and the Fred Hutch Cancer Research Centre. Preliminary analysis of the PHICS samples were performed by statistician Elizabeth Krantz. Subsequent analysis and the writing of the manuscript published was performed by me with the guidance of Dr. Soren Gantt and statistical help from Michael Irvine. Publication from work done with Dr. Soren Gantt is in the Journal of Infectious Diseases (2020) https://doi.org/10.1093/infdis/jiaa654.

Infectious mononucleosis blood specimen collection was approved by UBC's Clinical Research Ethics Board (H18-02031). The Luminex array on these samples was done by our collaborators in Augusta, Georgia. Raw data sent from Georgia was then analyzed by me with the help of statistical consultant, Michael Irvine at BC Children's Hospital. I developed the experimental design and set up with guidance from my supervisor Dr. Peter van den Elzen. Proofreading of the thesis was done by Dr. Dana Devine and my co-supervisor Dr. Bruce Verchere. I produced the written content and the figure for this thesis myself, with the exception of Figure 2 which was made by Michael Irvine, the statistician that supported the study with Dr. Soren Gantt.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

- ADCC Antibody dependent cellular cytotoxicity
- BRBC bovine red blood cells
- CI Confidence interval
- Cmah cytidine monophospho-N-acetylneuraminic acid hydroxylase enzyme
- CMAH gene that encodes Cmah enzyme
- EBV Epstein-barr virus
- HEU HIV exposed uninfected
- HIV Human immunodeficiency virus
- HR hazard ratio
- HUU HIV unexposed uiinfected
- IC50 inhibitory concentration
- IM Infectious mononucleosis
- IQR Interquartile range
- LIPS luciferase immunoprecipitation system
- LU Light units
- MFI Mean fluorescent intensity
- MHC major histocompatibility complex
- Mono Infectious mononucleosis
- MS Multiple sclerosis
- Neu5Ac N-acetylneuraminic acid
- OR Odds ratio

- PHICS Primary Herpesvirus Infection in Children Study
- qPCR quantitative polymerase chain reaction
- RFU Relative fluorescent units

Acknowledgements

I am incredibly thankful that I was given the opportunity to work on this project alongside Dr. Peter van den Elzen and Dr. Soren Gantt. Thank you, both, for the ongoing support, guidance and resources, especially during these unprecedented times brought on by the pandemic. I would also like to thank the Gantt and van den Elzen lab members for their guidance, especially Dong Jun Zheng and Bing Cai, your support in the laboratory was always unwavering.

I would also like to thank Dr. Dana Devine, Dr. Bruce Verchere and my other committee members (Dr Marc Horwitz, Dr. Angela Devlin) for their constant feedback and support with my thesis project. I would not have been able to finish my degree without all of you.

Last, but not least, I would like to thank my friends, family and fiancé for their unconditional support and encouragement.

Dedication

This thesis is dedicated to my parents and my mentor Dr. Soren Gantt. The dedication, drive and passion in your profession guides and inspires me.

Chapter 1: Introduction

1.1 Epstein-Barr Virus

Epstein-Barr virus (EBV) is an oncogenic human herpesvirus that is transmitted through saliva, and infects approximately 95% of the world's population[1]. EBV is responsible for approximately 200,000 cancers per year, and is the most common cause of infectious mononucleosis, a febrile syndrome responsible for substantial health care costs[2,3]. As such, a vaccine against EBV is a high-ranking public health priority [3,4].

1.1.1 Biology

EBV is initially thought to infect oral epithelial cells, transmitted through contact with infected saliva and other bodily fluids. Following extensive lytic replication in the oral epithelial cells, the virus will then infect naïve B cells in the underlying oral lymphoid tissue, resulting in the establishment of latency and lifelong infection [5,6]. EBV enters these B lymphocytes by binding the cellular receptor CD21 with viral glycoprotein gp350, followed by an interaction between viral glycoprotein gp42 and cellular major histocompatibility complex (MHC) class II molecules to facilitate membrane fusion and entry into the cell[7]. Since CD21 is necessary for infection, the concentration of CD21 on the surface of B cells may play a role in susceptibility to EBV infection.

Similar to other herpesviruses, EBV can take up lifelong latency in B lymphocytes and will reactivate if the lymphocyte differentiates into a plasma cell, differentiated B cells that produce antibodies[8]. On reactivation the virus will begin lytic replication and will be shed into the saliva enabling transmission to new hosts[9].

Neutralizing antibodies, antibodies from the adaptive immune system that defend the host cell against infection, inhibit EBV-induced proliferation or transformation of B cells[10]. B cell infection by EBV is initiated by the attachement through glycoproteins gp350 and gp220 (which we will not focus on for this study) to a complement receptor type 2 knowns as CD21 (cluster of differentiation 21)[11]. A glycoprotein complex gH/gL and gp42 noncovalently link to glycoprotein gB which mediates the fusion of the virus to the B cell membrane. Antibodies against the viral envelope glycoprotein gp350 in immune serum account for the majority of *in vitro* viral neutralizing activity in B cells, while gH/gL is the major target of epithelial cell neutralizing antibodies [12]. In summary, gp350 facilitates attachment of virions to CD21 positive B cells, while gH/gL is essential for fusion of the host and viral membranes.

1.1.2 Health Burden

1.1.2.1 EBV infection in Developed versus Developing Nations

Infection with EBV is incredibly common with >90% of the global adult population being infected[1]. In developing nations, most children become infected with EBV by the age of 3. However, in developed nations, EBV infection is delayed and is more commonly acquired in adolescence or early adulthood[1]. In this case, EBV infection is usually acquired through kissing [13,14]. This infection is usually asymptomatic, however, primary EBV infection in adolescents and adults can cause typical infectious mononucleosis[15].

Early EBV infection is seen in children in developing nations such as sub-Saharan Africa alongside other herpesvirus infections within the first few years of a child's life[16]. Many of these children are also born to human immunodeficiency virus type 1 (HIV)-infected mothers and therefore develop sequelae from EBV and other herpesvirus infections not usually seen outside

of this region[16]. HIV infection in women is a risk factor for EBV-associated lymphomas for their children, including non-Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric cancer[17]. EBV is also the most common cause of cancer among children in sub-Saharan Africa, specifically endemic Burkitt lymphoma[18].

1.1.3 Infectious Mononucleosis

The main risk factor for infectious mononucleosis (IM) is age: 75% of people aged 18 to 22 with a primary EBV infection will develop IM[15]. IM is a major health burden for the young adult population and is one of the main causes of absenteeism in US military recruits[19]. Common signs and symptoms of IM are fever, lymphadenopathy, pharyngitis, and severe fatigue[15]. In most people, fatigue lasts 2-3 weeks, but in 10% of cases, it can persist for 6 months or more[20]. Uncommon but severe complications of IM include encephalitis, hepatitis, hemolytic anemia, and splenic rupture.

Of note, there is a clear effect of age on the risk of IM: in young children in developed countries, EBV infection is nearly always asymptomatic, but in adolescents and adults, approximately 50% of primary EBV infections lead to IM. It is also important to recognize that IM is an immunopathologic syndrome that appears to result from an excessive immune response to EBV, rather than from the direct effects of the virus itself [21]. Even though young children appear to have higher EBV viral loads after infection than IM patients, they remain asymptomatic[16]. Similarly, even though antiviral treatment effectively reduces EBV viral replication during IM, it has no symptomatic benefit[22].

1.1.3.1 Diagnosis

Infectious mononucleosis can be diagnosed by the detection of heterophile antibodies, specifically those known as Paul-Bunnell antibodies. These were described by Paul and Bunnell when they observed that serum from patients with infectious mononucleosis could agglutinate animal red blood cells[23]. Heterophile antibodies are part of the natural antibody repertoire, produced by innate B cells without overt exposure to antigen[24], and are specific for non-human mammalian red blood cell antigens (e.g. antigens found on the surface of horse or cow erythrocytes[23,25]). The production of heterophile antibodies in infectious mononucleosis is a topic of interest since these antibodies are not specific to EBV.

The version of the agglutination test for infectious mononucleosis currently in use is termed the monospot test. This test is a latex agglutination test that uses latex beads coated with "Paul-Bunnell" carbohydrate antigens that have been purified from bovine erythrocytes. The beads are then mixed with patient serum. This test has 94% sensitivity and 93% specificity for the diagnosis of infectious mononucleosis[26].

The structure of the so-called "Paul-Bunnell" antigen has been proposed to be Neu5Gcα2-3Galβ1-3(Neu5Gc2-6)GalNAc(PCA)Thr-Pro-Gly-Pro-ProAsx.22 The terminal sialic acid, N-glycolylneuraminic acid (Neu5Gc), has been determined to be important for immunogenicity since desialylation led to a loss of reactivity[25].

1.1.3.2 Sialic Acids & Immunogenicity

Sialic acids are found diffusely throughout the human body. There are many types of sialic acids, all derivatives of neuraminic acid, but N-acetylneuraminic acid (Neu5Ac) is the most abundant in humans. In other mammals Neu5Ac can be converted to Neu5Gc by the

enzyme cytidine monophospho-N-acetylneuraminic acid hydroxylase (Cmah), which hydroxylates Neu5Ac to produce Neu5Gc. This enzyme is encoded by the gene CMAH. However, there is no functional CMAH gene in humans, and thus, we are lacking self-derived Neu5Gc. This means that Neu5Gc is a foreign antigen to humans[27]. Nevertheless, human cells do contain Neu5Gc and it is the current understanding that this Neu5Gc hails from dietary red meat and dairy that is then incorporated by some cells[28].

The similarity in structure between a foreign antigen, Neu5Gc, and a self antigen, Neu5Ac, leads to the question of whether anti-Neu5Gc antibodies are cross reactive towards Neu5Ac and therefore potentially prime the immune system for autoimmunity. Many myelin gangliosides contain Neu5Gc and Neu5Ac which could act as targets for autoimmunity[29].

1.2 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease in which the myelin sheath surrounding neuronal axons is destroyed by the patient's immune system. MS is the most common neurological disorder with its onset usually in young adults and a sex ratio of 3:1 women to men[30]. According to the MS Society of Canada, we have one of the highest rates of MS in the world, with an estimated 90,000 Canadians living with this disease. Characterized by a long survival but with progressive disability, MS requires continuous care which increases over time, rehabilitation, and expensive medication throughout its course.[30] As expected, the health-care burden of this disease is immense with over 2.2 million diagnosed worldwide, and a higher prevalence in developed countries.[31] Unfortunately, the individual drivers for MS are still unknown, although EBV infection is associated with a higher risk of developing MS and individuals who are EBV negative only have a 0.06 relative risk of developing MS (6%) [32].

Immunologically, it is known that MS involves both T cell and B cell mediated destruction of myelin and nerve fibers in the brain[33,34].

EBV is the strongest environmental risk factor for MS. Specifically, developing IM due to EBV infection increases the risk of developing MS by two- to three-fold[35,36]. Geographical differences in MS incidence closely mirror the typical age of EBV infection: where the incidence of MS is low, EBV is contracted early, and where the incidence of MS is high, EBV is contracted later and therefore has a higher risk of manifesting as IM[35]. The age association of EBV to MS is so striking that deliberate exposure of infants to EBV has been proposed as a means to prevent MS[32]. Exposing infants to EBV would reduce the incidence of IM and would prevent MS if IM was truly a causative factor[37]. Thus, a key question is: What are the immunologic responses in IM that confer susceptibility to MS?

1.3 Protection against EBV infection

1.3.1 Maternal Antibodies

A protective role for maternal antibodies has long been assumed, based on observations that EBV infection is typically delayed for the first 6 months after birth, after which maternal antibody levels wane and infants begin to acquire EBV infection at high rates [17,38–42]. Though there have been animal studies that show the ability of neutralizing antibody to protect against EBV lymphomagenesis or the rhesus lymphocryptovirus orthologue in animal studies, it remains unknown whether neutralizing antibody passed from mother to infant can protect against human EBV acquisition [3,4,43,44].

1.4 Hypothesis and Objectives

EBV is a ubiquitous virus that affects the immune system differently depending on the age of infection. My research has two separate but equally important objectives: (i) to elucidate the role that EBV infection, manifesting as IM, plays in the development of MS; and (ii) to determine which maternal antibodies, if any, protect infants from EBV infection.

1.4.1 Epstein-Barr Virus, Infectious Mononucleosis and Multiple Sclerosis

We hypothesize that during acute EBV infection in people with IM, the virus causes B cells to express natural antibodies which cross-react with gangliosides of the central nervous system, driving the autoimmunity to myelin. By further understanding the production of Paul-Bunnell antibodies in IM, we hope to unravel a potential mechanism of autoreactivity that would help explain the link between IM and MS. We aim to understand how the anti-glycan antibody repertoire in IM differs from the repertoire in uninfected and asymptomatic EBV-infected individuals without IM (Figure 1).

1.4.2 EBV infection in Sub-Saharan Africa

We hypothesized that maternal antibody can protect against EBV acquisition in infancy, and that HIV-exposed uninfected (HEU) infants become infected earlier than HIV-unexposed (HUU) infants as a result of having lower titers of EBV-specific maternal antibodies. We therefore attempted to determine whether neutralizing activity was correlated with protection against EBV infection. To examine the ability of maternal antibodies to protect against EBV infection, we took advantage of a longitudinal birth cohort study in which we characterized the precise timing of acquisition and risk factors for primary EBV infections in Ugandan infants [45]. In that cohort, EBV infection occurred significantly earlier among infants of HIV-infected,

compared to HIV-uninfected, women. Previous studies have shown impaired transplacental antibody transfer due to maternal HIV infection [46,47]. Identification of an immune correlate of protection would greatly facilitate efforts to develop an effective vaccine. It has been widely assumed that a vaccine able to confer sterilizing immunity to EBV would likely do so by inducing natural antibodies to one or more viral envelope proteins [3,4].

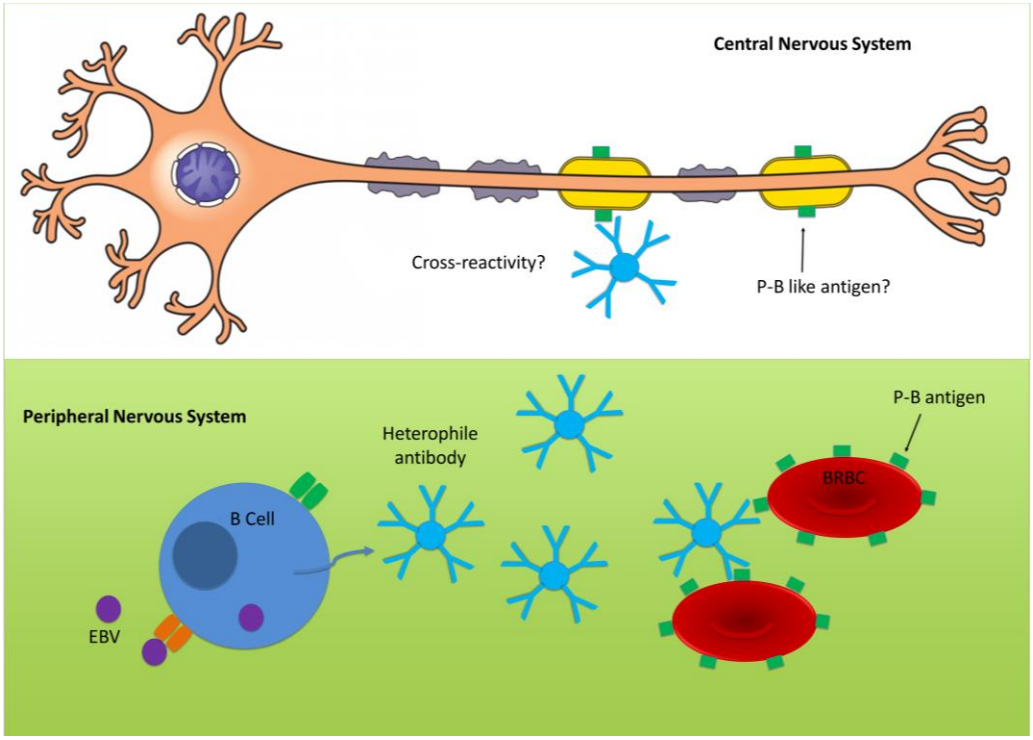

Figure 1. Possible mechanism of neuronal autoreactivity caused by IM. A simplified view of proposed autoreactivity due to EBV infection. B cells specific for the P-B antigen are infected by EBV and produce IgM heterophile antibodies. These heterophile antibodies bind and agglutinate bovine red blood cells (BRBC) that possess P-B antigen. Myelin gangliosides display antigens structurally similar to P-B antigen, possibly allowing heterophile antibodies to cross react and promote autoreactivity.

Chapter 2: Materials & Methods

2.1 Study Cohort and Data

Biological samples were collected as part of a previously described cohort of 32 mother-infant pairs in Uganda [45] (PHICS: Primary Herpesvirus in Children Study). All study procedures were approved by the relevant human subjects protection committees (in Kampala, Uganda; Seattle, Washington; and Vancouver, Canada), and all subjects provided informed consent. Seventeen of the mothers and none of the infants were HIV-infected. Oral swab specimens were collected from the mothers and infants followed from birth every week for EBV quantitative polymerase chain reaction (qPCR) testing to determine the infants' level of exposure and the week of acquisition, as described in Gantt et al [45]. Blood specimens were collected from mothers at the time of delivery, and from infants at 6 weeks of age and every 4 months thereafter for serologic testing. Only those infant samples collected prior to EBV infection were included in the analyses.

Leftover sera from monospot positive (IM) individuals were collected from St. Paul's Hospital, Vancouver, BC, with ethics approval from the University of British Columbia. The age range spanned from 3 years to 36 years with a mean age of 24 years (7 males and 2 females). Additionally, plasma samples from PHICS were previously studied and donated by Dr. Soren Gantt[45]. These samples were 6 mathced samples before and after EBV infection with the age range of 6 weeks to 1 year (3 males and 3 females). Positive and negative controls from an IM test kit (monospot) were used.

2.2 Measurement of antibodies to major neutralizing EBV antigens.

Using the luciferase immunoprecipitation system (LIPS) assay, as previously described [12,48], fusion proteins containing the EBV glycoproteins gp350 or gH/gL linked to Renilla luciferase gene were constructed in the mammalian expression vector pREN. 293T cells were transfected with the vector, cell lysates were incubated with human sera, immunoprecipitated with protein A/G beads, washed, and coelenterazine substrate was added to detect luciferase activity. Light units (LU) were measured in a luminometer which correspond to the level of EBV glycoprotein-specific antibodies [10].

2.3 B cell neutralization assay.

B cell neutralization activity was measured using infection of Raji (B cells) cells as described in [48]. Plasma was serially diluted in duplicate wells of 96-well round-bottom plates containing 25 µL of cRPMI in duplicate. B95-8/F virus (12.5 µL diluted to achieve an infection frequency of 1-5%) was added and incubated at 37°C for 1 hour. cRMPI (12.5 µL) containing 4×10^6 Raji cells/ml was added to each well and incubated for another hour at 37°C. The cells were then pelleted, washed once with cRPMI, and re-suspended in cRMPI. Antibody concentration or serum dilution is reported relative to the final infection volume (50 µL). After 3 days at 37°C, cells were fixed in 2% paraformaldehyde. The percentage of GFP+ Raji cells was determined on a BD LSRII cytometer. To account for any false-positive cells due to auto-fluorescence in the GFP channel, the average $\%GFP^+$ cells in negative control wells (n=5-10) was subtracted from each well. Percent neutralization in each well was defined as: [%GFP+ cells in the positive control wells containing virus alone (n=5 wells) – %GFP+ cells in the antibody containing well] / %GFP+ cells in the positive control wells × 100. The percent neutralization for

each well was plotted as a function of the $\log_{10}$ of the MAb concentration. The neutralization curve was fit using the log (inhibitor) vs response- variable slope (four parameters) analysis in Prism 7.03 (GraphPad Software).

2.4 Epithelial cell neutralization assay

AGS cells (1.5×10^4 per well) were seeded into a 96-well tissue culture plate. The following day, plasma was diluted 1:4 in complete F12 media in a final volume of 20 µl in a 96 well round bottom plate followed by the addition of 20 µl of 25X concentrated epithelial cell-tropic M81virus that expresses a luciferase reporter gene [49,50] and incubated for 15 minutes in triplicate. Media was aspirated from the AGS cells and replaced by the antibody-virus mixture and incubated at 37°C. Forty-eight hours later, the media was aspirated and replaced with 100 µl of Steadyglo luciferase reagent (Promega). Seventy five microlitres from each well was transferred to an opaque white-bottom 96-well plate and the relative luciferase units (RLU) in each well were determined using a FluoroSkan Ascent luminometer (ThermoFisher). To account for any background luciferase activity, the average RLUs from negative control wells (n=5-10) were subtracted from each well. Percent neutralization in each plasma containing well was defined as: RLUs in the positive control wells containing virus alone (n=5 wells) – RLUs in the plasma containing well] / RLUs cells in the positive control wells × 100. The percent neutralization for each well was plotted as a function of the $\log_{10}$ mAb concentration. The neutralization curve was fit using the $\log_{(\text{inhibitor})}$ versus response-variable slope (four parameters) and analyzed using Graphpad Prism 6 software.

2.5 Antibody-dependent cell-mediated cytotoxicity assay (ADCC)

Plates (96 well) were coated with recombinant EBV gp350 or gH/gL [12,51] at 400 ng/ml. Serial 8-fold (for gp350 ADCC) and 4-fold (for gH/gL ADCC) dilutions of sera were added to the wells, incubated for 15 minutes, and 5 x 10^5 NK-92-CD16 cells/wells were added and incubated for 5 hours at 37ºC. NK-92-CD16 cells express human CD16-176V and GFP [52]. The cells were washed with PBS, stained with APC-Cy7-conjugated anti-CD107a antibody for 30 minutes, and fixed with paraformaldehyde. The percentage of NK-92-CD16 expressing CD107a on their surface was analyzed by flow cytometry.

2.6 Antibody subclass binding

EBV proteins gp350, gH/gL, gp42, and gB, as well as tetanus toxoid, were each conjugated to MagPlex microspheres (beads) of different regions using an antibody coupling kit (Luminex Corp.) [53]. Antigen-bead conjugates were blocked, washed and mixed with serially diluted serum samples. After 1-hour incubation at room temperature, the beads were washed and mixed with secondary antibody conjugated to PE. Secondary antibodies used were specific to either: IgG1, IgG2, IgG3, IgG4, and IgA. After 1-hour incubation with secondary antibody, the beads were washed, and mean fluorescence intensity was measured using Luminex LX-200 instrument. Background was set as the mean fluorescent index (MFI) registered with antigen-beads incubated with secondary antibody (no sample). Background reading was subtracted from all experimental sample measurements. All samples were tested in duplicate.

2.7 Luminex bead-based glycan array

Both the monospot positive serum samples and the PHICS plasma samples were sent to Augusta University where a Luminex bead-based glycan array was used to measure the anti-glycan IgM antibodies present, as described by Purohit et al[54]. The glycan array includes approximately 200 glycans. The samples were sent in two batches. Batch 1: six PHICS matched pre- and post-EBV infection samples, and two monospot positive samples. Batch 2: seven monospot positive samples. The glycan array data was done in duplicates (biological replicate) in relative fluorescence units (RFU) and the average of the two runs presents the mean fluorescent units (MFI) which is how the results are presented in this study. The data was analyzed using R version 3.6.3, GraphPad Prism and JMP Pro 15.0.

2.8 Statistical analyses

As described in Gantt et al [45], the cumulative incidence of primary infection with EBV was calculated using Kaplan-Meier methods. Risk factors for primary infection were assessed by fitting Cox proportional hazards models. Antibodies were treated as time-dependent covariates in these models; for antibodies that were measured multiple times for each infant, values were carried forward no longer than three months. The proportional hazards assumption was assessed by testing for an interaction between the covariate and log-transformed time. For models in which the proportional hazards assumption was violated, we provided separate estimates for two time periods: 0-6 months and >6 months of age. SAS version 9.4 (SAS Institute, Cary, North Carolina), JMP and R statistical software were used to present the data. *P* values of <0.05 were considered statistically significant. Given the exploratory nature of the analyses and the small sample size, hazard ratios for EBV glycoprotein-specific antibody isotypes were not adjusted for

multiple comparisons. The values in the Cox model are predicted concentrations of antibody isotypes at a set serum dilution, therefore the hazard ratios are treated for a continuous variable. A post-hoc power analysis, using the Hsieh and Lavori method [55], as shown in Figure 2 is presented in order to show the effect size required given the study sample size. Assumptions for the post-hoc power analysis were that all infants would eventually become infected with EBV, and that there is a negative correlation of 50% between EBV infection any antibody of interest, after adjusting for other covariates.

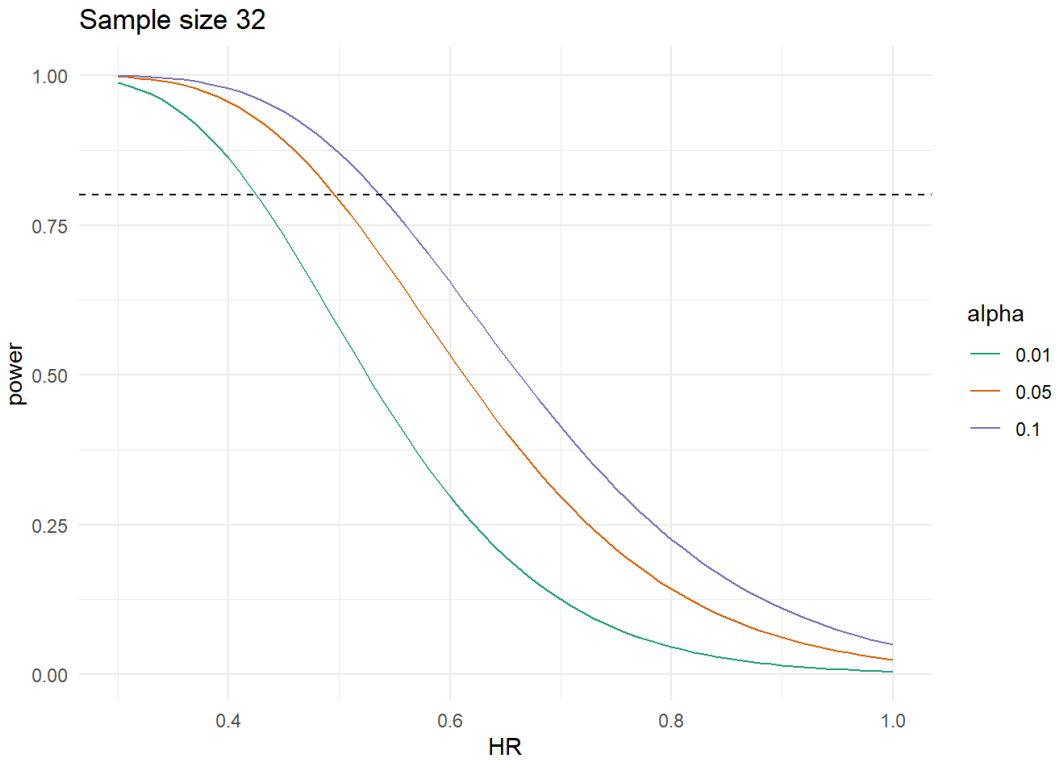

Figure 2. Power analysis of antibody effect size. Using the Hsieh and Lavori survival (2000) analysis method with 32 subjects. Power to detect protection for any antibody against Epstein-Barr virus infection is shown. We have approximately 80% power to detect at least a 50% protection (HR: 0.5) for any antibody (alpha: 0.05, orange line).

Chapter 3: Results

3.1 PHICS Study Subjects and samples

Thirty-two women and their full-term newborn infants were followed and weekly oral swabs were tested by EBV qPCR to determine the time of infant EBV infection and exposure to viral shedding (see [45] for additional details). As shown in Figure 3A, the cumulative incidence of infant EBV infection was 12.9% (95% CI, 5.1%-30.9%) at 6 months and 47.4% (95% CI, 31.3%-66.6%) at 12 months. As seen in Figure 3B, while no EBV infections occurred among HUU infants in the first 6 months, HEU infants began acquiring EBV infection within the first month; maternal HIV infection showed a hazard ratio (HR) of 7.2 (95% CI, 2.4-22.2; $P<0.001$) after adjusting for the intensity of shedding exposure [45]. Plasma samples obtained from study infants at 6 weeks of life and every 4 months thereafter, and from mothers at the time of delivery, were used for subsequent EBV-specific antibody assays.

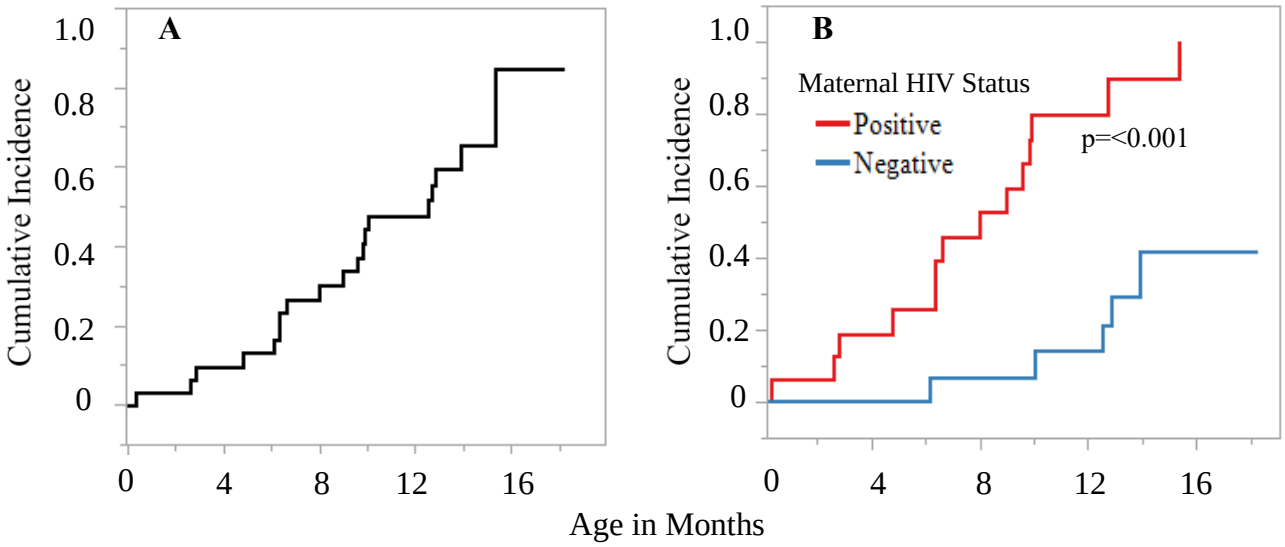

Figure 3. **Cumulative incidence of primary EBV infection in infants. A**, Postnatal infections occurring in the first 18 months of life for primary children are shown. **B**, Stratified data showing infants born to human immunodeficiency virus type 1 (HIV-1)–uninfected women (blue line) and HIV-1–infected women (red line). Kaplan-Meier methodology used to estimate cumulative incidence of infection and log-rank test used to compare curves.

3.2 Antibody binding to gp350 and gH/gL

Binding antibodies to the major targets of B cell and epithelial cell neutralization (gp350 and gH/gL, respectively) were significantly higher in HEU compared to HUU infants, and in HIV-infected compared to HIV-uninfected mothers (Figure 4). Median $\log_{10}$ antibody levels (measured in LU) to gp350 in HEU infants were 5.1 (IQR: 3.9 – 5.9) in the first (6-week) infant sample, 4.1 (IQR: 2.7 – 5.9) in all HEU infant samples, and 5.6 (IQR: 4.0 – 6.1) in HIV-infected mothers. Median $\log_{10}$ antibody levels to gp350 in HUU infants were 4.3 (IQR: 3.2 – 5.0) in the first infant sample, 3.2 (IQR: 2.6 – 5.0) in all HUU infant samples, and 4.6 (IQR: 3.1 – 5.6) in HIV-uninfected mothers (Figure 4A). Median $\log_{10}$ antibody levels to gH/gL in HEU infants were 5.6 (IQR: 4.6 – 6.0) in the first infant sample, 4.8 (IQR: 2.9 – 6.0) in all HEU infant samples, and 5.9 (IQR: 5.2 – 6.0) in HIV-infected mothers. Median $\log_{10}$ antibody levels to gH/gL in HUU infants were 5.0 (IQR: 3.7 – 5.4) in the first infant sample, 3.7 (IQR: 2.6 – 5.4) in all HUU infant samples, and 5.2 (IQR: 4.0 – 5.7) in HIV-uninfected mothers (Figure 4B). Using Cox regression, with adjustment for maternal HIV status, there was no evidence of protection by EBV binding antibodies (Table 1).

3.3 Neutralizing antibodies

Although antibody binding measures to gp350 and gH/gL have been shown to correlate well with neutralizing activity [10,48], we speculated that this may not hold true in HIV infection. As such, we assessed neutralizing activity using a functional assay in which antibody-mediated inhibition of infection of either B cells or epithelial cells by a recombinant GPF-EBV is measured by flow cytometry (Figure 4C and D). Using exact 2-sample Wilcoxon test, $\log_{10}$ of

the 50% inhibitory concentration (IC_{50}) of neutralizing antibody in B cells was significantly higher in HEU infants (1.8, IQR 0.7 – 2.6) compared to HUU infants (0.7, IQR 0.7 – 1.2; p<0.001). Similarly, percent neutralization in epithelial cells was significantly higher in HEU infants (94.4%, IQR 11.7% – 99.5%) than HUU infants (64.4%, IQR 0% – 89.5%; p=0.001). Among all infants, neutralizing antibody titers were positively correlated with risk of EBV acquisition in univariate analysis, but this association was no longer statistically significant after adjustment for maternal HIV status (Table 1).

3.4 Antibody-dependent cellular cytotoxicity

NK cells mediate classical antibody-dependent cellular cytotoxicity (ADCC). Activation of NK for cytotoxicity results in expression of CD017a on their surface, which is a marker for degranulation of the cells. Levels of NK cell activation from antibody bound to gp350 or gH/gL were not significantly different between HEU and HUU infants, unlike neutralization and binding antibody titers (Figure 5). Median levels of NK cell activation (%CD107a+) by gp350-binding were 1.9% (IQR 1.2% – 12.0%) in HEU infants and 2.3% (IQR 1.3% – 6.5%) in HUU infants (p=0.31). Median levels by gH/gL-binding were 0.43% (IQR 0.11% – 1.33%) in HEU infants and 0.29% (IQR 0.05% – 1.70%) in HUU infants (p=0.31). Because the proportional hazards assumption was violated for these models, we presented separate estimates for 0-6 month of age and >6 months of age. When adjusted for maternal HIV status, neither gp350 nor gH/gL ADCC levels were associated with the risk of EBV infection in infants, in either time period (Table 1).

3.5 IgG subclass-specific binding to EBV envelope glycoproteins

Immunoglobulin isotypes against EBV glycoprotein gp350, gH/gL, gp42 and gB, as well as tetanus toxoid, were measured by the Luminex method in infant blood at 6 weeks of age. As expected [46,47], levels of IgG subclasses against tetanus toxoid tended to be lower among HEU than HUU infants (Table 2**)**, and levels of IgA, which does not readily cross the placenta, were negligible (data not shown). In contrast, but consistent with other findings from this study, the opposite trend of higher titers among HEU infants was seen for several EBV-specific subclasses of IgG (Table 2). Using Cox regression, higher IgG2 to gH/gL was associated with an increased risk of EBV infection; none of the antibodies measured showed evidence of a protective effect against EBV acquisition.

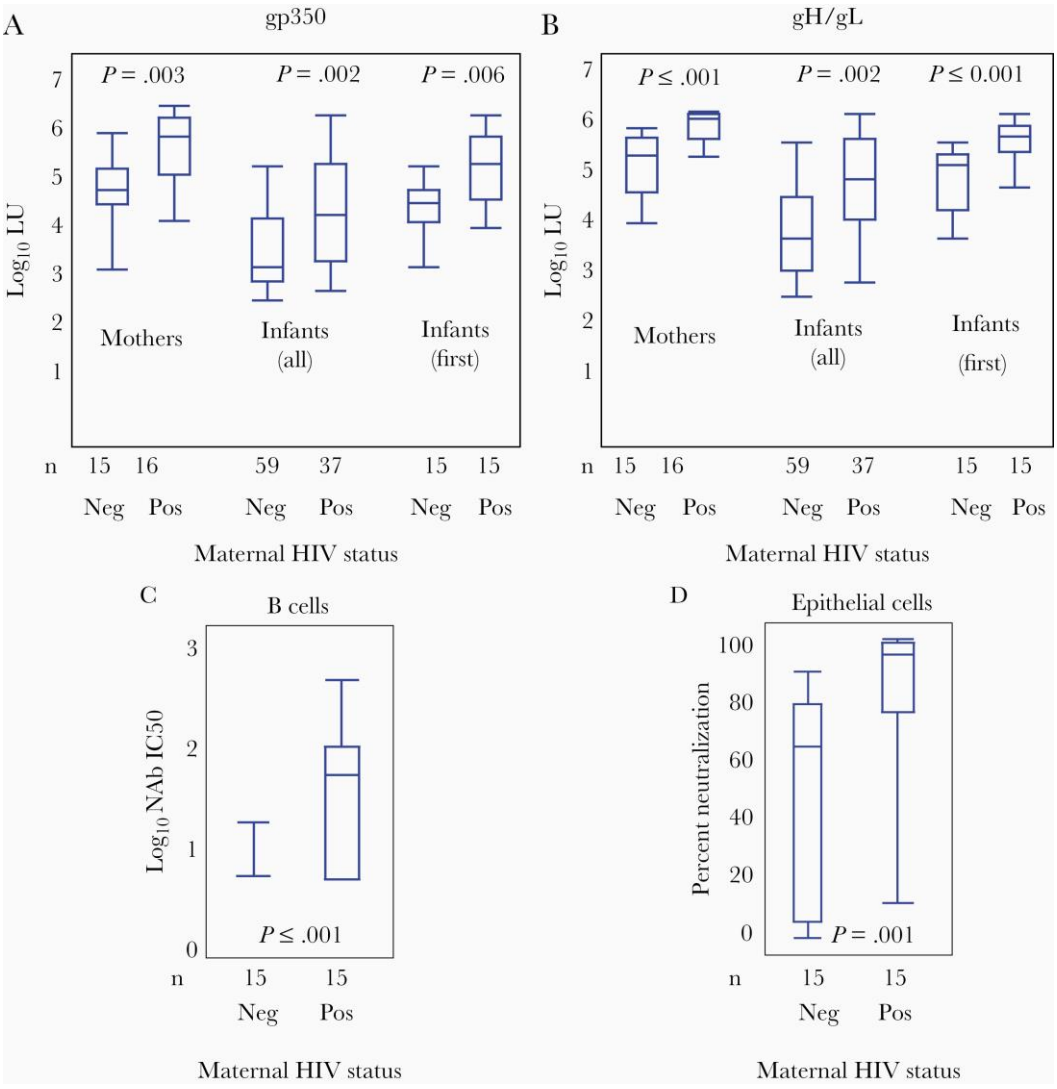

Figure 4. Binding antibody titers against gp350 and gH/gL and neutralization levels of EBV infection in B and epithelial cells. Distribution of $\log_{10}$ light units (LU) for gp350 (**A**) and gH/gL (**B**), a measure of the antibody titer, by maternal HIV status, positive (Pos) or negative (Neg). Data shown for first maternal samples at birth, all infant samples prior to EBV infection, and first infant sample (6 weeks). Neutralizing antibody levels in B cells (**C**) and epithelial cells (**D**) in infants based on the HIV status of their mothers. Boxes represent the interquartile range, whiskers represent the minimum and maximum values, and horizontal bars show the median values. Note that the left boxplot in Fig 4C has a small IQR and therefore the box is not visible. Exact 2-sample Wilcoxon test was used to compare both maternal data and data from first sample per infant by maternal serostatus. Generalized estimated equations were used to compare all infant pre-EBV infection samples against gp350 and gH/gL between HIV exposed and unexposed infants. P <0.05 considered significant.

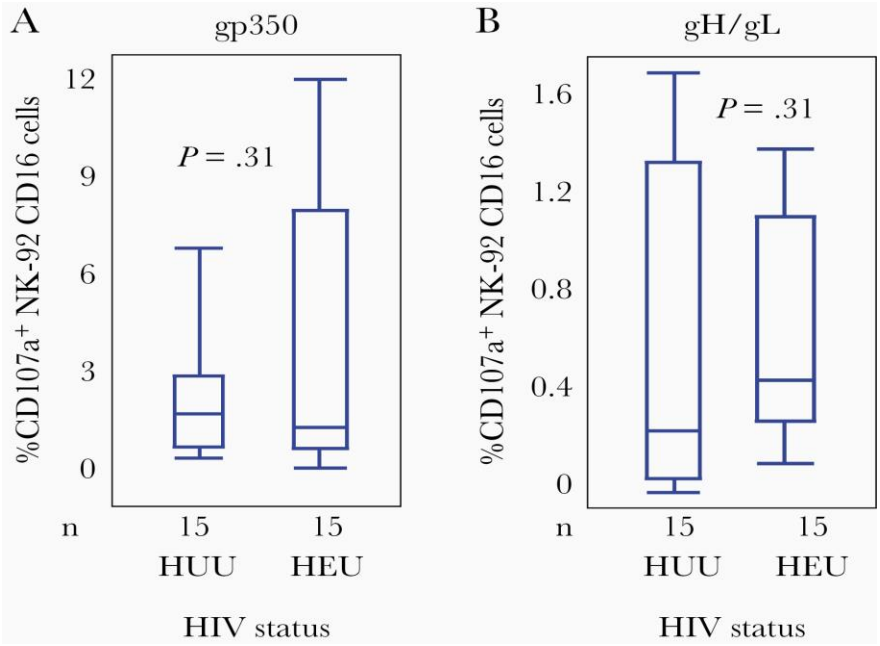

Figure 5. Antigen-dependent cellular cytotoxicity (ADCC) levels against gp350 and gH/gL. The percentage of NK-92 CD16 cells that express CD107a on their surface in response to gp350 (**A**) and gH/gL (**B**) is shown for HIV-exposed uninfected (HEU) and HIV-unexposed uninfected (HUU) infants. First sample used after birth (6 weeks, pre-EBV infection). Boxes represent the interquartile range, whiskers represent the minimum and maximum values, and horizontal bars show the median values. Exact 2-sample Wilcoxon test used for comparing groups. P <0.05 considered significant.

Table 1. Unadjusted and Adjusted Cox Model Estimates for Risk of EBV Acquisition[1]

Covariate	Unadjusted HR (95% CI)	p-value	Adjusted HR[2] (95% CI)	p-value
gp350 binding antibody (per log10 increase)	1.4 (0.6 – 3.2)	.417	1.0 (0.4 – 2.5)	.945
gH/gL binding antibody (per log10 increase)	2.0 (0.7 – 5.7)	.182	1.2 (0.4 – 3.5)	.704
B cell neutralization IC50 (per log10 increase)	3.0 (1.3 – 7.0)	.012	1.2 (0.4 – 3.2)	.734
Epithelial cell neutralization (per log10 increase)	1.02 (1.00 – 1.04)	.019	1.02 (1.00 – 1.03)	.100
gp350 ADCC CD107a+% (per one-unit increase)				
0-6 months	0.03 (0.0 – 6.9)	.209	0.1 (0.0 – 8.6)	.279
>6 months	1.3 (1.1 – 1.6)	.004	1.2 (1.0 – 1.4)	.086
gH/gL ADCC CD107a+% (per one-unit increase)				
0-6 months	0.5 (0.04 – 6.59)	.596	0.32 (0.01 – 8.18)	.488
>6 months	1.88 (0.78 – 4.51)	.158	2.39 (0.86 – 6.66)	.096

[1] Abbreviations HR = hazard ratio, CI = confidence interval.

[2] Multivariate analysis adjusted for maternal HIV status

Table 2. Unadjusted and Adjusted Cox Model Estimates for Risk of EBV Acquisition[1] Isotype specific binding to EBV glycoproteins

Antibody Isotype specific to EBV glycoprotein	HEU vs. HUU[2] *t* (p-value)	Unadjusted HR (95% CI)	p-value	Adjusted HR[3] (95% CI)	p-value
IgG1 to gp350	0.86 (.400)	1.00 (0.99 – 1.00)	.178	1.00 (0.99 – 1.00)	.405
IgG1 to gH/gL	**3.21 (.004)**	1.00 (0.99 – 1.01)	.231	1.00 (0.99 – 1.00)	.679
IgG1 to gp42	**2.25 (.033)**	1.00 (0.99 – 1.00)	.335	0.99 (0.99 – 1.00)	.746
IgG1 to gB	0.08 (.940)	0.99 (0.99-1.00)	.927	0.99 (0.99 – 1.00)	.773
IgG1 to TT	**-3.26 (.003)**	1.00 (0.99 – 1.00)	.101	1.00 (0.99 – 1.00)	.786
IgG2 to gp350	**2.53 (.023)**	**1.01 (1.00 – 1.02)**	**.012**	1.01 (0.99 – 1.01)	.210
IgG2 to gH/gL	-0.73 (.473)	1.12 (0.92 – 1.37)	.261	**1.29 (1.03 – 1.60)**	**.024**
IgG2 to gp42	1.03 (.319)	1.19 (0.90 – 1.58)	.230	1.05 (0.79 –1.39)	.743
IgG2 to gB	1.57 (.137)	1.00 (0.99 – 1.01)	.572	1.00 (0.99 – 1.01)	.665
IgG2 to TT	**-2.82 (.009)**	1.00 (0.99 – 1.00)	.254	1.00 (0.99 – 1.00)	.620
IgG3 to gp350	1.75 (.100)	1.01 (0.99 – 1.01)	.077	1.00 (0.99 – 1.00)	.367
IgG3 to gH/gL	**2.27 (.036)**	1.05 (0.95 – 1.16)	.354	0.98 (0.88 – 1.09)	.679
IgG3 to gp42	0.42 (.676)	0.93 (0.77 – 1.11)	.416	0.83 (0.67 – 1.03)	.089
IgG3 to gB	1.57 (.137)	1.00 (0.99 – 1.00)	.172	1.00 (0.99 1.00)	.651
IgG3 to TT	0.48 (.634)	1.00 (0.99 – 1.00)	.278	1.00 (0.99 – 1.00)	.448
IgG4 to gp350	1.72 (.096)	1.41 (0.60 – 3.3)	.432	0.84 (0.35 – 2.01)	.694
IgG4 to gH/gL	0.57 (.576)	1.09 (0.55 – 2.18)	.799	1.09 (0.55 – 2.17)	.803
IgG4 to gp42	0.19 (.847)	1.00 (0.30 – 3.4)	.990	1.94 (0.52 – 7.34)	.325
IgG4 to gB	0.75 (.460)	1.25 (0.91 – 1.73)	.170	1.27 (0.91 – 1.78)	.155
IgG4 to TT	-1.18 (.249)	1.00 (0.99 – 1.00)	.617	1.00 (0.99 – 1.00)	.467

[1] Abbreviations HR = hazard ratio, CI = confidence interval.
[2] HEU= HIV-exposed uninfected infants, HUU= HIV-unexposed uninfected infants
[3] Multivariate analysis adjusted for maternal HIV status
Bolded values show statistical significance

3.6 Glycan Array

Antibodies to over 200 glycans were measured in samples before and after EBV infection and IM samples from teens and adults. Median antibody levels to all the glycans in IM subjects were 157 (IQR: 43 – 538) which was significantly higher ($P<0.0001$) than pre-EBV and post-EBV infection median antibody levels 24 (IQR: 10 – 58), 25 (IQR: 10 – 74), respectively. As expected, there was no significant difference between the antibody levels before and after EBV infection ($P=0.17$). These values are represented by boxplot and Wilcoxon paired comparisons in Figure 6.

The reactivity to glycans in specific brain gangliosides was then compared. The glycan array contained the glycans of several gangliosides (Table 3). There was variability in the level of asialo GM2 reactivity across all samples (pre-EBV, post-EBV, and IM); however, there was a significant increase ($P<0.0001$) in the reactivity against GM1a (with Neu5Ac) and GD2 (with Neu5Gc) IM sera only, as shown in the heatmap in Figure 7. Quantitatively these reactivities were measured and the GM1a median MFI for the IM group was 336 (IQR: 135 – 1000) where as the median MFI for the pre- and post-EBV infection groups were 66 (IQR: 10 – 202) and 95 (IQR: 13 – 204), respectively. Similarly, the GD2 reactivity had the median MFI for the IM group of 446 (IQR: 102 – 1200) where as the median MFI for the pre- and post-EBV infection groups were 64 (IQR: 60 – 132) and 64 (IQR: 67 – 147), respectively.

Table 3. **Ganglioside glycans and their structure included in glycan array**

Glycan	Structure*
GM2 – asialo	GalNAc-β-1,4 -Gal-β-1,4-Glc-β-Sp1
GM2	GalNAc-β-1,4-(Neu5Ac-α-2,3)-Gal β-1,4-Glc-β-Sp
GM2 (KDN)	GalNAc-β-1,4-(KDN)-Gal β-1,4-Glc-β-Sp
GD2	GalNAc-β-1,4-(Neu5Ac-α-2,8-Neu5Ac-α-2,3)-Gal β-1,4-Glc-β-Sp
GD3	Neu5Ac-α-2,8-Neu5Ac-α-2,3-Gal β-1,4-Glc-β-Sp
GD3 (Gc-Ac)	Neu5Gc-α-2,8-Neu5Ac-α-2,3-Gal β-1,4-Glc-β-Sp
GD3 (KDN-Ac)	KDN-α-2,8-Neu5Ac-α-2,3-Gal β-1,4-Glc-β-Sp
GT3	α -2,8-Neu5Ac- α -2,8-Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal-β-1,4-Glc-Sp2
Neu5Ac-α-2,8-GT3	Neu5Ac-α-2,8-Neu5Ac-α-2,8-Neu5Ac-α-2,8 Neu5Ac-α-2,3-Gal β-1,4-Glc-β-Sp
GM1a	Gal β-1,3-GalNAc-β-1,4-(Neu5Ac-α-2,3)-Gal β-1,4-Glc-β- Sp1
GM1a (Gc)	Gal β-1,3-GalNAc-β-1,4-(Neu5Gc-α-2,3)-Gal β-1,4-Glc-β-Sp1
GD2 (Gc)	GalNAc-β-1,4-(Neu5Gc-α-2,8-Neu5Ac-α-2,3)-Gal β-1,4-Glc-β-Sp
T-antigen	Gal β-1,3-GalNAc-β-Sp1

Additional information on the glycans in the array can be found in Purohit et al[72]. When two glycans have the same name, the parentheses denote the change in structure. Gc indicates a Neu5Gc has replaced a Neu5Ac. Gc-Ac indicates that the order of the sialic acids is now reversed. KDN indicates that a Neu5Ac or Neu5Gc has become a deaminoneuraminic acid.

*KDN: deaminoneuraminic acid, Sp: OCH2CH2CH2NH2, Sp1: NH(CH3)OCH2CH2NH2, Sp2: O(CH2)3NHCOCH2(OCH2CH2)5CH2CH2NH2.

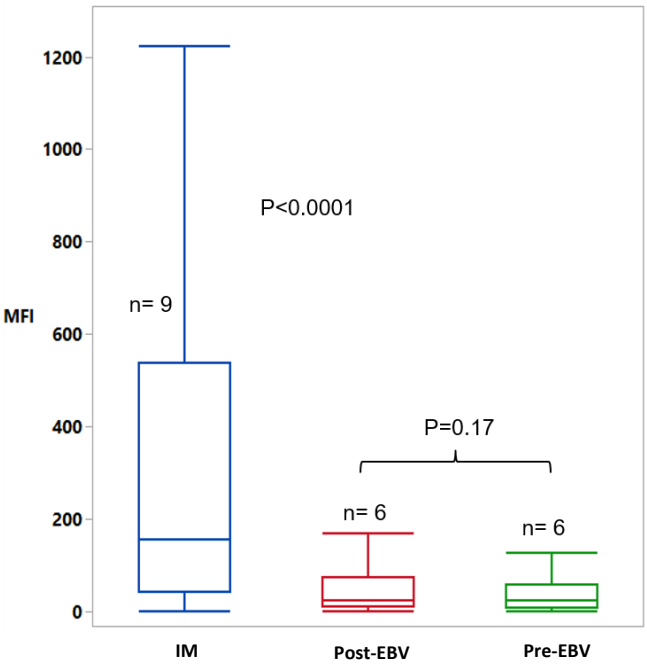

Figure 6. Antibodies against all glycans in IM patients versus pre and post EBV infected infants. Reactivity against total glycans in patients with IM (blue box plot) versus infants pre- and post-EBV infection (green and red boxplot, respectively). The glycan-binding reactivity was measured in relative fluorescent units and the mean fluorescent intensity (MFI) is shown from two runs. Boxes represent the interquartile range, whiskers represent the minimum and maximum values, and horizontal bars show the median values. Negative and extremely high outliers excluded from analysis. Non-parametric paired Wilcoxon test used for comparing groups. $P<0.05$ considered significant.

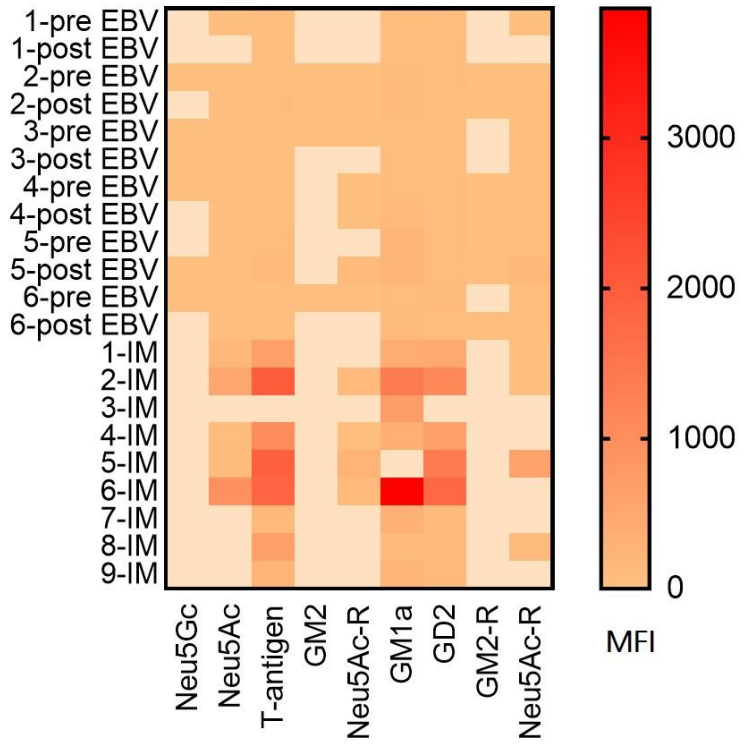

Figure 7. Heatmap – antibody reactivity against brain-specific glycans. The glycan-binding reactivities against brain-specific glycans were clustered using dendrogram algorithm in GraphPad Prism. Six samples from infants before and after EBV infection (pre/post-EBV) and 9 monospot positive samples were analyzed (IM). The glycan-binding reactivity is measured in mean fluorescence units (MFI).

Chapter 4: Discussion

The data generated from this study provide great insight into the immunopathology of EBV infection and its affects on both infants and adults. The not-/so protective role of maternal antibodies against EBV infection is discussed as well as the connection between increased anti-ganglioside antibodies during acute Epstein-Barr infection (IM) and MS.

4.1 Protection from Epstein-barr virus infection

An effective EBV vaccine is a priority due to its oncogenic burden on millions of children and adults in the developing world. A vaccine would also be able to decrease healthcare costs in the developed world by eliminating infectious mononucleosis, which has been associated with an increased risk of developing Hodgkin lymphoma and MS[40,56–58]. In this study, we evaluated a large panel of potential humoral correlates of protection against primary EBV infection in a cohort of Ugandan infants, beginning at birth. Almost all infants in this region are infected with EBV by the age of 3 years [1,39,45]. Within this cohort, HEU infants were infected as early as 2 weeks after birth, significantly earlier than HUU infants. Since maternal antibody levels are highest in the first 6 months of infancy, and maternal HIV-1 infection impairs transplacental antibody transfer [17,38,39,46,47,59], we hypothesized that HEU infants would have lower levels of maternal neutralizing EBV-specific antibodies.

Surprisingly, not only did we not find any evidence for protection against EBV acquisition from neutralizing antibodies, HEU infants had significantly higher titers than HUU infants. Pathogen-specific differences in the level and type of antibody that is transferred across the placenta have been described [60–62]. With the advantage of having the strong perturbation of maternal HIV-1 infection on infant EBV acquisition risk, our results argue that neutralizing maternal antibodies are not strongly protective against EBV infection, rather than simply being

unable to detect an association. Incomplete protection by humoral immunity would be consistent with EBV superinfection, which may occur in healthy individuals as with other viruses [63]. It should be noted that due to the small sample size we may not have been able to discern small protective effects, nor would be able to assess the possible impact of combinations of modestly protective antibodies. However, we estimate reasonable power to detect an antibody measure that conferred >50% protection against EBV acquisition (Figure 2).

The temporal pattern of EBV infection during infancy, and the effect of earlier acquisition in infants of mothers with HIV or malaria infections, strongly suggests that maternal antibody provides protection [1,39,45]. Thus, although the ability of maternal antibody to prevent infant EBV infection has not been formally proven, our findings indicate the potential protective role of non-neutralizing antibody functions. Although we did not detect a significant association between ADCC activity and risk of EBV acquisition, it is interesting that the levels of cytotoxicity were relatively similar between the HEU and HUU infants, in contrast to neutralizing antibodies. Of note, these assays were limited to only two viral antigens, which may not reflect ADCC responses against infected cells or viral particles *in vivo*. Of specific IgG subtypes binding EBV glycoproteins, one positive correlation between antibody level and EBV infection (IgG2 binding to gH/gL) was observed. This association is tenuous given the small sample size and large number of comparisons; however, it is conceivable that some antibody functions might increase risk of EBV acquisition, as has been noted for HIV-1 [64]. This would again be highly valuable information for EBV vaccine development.

In conclusion, although it is still unclear which maternal antibodies might provide protection against EBV infection during early infancy, our study indicates that neutralizing antibodies did not play a major role. Additional studies are needed to further characterize non-

neutralizing functions of maternal that may be protective, the identification of which would be invaluable for the development of a prophylactic EBV vaccine. Importantly, our findings do not preclude the possibility that a vaccine might be able to protect against EBV infection through induction of highly neutralizing antibodies [44]. Furthermore, a vaccine that is unable to provide sterilizing immunity but that is able to modulate EBV infection to prevent disease might be equally valuable [3,4].

4.2 Infectious mononucleosis and multiple sclerosis

Dramatic increases were observed in natural IgM antibodies against multiple glycans including blood group antigens (T-antigen) and gangliosides that are also found to correlate with MS [65]. Additionally, the high reactivity against GM1a and GD2 glycans in IM is an important result that warrants further investigation. Patients with MS have been found to exhibit higher GM1a and GD2 reactivity in their sera as well [66–68]. There is also evidence to suggest that the presence of anti-ganglioside antibodies may be correlated with more rapidly progressing forms of MS[67,69]. Recently, Boligan et al. demonstrated that patients with multiple sclerosis have serum IgG with higher reactivity to Neu5Gc and Neu5Ac than patients with other inflammatory and noninflammatory neurological diseases[70]. These findings support the idea that these sialic acids are playing a role in both IM and MS and may be an important connecting factor between the two diseases.

There remain, however, some limitations to interpreting the glycan array data. Due to the batch to batch variability, it is difficult to compare the results of samples run on different days. Additionally, the second batch of monospot positive samples were analyzed without an EBV

uninfected and infected control. In future, we would analyze all samples concurrently, enabling us to compare the all monospot-positive samples to the pre- and post-EBV infection samples. It is important to note that the glycan array used in our study was only able to measure IgM antibodies, which are the short lives antibodies that indicate recent primary infection with EBV which tend to disappear after 4 to 6 weeks. After this time point, IgG antibodies to EBV remain indefinitely in the serum and can indicate past infection[71]. IM is an autoimmune reaction of the body to acute EBV infection in the first 4 to 6 weeks, it is possible that were we to measure IgG antibodies instead of IgM, that the results would be different between IM patients and pre and post EBV infected infants.

This project laid the foundation for future research into the specificities of EBV-infected B cells and the presence of immunoglobulin targeting the glycans of gangliosides in infectious mononucleosis. The discovery of antibodies targeting the glycans of GM1a and GD2 in IM provides a new link of EBV infection to MS.

4.3 Future Directions

A prospective cohort study of late-onset EBV infection and IM would be a very informative addition to this thesis. Few prospective cohort studies have captured incident EBV infection in adolescents or young adults and quantified the exact changes in the immune system before and after EBV infection. It would also be useful to follow this cohort for an extended period of time to see whether any of the adolesents who developed IM become diagnosed with MS later in life.

References

1. de-The G, Day N. E., Geser A., et al. Sero-epidemiology of the Epstein-Barr virus: preliminary analysis of an international study – a review. *IARC Sci. Publ.* **11**, 3–16 (1975).
2. Stratton, K; Durch, J; Lawrence, R. Overview of Analytic Approach and Results. in *Vaccines for the 21st Century* (ed. Stratton K; Durch J; Lawrence R) (National Academies Press, 2000). doi:10.17226/5501
3. Cohen, J. I., Fauci, A. S., Varmus, H. & Nabel, G. J. Epstein-Barr Virus: An Important Vaccine Target for Cancer Prevention. *Sci. Transl. Med.* **3**, 107fs7 LP-107fs7 (2011).
4. Cohen, J. I., Mocarski, E. S., Raab-Traub, N., Corey, L. & Nabel, G. J. The need and challenges for development of an Epstein-Barr virus vaccine. *Vaccine* **31 Suppl 2**, B194–B196 (2013).
5. Odumade, O. A., Hogquist, K. A. & Balfour, H. H. Progress and Problems in Understanding and Managing Primary Epstein-Barr Virus Infections. *Clin. Microbiol. Rev.* **24**, 193 LP – 209 (2011).
6. Hadinoto, V. *et al.* On the dynamics of acute EBV infection and the pathogenesis of infectious mononucleosis. *Blood* **111**, 1420–1427 (2008).
7. Hutt-Fletcher, L. M. & Chesnokova, L. S. Integrins as triggers of Epstein-Barr virus fusion and epithelial cell infection. *Virulence* **1**, 395–398 (2010).
8. Taylor, G. S., Long, H. M., Brooks, J. M., Rickinson, A. B. & Hislop, A. D. The immunology of Epstein-Barr virus-induced disease. *Annu. Rev. Immunol.* **33**, 787–821 (2015).
9. Laichalk, L. L. & Thorley-Lawson, D. A. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J. Virol.* **79**, 1296–1307 (2005).
10. Bu, W. *et al.* Kinetics of Epstein-Barr Virus (EBV) Neutralizing and Virus-Specific Antibodies after Primary Infection with EBV. *Clin. Vaccine Immunol.* **23**, 363–369 (2016).
11. Borza, C. M., Morgan, A. J., Turk, S. M. & Hutt-Fletcher, L. M. Use of gHgL for attachment of Epstein-Barr virus to epithelial cells compromises infection. *J. Virol.* **78**, 5007–5014 (2004).
12. Bu, W. *et al.* Immunization with Components of the Viral Fusion Apparatus Elicits Antibodies That Neutralize Epstein-Barr Virus in B Cells and Epithelial Cells. *Immunity* **50**, 1305-1316.e6 (2019).
13. HOAGLAND, R. J. The transmission of infectious mononucleosis. *Am. J. Med. Sci.* **229**, 262–272 (1955).
14. Balfour Jr, H. H. *et al.* Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. *J. Infect. Dis.* **207**, 80–88 (2013).
15. Dunmire, S. K., Hogquist, K. A. & Balfour, H. H. Infectious Mononucleosis. *Curr. Top. Microbiol. Immunol.* **390**, 211–240 (2015).
16. Gantt, S. *et al.* Prospective Characterization of the Risk Factors for Transmission and Symptoms of Primary Human Herpesvirus Infections Among Ugandan Infants. *J. Infect. Dis.* **214**, 36–44 (2016).
17. Slyker, J. A. *et al.* Clinical and Virologic Manifestations of Primary Epstein-Barr Virus

(EBV) Infection in Kenyan Infants Born to HIV-Infected Women. *J. Infect. Dis.* **207**, 1798–1806 (2013).
18. De-The, G. IS BURKITT'S LYMPHOMA RELATED TO PERINATAL INFECTION BY EPSTEIN-BARR VIRUS? *Lancet* **309**, 335–338 (1977).
19. Evans, A. S. Infectious mononucleosis in the Armed Forces. *Mil. Med.* **135**, 300–304 (1970).
20. Rickinson, A. B., Long, H. M., Palendira, U., Münz, C. & Hislop, A. D. Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory. *Trends Immunol.* **35**, 159–169 (2014).
21. Silins, S. L. *et al.* Asymptomatic primary Epstein-Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load. *Blood* **98**, 3739–3744 (2001).
22. Tynell, E. *et al.* Acyclovir and prednisolone treatment of acute infectious mononucleosis: a multicenter, double-blind, placebo-controlled study. *J. Infect. Dis.* **174**, 324–331 (1996).
23. Paul, J. R. & Bunnell, W. W. The presence of heterophile antibodies in infectious mononucleosis. *Am. J. Med. Sci.* **267**, 178–188 (1974).
24. Palma, J., Tokarz-Deptuła, B., Deptuła, J. & Deptuła, W. Natural antibodies - facts known and unknown. *Cent. J. Immunol.* **43**, 466–475 (2018).
25. Golaszewska, E., Kurowska, E., Duk, M. & Koscielak, J. Paul-Bunnell antigen and a possible mechanism of formation of heterophile antibodies in patients with infectious mononucleosis. *Acta Biochim. Pol.* **50**, 1205–1211 (2003).
26. Tilton, R. C., Dias, F. & Ryan, R. W. Comparative evaluation of three commercial tests for detection of heterophile antibody in patients with infectious mononucleosis. *J. Clin. Microbiol.* **26**, 275–278 (1988).
27. Altman, M. O. & Gagneux, P. Absence of Neu5Gc and Presence of Anti-Neu5Gc Antibodies in Humans-An Evolutionary Perspective. *Front. Immunol.* **10**, 789 (2019).
28. Banda, K., Gregg, C. J., Chow, R., Varki, N. M. & Varki, A. Metabolism of vertebrate amino sugars with N-glycolyl groups: mechanisms underlying gastrointestinal incorporation of the non-human sialic acid xeno-autoantigen N-glycolylneuraminic acid. *J. Biol. Chem.* **287**, 28852–28864 (2012).
29. Schnaar, R. L., Gerardy-Schahn, R. & Hildebrandt, H. Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiol. Rev.* **94**, 461–518 (2014).
30. Stenager, E. A global perspective on the burden of multiple sclerosis. *Lancet Neurol.* **18**, 227–228 (2019).
31. Kaufmann, M. *et al.* 60/30: 60% of the Morbidity-Associated Multiple Sclerosis Disease Burden Comes From the 30% of Persons With Higher Impairments. *Front. Neurol.* **11**, 156 (2020).
32. Ascherio, A., Munger, K. L. & Lunemann, J. D. The initiation and prevention of multiple sclerosis. *Nat. Rev. Neurol.* **8**, 602–612 (2012).
33. Brosnan, C. F. & Raine, C. S. Mechanisms of immune injury in multiple sclerosis. *Brain Pathol.* **6**, 243–257 (1996).
34. Claes, N., Fraussen, J., Stinissen, P., Hupperts, R. & Somers, V. B Cells Are Multifunctional Players in Multiple Sclerosis Pathogenesis: Insights from Therapeutic Interventions. *Front. Immunol.* **6**, 642 (2015).

35. Thacker, E. L., Mirzaei, F. & Ascherio, A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann. Neurol.* **59**, 499–503 (2006).
36. Nielsen, T. R. *et al.* Multiple Sclerosis After Infectious Mononucleosis. *Arch. Neurol.* **64**, 72–75 (2007).
37. Endriz, J., Ho, P. P. & Steinman, L. Time correlation between mononucleosis and initial symptoms of MS. *Neurol. Neuroimmunol. NeuroInflammation* **4**, 1–8 (2017).
38. Biggar, R. J. *et al.* Primary epstein-barr virus infections in african infants. I. Decline of maternal antibodies and time of infection. *Int. J. Cancer* **22**, 239–243 (1978).
39. Piriou, E. *et al.* Early age at time of primary epstein-barr virus infection results in poorly controlled viral infection in infants from western Kenya: Clues to the etiology of endemic Burkitt lymphoma. *J. Infect. Dis.* **205**, 906–913 (2012).
40. Rickinson, A. B. & Fox, C. P. Epstein–Barr Virus and Infectious Mononucleosis: What Students Can Teach Us. *J. Infect. Dis.* **207**, 6–8 (2012).
41. Chan, K. H., Tam, J. S. L., Peiris, J. S. M., Seto, W. H. & Ng, M. H. Epstein–Barr virus (EBV) infection in infancy. *J. Clin. Virol.* **21**, 57–62 (2001).
42. Smith, N. A. *et al.* Differences in the Epstein-Barr Virus gp350 IgA Antibody Response Are Associated With Increased Risk for Coinfection With a Second Strain of Epstein-Barr Virus. *J. Infect. Dis.* **219**, 955–963 (2019).
43. Sashihara, J. *et al.* Soluble Rhesus Lymphocryptovirus gp350 Protects against Infection and Reduces Viral Loads in Animals that Become Infected with Virus after Challenge. *PLOS Pathog.* **7**, e1002308 (2011).
44. McGuire, A. T. *et al.* Neutralizing antibodies protect against oral transmission of lymphocryptovirus. *Cell reports Med.* **In Press**, (2020).
45. Gantt, S. *et al.* Prospective Characterization of the Risk Factors for Transmission and Symptoms of Primary Human Herpesvirus Infections among Ugandan Infants. *J. Infect. Dis.* **214**, 36–44 (2016).
46. Jones, C. E. *et al.* Maternal HIV Infection and Antibody Responses Against Vaccine-Preventable Diseases in Uninfected Infants. *JAMA* **305**, 576–584 (2011).
47. Cumberland, P. *et al.* Maternal HIV Infection and Placental Malaria Reduce Transplacental Antibody Transfer and Tetanus Antibody Levels in Newborns in Kenya. *J. Infect. Dis.* **196**, 550–557 (2007).
48. Sashihara, J., Burbelo, P. D., Savoldo, B., Pierson, T. C. & Cohen, J. I. Human antibody titers to Epstein–Barr Virus (EBV) gp350 correlate with neutralization of infectivity better than antibody titers to EBV gp42 using a rapid flow cytometry-based EBV neutralization assay. *Virology* **391**, 249–256 (2009).
49. Tsai, M.-H. *et al.* Spontaneous lytic replication and epitheliotropism define an Epstein-Barr virus strain found in carcinomas. *Cell Rep.* **5**, 458–470 (2013).
50. Bilger, A. *et al.* Leflunomide/teriflunomide inhibit Epstein-Barr virus (EBV)- induced lymphoproliferative disease and lytic viral replication. *Oncotarget* **8**, 44266–44280 (2017).
51. Kanekiyo, M. *et al.* Rational Design of an Epstein-Barr Virus Vaccine Targeting the Receptor-Binding Site. *Cell* **162**, 1090–1100 (2015).
52. Binyamin, L. *et al.* Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. *J. Immunol.* **180**, 6392–6401 (2008).

53. Snijder, J. *et al.* An Antibody Targeting the Fusion Machinery Neutralizes Dual-Tropic Infection and Defines a Site of Vulnerability on Epstein-Barr Virus. *Immunity* **48**, 799-811.e9 (2018).
54. Purohit, R., Zheng, S., Cavinaw, D. J. & Elzen, M. Van Den. Gangliosides are recognized by Natural Killer T cells and B cells in infectious mononucleosis – its implications for MS. **430**, 221242 (2018).
55. Hsieh, F. Y. & Lavori, P. W. Sample-size calculations for the cox proportional hazards regression model with nonbinary covariates. *Control. Clin. Trials* **21**, 552–560 (2000).
56. Levin, L. I., Munger, K. L., O'Reilly, E. J., Falk, K. I. & Ascherio, A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann. Neurol.* **67**, 824–830 (2010).
57. Handel, A. E. *et al.* An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PLoS One* **5**, (2010).
58. Hjalgrim, H. *et al.* Characteristics of Hodgkin's lymphoma after infectious mononucleosis. *N. Engl. J. Med.* **349**, 1324–1332 (2003).
59. Chan, K. ., Tam, J. S. ., Peiris, J. S. ., Seto, W. . & Ng, M. . Epstein–Barr virus (EBV) infection in infancy. *J. Clin. Virol.* **21**, 57–62 (2001).
60. Martinez, D. R. *et al.* Fc Characteristics Mediate Selective Placental Transfer of IgG in HIV-Infected Women. *Cell* **178**, 190-201.e11 (2019).
61. Jennewein, M. F. *et al.* Fc Glycan-Mediated Regulation of Placental Antibody Transfer. *Cell* **178**, 202-215.e14 (2019).
62. Pou, C. *et al.* The repertoire of maternal anti-viral antibodies in human newborns. *Nat. Med.* **25**, 591–596 (2019).
63. Walling, D. M., Brown, A. L., Etienne, W., Keitel, W. A. & Ling, P. D. Multiple Epstein-Barr Virus Infections in Healthy Individuals. *J. Virol.* **77**, 6546 LP – 6550 (2003).
64. Kim, J. H., Excler, J.-L. & Michael, N. L. Lessons from the RV144 Thai Phase III HIV-1 Vaccine Trial and the Search for Correlates of Protection. *Annu. Rev. Med.* **66**, 423–437 (2015).
65. Freedman, M. S. *et al.* Anti-alpha-glucose-based glycan IgM antibodies predict relapse activity in multiple sclerosis after the first neurological event. *Mult. Scler.* **15**, 422–430 (2009).
66. Arnon, R. *et al.* Anti-ganglioside antibodies in multiple sclerosis. *J. Neurol. Sci.* **46**, 179–186 (1980).
67. Sadatipour, B. T., Greer, J. M. & Pender, M. P. Increased circulating antiganglioside antibodies in primary and secondary progressive multiple sclerosis. *Ann. Neurol.* **44**, 980–983 (1998).
68. Marconi, S. *et al.* Anti-GD2-like IgM autoreactivity in multiple sclerosis patients. *Mult. Scler.* **12**, 302–308 (2006).
69. Acarín, N. *et al.* Different antiganglioside antibody pattern between relapsing-remitting and progressive multiple sclerosis. *Acta Neurol. Scand.* **93**, 99–103 (1996).
70. Boligan, K. F. *et al.* Xenogeneic Neu5Gc and self-glycan Neu5Ac epitopes are potential immune targets in MS. *Neurol. - Neuroimmunol. Neuroinflammation* **7**, (2020).
71. De Paschale, M. & Clerici, P. Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. *World J. Virol.* **1**, 31–43 (2012).
72. Purohit, S. *et al.* Multiplex glycan bead array for high throughput and high content

analyses of glycan binding proteins. *Nat. Commun.* **9**, (2018).