THE IMMUNOLOGY AND EPIDEMIOLOGY OF VIRAL INFECTIONS IN HUMANS

by

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the degree of	Doctor of Philosophy	
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Abstract

Viral infectious disease is of vast global importance, specifically human herpesviruses and coronaviruses, which have implications in human health and disease, along with the risk of emergence as a pandemic. Understanding the relationship between viral pathogenesis and immune responses can help unravel the complex immunopathology that results from infection by these viral pathogens and their contribution to morbidity and mortality in different age groups. Human herpesviruses have been associated with development of several diseases in adulthood and old age, including autoimmune disease, neurodegenerative disease, and malignancies. Much less is known about previous exposures to coronaviruses and their role in the development of COVID-19 immunopathology during SARS-CoV-2 infection.

My research findings have identified that healthy adults and neonates share remarkedly similar populations of immune cells. Furthermore, while there are subtle differences in the expression of different immune related genes, their response to stimulus results in very comparable gene expression signatures. In addition, there was also no discernible difference between the detection of human herpesviruses between the brains of healthy donors and those with Alzheimer's disease. Finally, this research also noted that previous infection with coronavirus is ubiquitous and initial infection typically occurs during childhood in the population.

By researching the complex relationship between immunity and viral response, it is evident that while viruses like herpesvirus can be associated with the development of disease like Alzheimer's disease, determining causality is challenging. Conversely, coronavirus, a forgotten pathogen during respiratory virus testing, is found to be quite a common infection throughout the population.

Viral infectious disease is a globally important subject of health research that requires linking the biology of the pathogen to the pathology of disease and contribution of the immune system to disease outcome. My research reflects the complexity of studying immune ontogeny, attributing early life viral exposure to disease development, and understanding the role of past viral exposure's influence on immunopathology in related viruses.

Lay Summary

Human herpesviruses and coronaviruses are viral pathogens of global importance, causing a spectrum of illnesses from mild asymptomatic disease to malignancies and death. Understanding the role of the immune system in response to infection is key to unravelling the complex relationship that past exposures to viruses may contribute to human health and disease. Through investigating the presence of virus or antibodies against viruses, the infection history of a person can be revealed and correlated to disease. The results of my research found that the immune systems between healthy adults and babies are quite similar; that it is hard to attribute human herpesviruses to the development of Alzheimer's disease; and that humans are exposed to coronaviruses starting at a young age. Together, this thesis highlights the complexity in studying immune response to disease between different ages and attributing viral exposure to disease development and outcomes.

Preface

All of the work presented in this dissertation was conducted at the British Columbia Center for Disease Control – Public Health Laboratory and British Columbia Children's Hospital Research Institute, both institutes are affiliated with the University of British Columbia. All projects and associated methods were approved by the University of British Columbia's Research Ethics Board (H20-00653; H20-01431; and H18-01561)

Chapter 2. The work conducted in this chapter is unpublished, independent work by me, A. Liu.

Chapter 3. A version of this material and its associated figures and tables has been submitted and is under revision. The experimental design and planning were done primarily by W. Panenka, G. Tanunliong, and me. The data analysis in this chapter was done by K. Gricas and samples provided by collaborators as part of the Rush Memory and Aging Project.

Chapter 4. A version of this material and its associated figures and tables is being prepared as a manuscript for submission. The experimental design and planning were done primarily by A. Jassem, S. Gantt, I. Sekirov, G. Tanunliong, and me. The data analysis in this chapter was primarily done by me, with the assistance of M. Irvine and D. Skowronski. Samples in this study were provided by D. Skowronski.

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List of Abbreviations

AB – Amyloid Beta

ACE2 – Angiotensin-Converting Enzyme 2

AD – Alzheimer's Disease

BCR – B-Cell Receptor

CBMC – Cord Blood Mononuclear Cell

cDC – Conventional Dendritic Cell

CLIA – Chemiluminescent Immunoassay

CMV – Cytomegalovirus

CNS – Central Nervous System

CoV – Coronavirus

COVID-19 – Coronavirus Disease 2019

CTL – Cytotoxic T Lymphocyte

DAMPS – Damage Associated Molecular Patterns

DC – Dendritic Cell

DEM – Dementia

DENV – Dengue Virus

EBV – Epstein-Barr Virus

EOS – Early Onset Sepsis

FACS – Fluorescence-Activated Cell Sorting

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HCoV – Human Coronavirus

HHV – Human Herpesvirus

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HPV – Human Papillomavirus

HS – Hippocampal Sclerosis

HSV – Herpes Simplex Virus

IFN – Interferon

IL – Interleukin

ISG – Interferon Stimulated Gene

KSHV – Kaposi Sarcoma-Associated Herpesvirus

LAL – Limulus Amebocyte Lysate

LOS – Late Onset Sepsis

MAP – Memory and Aging Project

MCI – Mild Clinical Impairment

MHC – Major Histocompatibility Complex

MIA – Multiplex Immunoassay

mRNA – Messenger Ribonucleic Acid

MSD – Mesoscale Discovery

NK – Natural Killer

NLR – Nod-Like Receptor

PAMP – Pathogen Associated Molecular Pattern

PBMC – Peripheral Blood Mononuclear Cell

PCR – Polymerase Chain Reaction

pDC – Plasmacytoid Dendritic Cell

PRR – Pattern Recognition Receptor

qPCR – Quantitative Polymerase Chain Reaction

RBD – Receptor Binding Domain

RIG-I – Retinoic Acid Inducible Gene I

ROS – Religious Orders Study

RSV – Respiratory Syncytial Virus

scRNAseq – Single-Cell RNA Sequencing

SHM – Somatic Hypermutation

SOCS3 – Suppressor of Cytokine Signalling 3

Tc – Cytotoxic T-Cell

TCR – T-Cell Receptor

Tfh – Follicular Helper T-Cell

TGFβ – Tissue Growth Factor Beta

Th – Helper T-Cell

TLR – Toll-Like Receptor

TNFα –Tissue Necrosis Factor Alpha

VDJ – Variable-Diversity-Junctional

VZV – Varicella Zoster Virus

WNV – West Nile Virus

Acknowledgements

I would like to acknowledge the support of my supervisors, Drs. Soren Gantt and Agatha Jassem, who were instrumental not only in my research, but also my growth as a person. Their patience as I found my way, will not have gone by without my deepest appreciation; their encouragement gave me the opportunity to find value in myself. While the challenges of research and life wore, their mentorship and understanding never took the enjoyment out of science for me. Furthermore, I would like to thank the unwavering support of my supervisory committee, including Drs. Mel Krajden, Manish Sadarangani, and Scott Tebbutt, and their understanding of the events that have occurred during my time as a PhD student and how they have affected the trajectory and development of my career.

In addition, I would also like to thank the funding agencies that have made this research and PhD possible. This includes the BC Children's Hospital Foundation and the University of British Columbia, who have provided me with fellowship funding for the duration of my studies. In addition, the Michael Smith Foundation for Health Research, who provided the majority of funding towards the COVID-19 related research.

Finally, I would like to recognize the support of my fellow students, colleagues, and collaborators who have made this work possible. Your support has been invaluable in the success of my research.

Dedication

I would like to dedicate this thesis to my mother, Yvonne Liu, who during my studies passed away after a long battle with relapsing-remitting endometrial cancer. Her perseverance and resolution found me immeasurable strength and gave me pause to seek the life that I wanted to live. Without the encouragement of her in the midst of the challenges she faced, I would not be here today. For this, I am forever grateful for the moments and memories that I have been able to share during the finite amount of time she was here.

Chapter 1 – Introduction

1.1 Overview

Not everything goes according to plan, in life or research. Certainly not my PhD, during which I had not one, but two supervisors leave UBC during my tenure. However, the impacts of having to switch labs and projects twice on my academic career, including a shift to respond to the coronavirus disease 2019 (COVID-19) pandemic, was far less than the experience of caring for my now deceased mother during her battle with cancer. Nonetheless, during my time as a doctoral student, I have been overwhelmed by the support of my mentors and how they buoyed my development as a researcher. The lack of continuity has resulted in a somewhat loose relatedness between the projects that I have worked on. Ultimately, however, as presented in this thesis, these projects are all linked to viral infection and the immune system, subjects that I am extremely passionate about.

1.2 Selected Viruses of Global Importance

Viruses represent a diverse group of pathogens that infect all forms of life on Earth, including plants, animals, bacteria, and archaea. Structurally, viruses contain (1) genetic material, i.e., DNA or RNA that encodes for proteins needed for replication, (2) a protein coat or capsid, that protects the genomic material, and in some cases (3) an envelope of lipids to adapt and evade the immune system. Unlike other pathogens, viruses require a host to replicate their genome, thus on their own, they are incapable of

producing offspring. Although viruses cause disease through direct effects, e.g., cytolytic destruction of tissues, indirect damage from the host inflammatory response to infection is frequently even more pathological.

In 2012, it was estimated that there were 219 virus species known to infect humans, with 3-4 new virus species discovered annually [1]. Many of the emergent processes that viruses are known to undertake to jump into humans have been studied. These typically include non-human reservoirs, which account for 2/3 of human viruses, which are typically zoonotic in origin [1,2]. Others are specialist viruses that have co-evolved with humans over time, i.e., hepatitis B and herpesviruses [3,4], or have more recent origins like human immunodeficiency virus (HIV) [5]. Most often, the strongest determinant of a whether a virus can jump into another species is their use of a conserved receptor between different hosts [1,6]. Of principal interest in this thesis are the *Herpesviridae* and *Coronaviridae*, two virus families of vast global importance.

1.2.1 Human Herpesviruses

Human herpesviruses HHVs are part of a large family of viruses that infect a plethora of hosts including other mammals, birds, fish, and other invertebrates [7]. Members of the *Herpesviridae* family are morphologically similar spherical shaped viruses comprising of four major components: the core, capsid, tegument, and envelope [7]. Containing a linear piece of double-stranded DNA, genomes for herpesviruses are structurally within a range from 125-230 kbp [8]. However, the natural host range of viruses tends to be

restricted to single species, due to their high adaptivity and long co-evolution with their hosts [7,9]. This typically limits the severity of their symptoms to those who are very young or immunocompromised [9].

A unique aspect of HHVs are their complex life cycles that involve lytic and latent programmes, allowing virus to reside quietly in reservoirs in the host after primary infection. Productive cytolyic infection by HHVs involve the coordination and expression of many genes, some of which manipulate the infected cells and others the immune response [10]. In order to remain in reservoirs undetected, HHVs resort to establishing latency and evade host immune responses by forming episomes [10,11]. During latency, however, HHVs are not completely silenced and continue to express certain proteins to maintain latency and host detection [10]. HHV reactivation can be induced by certain types of stressors and local trauma, resulting in the appearance of cold sores [11] or herpes zoster [12].

1.2.1.1 Alpha, Beta and Gamma HHVs

HHVs belong mainly to 3 different subfamilies defined by biological properties, replication cycles, and genomic analyses: *Alpha*, *Beta*, and *Gammaherpesvirinae*. Herpes simplex virus 1 (HSV-1), 2 (HSV-2) and varicella zoster virus (VZV) are *Alphaherpesvirinae*; Human cytomegalovirus (CMV or HHV-5), HHV-6, and HHV-7 are *Betaherpesvirinae*; Epstein-Barr virus (EBV or HHV-4) and Kaposi sarcoma-associated herpesvirus (KSHV or HHV-8) are *Gammaherpesvirinae* [7].

Typically, humans will be infected by multiple HHVs in their lifetime which establishing latency in host cells, allowing the viruses to remain within reservoirs for their lifetime [9,13]. In general, the mild symptoms of HHV infection lends themselves to long and close association with humans to perhaps provide immunomodulatory effects against other infections and malignancies [14]. Due to their relatedness, members of each subfamily also show unique tropism where they establish latency, for *Alphaherpesvirinae*, the neuron; *Betaherpesvirinae*, the monocytic lineage; and *Gammaherpesvirinae*, the lymphocytes [13].

1.2.1.2 Epidemiology and Seroprevalence

Infection with HHVs is among the most ubiquitous of infections, with ~90% of the population having been infected with one or more HHVs. For alpha-herpesviruses, HSV-1 and HSV-2, ~90% of the world has been infected with either or both viruses [4]. For HSV-1, seroprevalence (i.e. the presence of specific antibodies to HSV-1) is estimated to be 50-65% in developed nations and close to universal for developing nations, acquired early in childhood from contact with family [15]. There is data to suggest that between developed and developing nations, HSV-1 acquisition is delayed into adolescence rather than early childhood [16] in more developed nations. In contrast, HSV-2 infections are less frequent with 15-80% of people in different populations infected [4]. As with HSV-1, developing nations have a markedly higher burden of HSV-2 infection, with African nations having >50% seroprevalence [17]. As

HSV-2 infections are extensively transmitted during sexual activity, acquisition typically increases during sexual debut and levels off in the 40s [18]. For VZV, the remaining alpha-herpesvirus, annual incidence is estimated to occur in the general population at 1.5-3.0 cases per 1000 persons in up to 20% of individuals in their lifetime [19]. Of note, is that 90% of cases of VZV occur during adolescence and VZV is the only HHV for which there is a vaccine. Thus case rates of Varicella have been declining overall in nations with access to the vaccine[20].

Among the *Betaherpesviruses,* the most well studied is HHV-5 or CMV, acquisition is characterized similarly with an age dependent rise in seroprevalence, correlated to socioeconomic level and ethnicity [21]. In developed nations, seroprevalence can range between 50-70% of the population, whereas in lower income nations it can be >70%. As a result, 50% of women of childbearing age are typically sero-negative in developed nations [21]. In comparison for developing nations, CMV is typically acquired very early in life due to breastfeeding and close living conditions with other family members, leading to higher seroprevalence in women [22]. This increases the overall risk for maternal transfer and *in utero* CMV infection [22,23]. Similarly, for HHV-6 and HHV-7, infection occurs during early in life, with peak seroconversion occurring between 1-2 years of age, with infections distributed globally and being nearly universal amongst the adult population [24,25].

Like other HHVs, the *Gammaherpesviruses*, HHV-4 or EBV, is highly prevalent within the healthy population, with infection rates ~90% of individuals worldwide [26]. As with other HHVs, typical age of acquisition is highly correlated with socioeconomic factors that reflect hygiene and crowded living quarters [27]. In developed nations, peak infection occurs during early childhood (2-4 years) or during teenage adolescence (14-18 years), while in developing nations, acquisition occurs during early childhood and individuals are universally positive by age 6 [26,28]. KHSV instead shows several patterns of seroprevalence: high, intermediate, and non-endemic. In many parts of Africa, seroprevalence is between 30 to 70%, typically in areas with high incidence of HIV. Moreover, KSHV is a common malignancy in many areas there [29]. Other areas showing intermediate seroprevalence of KSHV, include countries in the Mediterranean, where rates vary between 10 to 25% [30]. In non-endemic areas, seroprevalence remains under 10%, however within certain populations, seroprevalence can rival highly endemic regions. These typically involve groups highly at risk for HIV, such as men who have sex with men, where rates approach 60% in HIV infected individuals [29,31].

1.2.1.3 Clinical Importance

Although most infections with HHVs are innocuous, in cases involving specific age groups or immunocompromised individuals, primary infection or reactivation of HHVs can have major consequences. For VZV, primary infection (varicella) during childhood causes mild to moderate symptoms that typically resolve without intervention [19]. However, in adolescents and adults, primary infection can cause major neurological and respiratory disease, leading to a high risk of hospitalization and death [32]. Furthermore,

reactivation of VZV in adulthood results in herpes zoster (shingles), which consists of blistering rash and pain that can be debilitating. In contrast, HSV-1 infections are mostly mild causing ulcerations or “cold sores” in the oral cavity, and in some cases the genital tract [33]. HSV-2, however, increases the likelihood of acquiring HIV in at-risk subjects, due to the increased infiltration of CD4 T-cells into genital tissues in the presence of ulcerations [34]. There is, however, increased risk for vertical transmission of HSV-1 and HSV-2, leading to congenital infection during childbirth [35]. This can involve invasion of the central nervous system (CNS) or dissemination into different organs including the liver in the infant [36].

CMV causes different pathologies in different at-risk populations, including fetuses and infants, immunocompromised adults, and in some cases even immunocompetent adults [22]. Most notably, CMV is the most common vertically-transmitted infection and a major cause of congenital abnormalities [21,22]. Complications of congenital CMV infection include low birth weight, microcephaly, and jaundice, while long-term sequelae include sensorineural hearing loss and learning disability [21]. For immunocompromised adults, such as those who are recipients of organ transplants, infection or reactivation can cause inflammation of mucosal membranes and the nervous system [37]. In some cases, CMV can manifest with mononucleosis (expanded in section below) like symptoms in immunocompetent subjects, or Guillain-Barré syndrome [38,39]. While HHV-6 and HHV-7, other beta-herpesviruses are universally found in adult populations, they can cause Roseola or “sixth disease” in children [40,41]. Although typically mild,

this infection can result in rash and fever in children with febrile seizures a resulting complication [41].

As with other HHVs, EBV and HHV-8 are typically acquired asymptomatically in childhood [26]. Notably, although infection with EBV in childhood is typically subclinical, acquiring infection during teenage years and adulthood frequently manifests as infectious mononucleosis, an immunopathologic syndrome that consists of pharyngitis, lymphadenopathy, and a fever that lasts weeks to months [42]. Furthermore, EBV and HHV-8 are oncogenic viruses. EBV can cause several malignancies, such as Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma [42,43]; HHV-8 causes Kaposi's sarcoma and primary effusion lymphoma [29]. In addition, EBV has been linked to other disease such as multiple sclerosis, Parkinson's disease, and lymphomatoid granulomatosis [42].

1.2.2 Coronaviruses

Coronaviruses (CoVs) are a group of enveloped viruses that contain a non-segmented, single-stranded positive stranded RNA genome that are known to infect several vertebrates, including 7 that infect humans (HCoVs) [44]. Structurally, the genome encodes for several core proteins, including replicase (ORF1a/b), spike (S), envelope (E), membrane (M), and nucleocapsid (NP), in addition to other accessory proteins interspersed within these genes [44,45]. Phylogenetic analysis of genomes has separated the CoVs into 4 distinct genera: *Alpha, Beta, Gamma* and *Deltacoronavirus*.

Within the Beta-CoV genus, there is further assignment of subgenera or lineages from A-D. In general, human-relevant endemic CoVs can be found within the Alpha-CoV (HCoV-229E and HCoV-OC43) and Beta-CoV lineage A (*Embecovirus*) (HCoV-HKU1 and HCoV-NL63), while emergent CoVs lie completely within Beta-CoV lineage B (*Sarbecovirus*) (SARS-CoV-1 and SARS-CoV-2) and C (*Merbecovirus*) (MERS-CoV) [44].

Initial discovery of the two endemic CoVs, HCoV-229E and HCoV-OC43, occurred prior to SARS-CoV-1 in 2002, whereas HCoV-HKU1 and HCoV-NL63 were found later in 2004 and 2005, almost 50 years later [44,45]. In that time, two new emergent CoVs became a public health concern, MERS-CoV, which caused isolated outbreaks in the Middle East and South Korea with high mortality rates and SARS-CoV-2, which emerged as a pandemic in 2020. Amino acid analyses of the spike and nucleocapsid proteins from SARS-CoV-2 reveals conservation between different HCoVs, with SARS-CoV-1 and SARS-CoV-2 having the highest similarity (Table 1). However, only the receptor-binding domain (RBD) is conserved between SARS-CoV-1 and SARS-CoV-2, reflecting the differences in cell surface receptors used for internalization between HCoVs [46]. Of note, genomic analyses have also revealed that many Alpha-CoVs and Beta-CoVs and most importantly, all emergent CoV species have been found in diverse bat species [47–49]. Thus, suggesting that bats serve as an important reservoir for recombination and mutations, with eventual spill over into intermediate hosts that ultimately lead to their ability to infect humans [47,49].

Table 1 - SARS-CoV-2 Antigen Amino Acid Similarity

	Spike (Identity)	S1 RBD	Nucleocapsid	Entry Receptor	Source
Beta CoV					
HCoV-HKU1	40% (29%)		34%	N-acetylneuraminic acid	[50,51]
HCoV-OC43	41% (30%)		34%	N-acetylneuraminic acid	[50,51]
SARS-CoV-1	87% (76%)	73%	90%	Angiotensin-converting enzyme 2	[50,51]
MERS-CoV	58% (42%)		49%	Dipeptidyl peptidase-4	[50,51]
Alpha CoV					
HCoV-229E	30%		28%	CD13 (Aminopeptidase N)	[51,52]
HCoV-NL63	28%		29%	Angiotensin-converting enzyme 2	[51,53]

1.2.2.1 Endemic Coronaviruses

Four endemic CoVs—2 alpha (HCoV-229E, HCoV-NL63) and 2 beta (HCoV-HKU1, and HCoV-OC43)—have been estimated to cause between 15-30% of all common cold cases in humans [54]; noting their relatively mild respiratory symptoms and self-limiting illness in healthy immunocompetent humans [55]. However, in infants, elderly, and immunocompromised hosts, these viruses can develop into severe lower respiratory infections [56].

As with many respiratory viruses in temperate regions, such as influenza and human respiratory syncytial virus, HCoVs typically follow a seasonal pattern, with the winter months (December – April in the Northern Hemisphere), showing the highest incidence [57–59]. In the United States, it has been reported that typically, all four endemic CoVs peak through January and wane into the summer months, with HCoV-OC43 the most commonly detected HCoV [60]. Clinical detections also show that the age distribution of PCR confirmed infections was most commonly found in adults 18-65 years of age and were commonly found with co-detected viral species [54,60]. Long term immunity against endemic CoVs has not been well studied, but recent reinfection studies have found that protection lasts for about one year [61]. Furthermore, in a small study, heterologous Alpha-CoV challenge using HCoV-229E after HCoV-NL63 priming resulted in productive infection, suggesting that there is limited cross-protection between strains [62].

1.2.2.2 Emergent Coronaviruses

All emergent CoVs today to date are from the Beta-CoV genus, therefore they show quite high sequence identity between viral strains [44]. Due to their similarity, both lineage B CoVs, SARS-CoV-1 and SARS-CoV-2 share 79% genome sequence identity, while with lineage C MERS-CoV, there is 50% similarity [63,64]. Furthermore, between non-structural genes of SARS-CoV-1 and SARS-CoV-2, there is >85% amino acid sequence identity [64]. Due to the relatedness of strains, SARS-CoV-1 and SARS-CoV-2 share the use of angiotensin-converting enzyme 2 (ACE2) to gain entry into lung epithelial and other cells [65]. In contrast, MERS-CoV uses the dipeptidyl-peptidase 4 (DPP4) receptor to enter lung epithelial and other cells [66].

The resulting clinical symptoms of SARS-CoV-1, SARS-CoV-2, and MERS-CoV infection can be quite varied, from relatively mild symptoms to severe respiratory failure requiring ventilation [44]. For coronavirus disease 2019 (COVID-19), where the mortality rate is lower than SARS and MERS, the elderly (60+ years), men, and people with existing co-morbidities, such as diabetes, hypertension, and other chronic pulmonary disease, are at higher risk to develop severe illness and death [44,64]; younger age groups tend to only have mild or asymptomatic disease [64]. As with many respiratory viruses, symptoms tend to be non-specific to SARS-CoV-2 including fever, fatigue, and non-productive cough [64]. Interestingly, there have been several studies reporting olfactory and taste related symptoms by some patients suffering early from SARS-CoV-2 infection [67,68]. The incubation period for those infected with emergent CoVs has been reported to be between 1-14 days [69].

Currently, there are currently no highly-effective virus-specific therapies for COVID-19, although some treatments have been beneficial in some cases [64]. Controversial therapies such as hydroxychloroquine ultimately showed no benefit in well-controlled studies [70,71]. Remdesivir, which inhibits viral replication, has been shown to reduce recovery times in selected patients, but without any clear reduction in mortality [72]. However, in severe COVID cases, much of the associated morbidity and mortality is immunopathologic; lymphopenia and an elevated neutrophil-lymphocyte ratio are found in severe cases and indicated in poor clinical outcomes [73]. As such, immunomodulatory therapies have been effective in hypoxemic patients, with dexamethasone showing a reduction in mortality [74], with benefits of interleukin (IL)-1 and IL-6 blockers [75].

Vaccines are currently the most effective long-term strategy to prevent and control the spread of SARS-CoV-2. Currently available COVID-19 vaccines rely on presenting the spike antigen of SARS-CoV-2 using a variety of methods, including using adenovirus vectors [76], mRNA nanoparticles [77], recombinant protein [78], and inactivated whole virus [79]. Overall, vaccines have had high success in phase 3 trials, however, the emergence of new genotypic variants of SARS-CoV-2 have been a concern, with some vaccines showing reduced effectiveness with new variants [80].

1.3 Immune-Mediated Protection and Pathogenesis in Viral Infections

Detection and elimination of viral pathogens are key functions of the immune system. These processes involve distinct immunologic functions, including physical barriers as well as innate and adaptive immune responses, which initialize the host's response to a foreign pathogens and generate long-term protection against a pathogen (Fig. 1) [81]. Primary recognition is provided by the class-specific and germ-line encoded receptors of the innate immune cells that begin the generalized response. Then the adaptive immune system follows by developing both cellular and humoral pathogen-specific responses in the form of cytotoxic T-cells and antibodies, respectively, through somatic mutations [82]. However, the delicate balance of these immune response and their resolution to viral infection are crucial to the host and deleterious if left unabated (e.g., in severe COVID-19). Therefore, understanding the processes of the immune system involved in viral infections can provide key insights into the ability of the host to control infection and prevent dissemination.

1.3.1 Innate Immunity

The innate immune system is indispensable for detecting viral pathogens and activating the adaptive immune system. The innate response involves pattern recognition receptors that detect viral components. These include double stranded RNA, single stranded RNA, and DNA, that initiate the production of type I interferons and proinflammatory cytokines in a cell type-specific manner [83]. Most known are the Toll-like receptors (TLR), Nod-like receptors (NLR), and retinoic acid-inducible gene I like

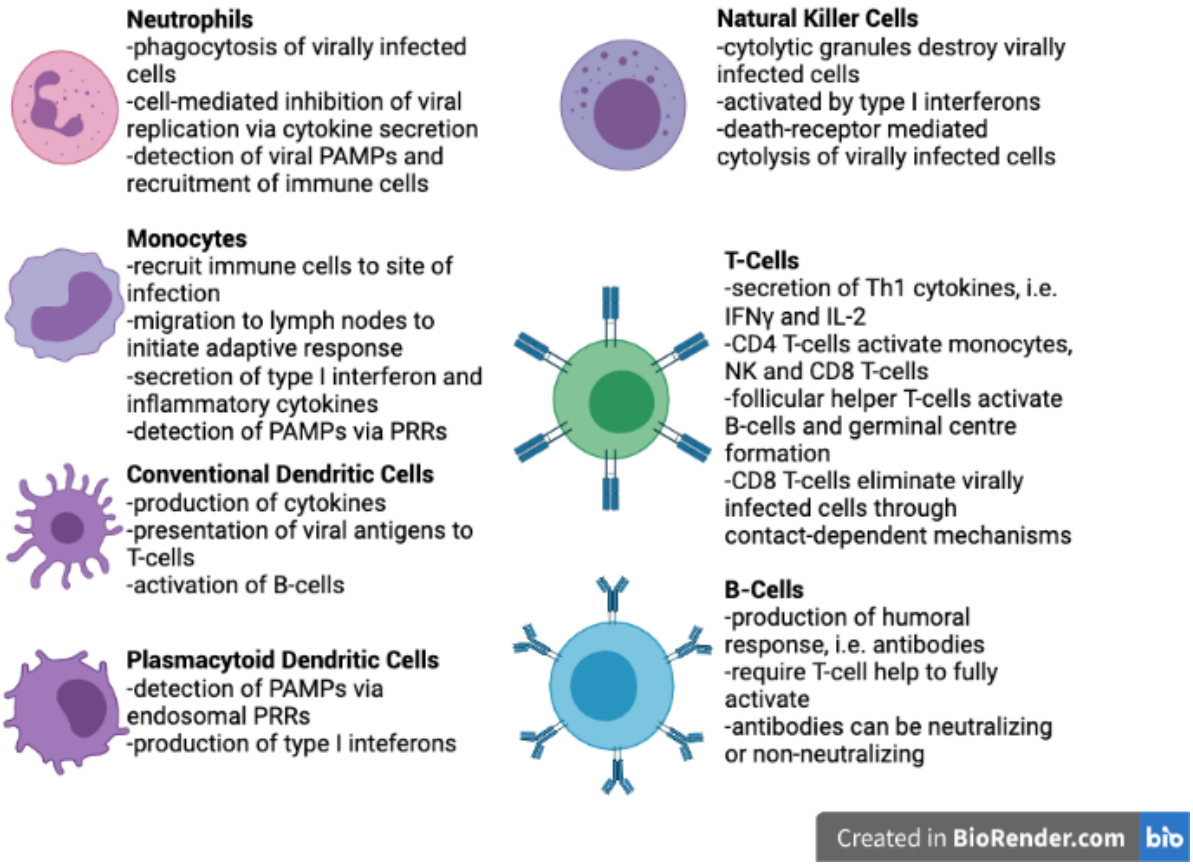


Figure 1 - Overview of Immune Cell Functions During Viral Infection

receptors (RIG-I), which are used by innate immune cells to sense viral components and activate signalling cascades leading to a type I interferon (IFN) response [84]. Production of these proinflammatory cytokines and chemokines are important for eliminating viral infections by recruiting innate and adaptive immune cells by acting as co-stimulatory molecules for T-cell and B-cells to mediate the memory response [84].

Several cell types are involved in innate immunity, including professional (i.e. immune cells) and non-professional cells (i.e. epithelial cells), that mediate the initial response to viral pathogens [82]. Detection of viral pathogen often occurs on the surface of the mucosal pathways by epithelial cells, which can produce type I IFNs and chemokines [81]. Recruitment of professional innate immune cells, such as macrophages and dendritic cells, then proceed to not only eliminate virally infected cells, but also initiate the recruitment, activation, and maturation of adaptive immune cells by presenting viral antigens [85]. Other cells, including neutrophils and natural killer cells, are able to phagocytose virions and virally infected cells, or mediate a cytotoxic response in contact with a virally infected cell [84]. Together, these cells are crucial to the initial detection and elimination of the viral pathogen from the host, while also initiating memory responses by adaptive immune cells.

1.3.1.1 Neutrophils

While neutrophils are the most abundant leukocyte subset and their roles are well characterized in the control of bacterial and fungal infections, their role in immunity

against viral pathogens is less well defined. In early infection, neutrophil infiltration has been seen recruited locally to the site of the virus, following infection with influenza or RSV [86]. Furthermore, in mouse models, depletion of neutrophils before influenza, results in higher viral loads and antigens circulating. Recent studies have also demonstrated antiviral roles for neutrophils, including direct phagocytosis of HSV- and VZV-infected cells, or virions themselves [87]. Neutrophils are not only able to phagocytose infected cells and pathogens, but sense pathogens using PRRs and limit their replication [88]. In addition, neutrophils can also secrete cytokines, chemokines, and other antimicrobial agents, including myeloperoxidase, defensins, and reactive oxygen species, that can mediate immune recruitment and elimination of pathogens [89]. However, it has also been noted that paradoxically neutrophil recruitment can also be detrimental to the host if left unchecked, due to collateral damage of host cells by their cytotoxic functionality [87].

Other viruses, however, are able to use neutrophils as a “Trojan” horse to gain entry into cells and migrate into different physiological compartments (i.e. organs) in the host. Most notably, West Nile virus not only hijacks neutrophils, but uses them to replicate, thus spreading the virus in the host. Of HHVs, CMV exploits neutrophils to spread through the body, causing them to secreting chemo-attractants to recruit other neutrophils, monocytes and DCs. EBV binds to and infects neutrophils and its genomic DNA is found in cells from patients with mononucleosis [87].

1.3.1.2 Monocytes and Dendritic Cells

Monocytes form the other important compartment of leukocytes involved with inflammation and elimination of pathogens. These highly plastic non-dividing cells can undergo further differentiation into highly specialized tissue resident and antigen presenting subsets, such as macrophages and dendritic cells (DCs). As circulating cells, monocytes have been found to play a role in recruitment to sites of infection where they secrete cytokines, act as regulatory cells, and help aid in the viral clearance [81]. When encountering a virus, monocytes will then migrate towards lymph nodes and mature as DCs, presenting antigen to naïve T-cells [90]. Dendritic cells can be divided into 2 main subsets: conventional dendritic cells (cDCs) or plasmacytoid dendritic cells (pDCs). cDCs are more potent antigen presenting cells located in tissue and blood, while pDCs are found mostly in the blood and are specialized for secreting large quantities of type I IFN [91]. During initial infection, cDCs are likely to encounter the virus before pDCs are able to interact and contact virus in the secondary lymphoid organs or peripheral blood [91].

As with other innate immune cells, monocytes are able to recognize many different PAMPs and DAMPS using PRRs such as TLRs and RIG-I, which can sense viral DNA, RNA, and glycoproteins, which leads to productions of type I IFNs and pro-inflammatory cytokines TNFα and IL-1β [92]. cDCs being closely related to monocytes, also recognize many of the same PAMPs and produce type I IFNs. However, pDCs typically only express endosomal nucleic acid sensors TLR-7, TLR-8, and TLR-9, which initiate type I IFN production [91].

One of the most important function of monocytes and DCs, however, is their role as antigen presenting cells. In this role, these phagocytotic cells present processed antigens on MHC class II molecules to activate T-cells and stimulated them to become T-helper cells and memory cells in lymph nodes [93]. Specifically, to fully activate B-cells, interactions with both DCs and follicular helper T-cells are needed to support the activation signals to produce antibodies. Finally, activated cells are additionally able to cross present antigens to CD8 T-cells and induce their activation [91,93].

1.3.1.3 Natural Killer Cells

Natural killer (NK) cells are a unique type of innate lymphoid cell that lack a clonal antigen-specific receptor. Initially identified by their ability to destroy tumour cells without prior sensitization, NK cells were found to be non-reactive in the presence of MHC class I presentation, leading to the “missing-self” hypothesis [94]. In addition to their tumour killing abilities, NK cells are indispensable for the control of many viral infections, including HHVs, poxvirus and HPV. Individuals with primary NK cell deficiencies are predisposed to severe and recurrent viral infections [95]. During infection, NK cells can be activated by stress-induced ligands, through the NKG2 family, which overcome inhibitory signals from KIRs. The balance of these signals determines the action of the cell. Furthermore, cytokines can serve to recruit and activate NK cells, including type I IFNs produced by activated dendritic cells or macrophages [95]. NK cells migrate towards sites of infection and upon activation, secrete additional cytokines, cytotoxic

granules, and use death receptor-mediated cytolysis to destroy virally infected cells. The granules used by NK cells are similar to those used by cytotoxic T-cells, and contain perforin and granzyme [95].

1.3.2 Adaptive Immunity

If the innate immune system is unable to clear a viral pathogen and infection persists, the adaptive immune system is required. The adaptive immune system has both humoral and cellular components that help in eliminating viruses. B-cells are crucial to this process as they produce high affinity immunoglobulins that are specific for viral antigens, and they can also act as antigen-presenting cells to activate T-cells. T-cells are the other important cellular component of the adaptive immune system and are divided into 2 main types, CD4+ or CD8+ T-cells. Helper (CD4+), regulatory (CD4+ Foxp3+), or follicular helper (Tfh) T-cells require co-stimulation by an antigen presenting cell for activation [96]. Cytotoxic (CD8+) T-cells, however, can be directly activated by all cells presenting the Major Histocompatibility (MHC) I complex, but require additional co-stimulation by antigen presenting cells [96].

A key hallmark of B-cells and T-cells are their antigen-specificity, which is determined by recombination of variable, diversity and joining (VDJ) gene segments to form the B-cell receptor (BCR) or the T-cell receptor (TCR), respectively. Due to the random nature of the recombination events, an enormously diverse repertoire of receptors is created during the development of T- and B-cells. However, this process also produces a

number of receptors that will recognize self-proteins [96]. Thus, prior to reaching the periphery, B-cells go through a process of maturation in the bone marrow that results in negative selection of self-recognizing receptors and positive selection of antigen-independent signalling of the BCR. T-cells, likewise, go through a similar process of negative and positive selection in the thymus, where TCRs must bind to MHC I or MHC II with enough strength to be positively selected, while not enough to receive an apoptotic signal [96,97].

1.3.2.1 T-Cells

T-cells have a membrane bound TCR that is able to recognize a unique antigen that is formed by recombination of VDJ regions, presented by MHC I or MHC II [96]. Classified based on the interaction with specific MHC proteins, T-cells are made up of 2 major populations: CD4+ helper T-cells (Th), which interact with MHC II, while CD8+ cytotoxic T-cells (Tc), form interactions with MHC I. In addition, all T-cells have a TCR-CD3 complex and co-receptor that interacts with both co-stimulatory and co-inhibitory ligands [96]. While typically the TCR complex is formed of alpha and beta TCR chains, there is a subpopulation of T-cells that contain a distinctive gamma and delta chains and are found in high abundance in the gut mucosa [98]. These distinct gamma-delta T-cells are unique in that they both contain characteristics of the innate and adaptive system, responding more rapidly than CD4+ and CD8+ T-cells and have shown to be important in protecting against certain HHV infections [96].

CD4+ helper T-cells are the largest population of T-cells and serve a helper function by activating B-cells and CD8+ T-cells. Once activated, these helper T-cells secrete a host of cytokines and can be further classified by the cytokines secreted into Th1 and Th2 populations [96]. Of relevance to viral infections are Th1 cells, which produce interferon gamma (IFNγ) and interleukin (IL)-2, which drive cell mediated response, activating mononuclear cells, NK cells, and CD8 T-cells [93]. In addition, a specialized subset of the T-helper cells, follicular helper T-cells (Tfh), reside in the lymph nodes triggering B-cell activation and germinal centre formation [93].

The other fraction of T-cells includes the CD8+ T-cells which function to eliminate virally infected cells, using the MHC I complex. CD8+ cytolytic T-cells (CTL) through MHC I primarily recognize cytosolic antigens produced by viruses and kill target cells through a contact-dependent mechanism [96]. Once activated, CTLs cause cell death in the target cell, by releasing granules that contain granzymes and perform, which begins the apoptotic process [96].

1.3.2.2 B-Cells

Adaptive humoral immunity to viral pathogens is mediated by antibodies produced by B-cells under the direction of signals from T-cells and antigen presenting cells, such as monocytes and DCs [99]. After encountering an antigen and stimulation by various cytokines, the B-cell will become a memory cell or a plasma cell producing large amounts of antibody. While B-cells can be activated by T-independent antigens, for viral

infections, the vast majority of antibody responses require T-cell help [100]. Circulating B-cells require 2 signals to become activation, first cross linking of the immunoglobulin receptor by antigen, then interacting with T-cells to receive signal 2 [100]. From there, the B-cells can become short lived plasma cells, or enter a follicle to establish a germinal centre, changing production of IgM to other isotypes [100]. This process of class-switch recombination results in changes in the heavy chains that produce different isotypes of immunoglobulin all while keeping the same variable domains as the original [99]. Concurrently, a process called somatic hypermutation (SHM) occurs on the heavy and light chain variable regions where mutations occur [101]. This process allows for the production of higher affinity antibodies towards the antigen through what is known as affinity maturation [100]. Initial exposure to an antigen results in a primary response that is relatively slow and predominantly low affinity IgM, before maturation and SHM leads to higher affinity immunoglobulins [82,102]. Upon repeat exposure, high affinity immunoglobulins are established.

1.3.2.2.1 Neutralizing and Non-Neutralizing Antibodies

For every pathogen encountered, numerous antibodies are produced by B-cells. Importantly, the functions of these antibodies contribute to their ability to prevent infection. Namely, antibodies can be non-neutralizing or neutralizing in function, whereby they are able to inhibit the infectivity of a virion by binding to conserved sites needed for invasion [103]. Several models exist on how they are able to inactivate the virus, requiring either a single antibody clone or engagement by numerous clones [104]. While some studies suggest that the number of antibodies required for neutralization is

correlated with the size of the virion, this is controversial [104]. Nonetheless, neutralizing antibodies must be able to block infection at any number of key steps during entry: this can be attachment to the cell surface, receptors or co-receptors, fusion, membrane penetration or genome injection, or viral genome uncoating [105]. Some of these methods of neutralization have been studied through inhibiting receptor engagement in those with dengue virus (DENV) immune sera [106]. While this receptor binding inhibition is considered a reversible process, some HIV-1 monoclonal antibodies induce shedding of glycoprotein which renders the virion non-infectious [104]. However, it is important to note that *in vitro* neutralization activity is not the sole determinant of *in vivo* protection against viruses.

Non-neutralizing antibodies can also offer some protection from viral infection through interactions with other cells and proteins: antibodies can bind to infected cells to enhance antibody-dependent cellular cytotoxicity, promote opsonization, mast cell activation, and complement activation [103]. Studies have shown that in models of West Nile virus (WNV) and HIV, non-neutralizing antibodies can activate the complement cascade and recruit NK cells to kill infected cells [103,104].

1.3.2.3 Immunopathology

Over time, exposure to different viruses results in different clinical symptoms, most often the infection is resolved with minimal damage to the host. Viral infections cause the recruitment of inflammatory cells and in some cases cause the release of molecules

that induce tissue damage [107]. These inflammatory cells can be from both branches of the immune system, innate and adaptive, causing collateral damage from processes used to eliminate pathogens and infected cells. While the host has counteractive anti-inflammatory processes and cells have inhibitory receptors, when these factors are out of balance, the host tissues can be inadvertently targeted.

1.3.2.3.1 Viral Induced Inflammation and Hyper-Inflammation

As the first line of defence against viral pathogens, the innate immune receptors expressed on the cell's surface or cytoplasm can detect PAMPs. Activation of PRRs, such as TLR, RIG-I, and NLRs, causes the production of pro-inflammatory cytokines and IFNs, in addition to chemokines that recruit and activate other innate immune cells [107]. Of viruses that are able to persist, many are also able to induce innate cells to produce anti-inflammatory molecules such as IL-10 and TGFβ, which reduce host tissue damage [107]. Viruses that can circumvent or interfere with innate immune defences, tend to result in a more damaging response. This is also seen with the adaptive immune responses, especially towards non-cytopathic viral infections, such as HCV and HBV, where host CD8 CTLs cause the most damage to the liver [107,108]. In the case of HIV, HCV, and influenza, recruitment of Th1 and Th17 cells have also been a source of tissue damage as they recruit neutrophils locally [109–111]. Antibodies are also hypothesized to contribute to tissue and organ damage. Immune complex lesions have been found in livers of those with chronic HBV and HCV, due to viral persistence and poor neutralizing IgG [112].

Other factors can also favour the development of immunopathology including the age at which the infection occurs, such that the young and elderly suffer the most severe consequences of infection. Influenza and RSV are two examples where there are disproportionate effects on the young and old [113,114]. In infants, RSV tissue damage results as a failure to mount a proper type I IFN response that prime DCs to mediate CD8, and Th1 CD4 responses [115]. The dose and route of infection has also been hypothesized as contributing to the immunopathology of viral infections as well. The location and amount of virus at the beginning of infection can initially overwhelm the immune system and lead to a damaging response to the host. In addition, the host genetics can play a part in immunopathology [107]. Studies of chemokine receptor CCR5 have found variants with higher resistance to HIV than others [116], while mutations in UNC93B result in children with herpes simplex encephalitis [117].

1.3.2.3.2 Immune Imprinting and Antibody-Dependent Enhancement

It is common for humans to be infected by the same virus more than once in their lifetime. For example, infections with respiratory viruses such as Influenza and Rhinovirus can occur annually. This development of immunity and recognition of a pathogen after first exposure was once called “Original Antigenic Sin” but is now renamed to “immune imprinting”, to account for both the beneficial and harmful effects of immune recall [118]. The most well studied example of this phenomenon is influenza, due to annual reinfections by the virus. Antigenically drifted strains of influenza

dominate each year and antibodies produced from prior infections only provide some protection to a second strain of the same subtype [118]. However, it was found in several studies that during the 2009 H1N1 pandemic that those vaccinated previously or who were born between 1957 and 1968 were linked with greater incidences of medical intervention [119,120]. As H1N1 had been the dominant strain during flu seasons before 2009, much of the population had been exposed to an earlier H1N1 strain as their first flu, therefore producing a strong neutralizing cross-reactive response [121]. In porcine models, heterologous reinfection with H1N1 after H1N2 infection revealed enhanced lung immunopathology, possibly due to the enhanced binding of non-neutralizing antibodies that accelerated fusion [121]. Therefore, the strain of influenza that an individual is first infected by can have a strong influence on their response to future influenza strains that circulate.

DENV is another viral pathogen that has been shown to produce a more severe disease, dengue haemorrhagic fever, when pre-existing poorly-neutralizing cross-reactive antibodies against a heterologous serotype are present [119]. Specifically, because DENV targets monocytes, macrophages, and DCs, all cells expressing Fc receptors, entry of virus can be enhanced in the presence of non-neutralizing response, thus heterologous infection with a different strain can lead to severe infection [119,122]. Most notably, the quadrivalent live attenuated dengue vaccine (Dengvaxia), led to higher hospitalization rates and 14 deaths for those initially vaccinated with no previous exposure in the Philippines [123]. However, the related flavivirus, Zika virus (ZIKV), infection was less common in those with previous history of dengue infection [124].

Similarly, those who received the yellow fever vaccine after the Japanese encephalitis vaccine [125] or DENV vaccine also showed enhanced efficacy [126]. Thus, the benefits and disadvantages of producing heterologous cross-reactive immune responses to viral pathogens is a complicated subject of study.

1.4 Age-Dependence of Viral Infectious Disease and Immunity

The burden of viral infections often disproportionately affects the young and old, with hospitalization and long-term sequelae because of infection. Due to differences in immune function and senescence, responses in both innate and adaptive immunity often lead to improper or exuberant response to viral insult, this increasing the likelihood of immunopathology at extreme ends of ages [127]. For infants, the womb environment during gestation and its encounter with the mother's immune system leads to a tolerant state to avoid rejection [127,128]. However, in the elderly, the decrease of functional immune cells, along with the decline in adaptive responses often leads to a deficiency in recognizing pathogens and mounting a productive response [127]. Notably, HHVs and SARS-CoV-2 are known to show age dependency, causing higher morbidity and mortality in certain age groups, while mild symptomatic infection in others.

1.4.1 Differences in Severity of Viral Infection at Extremes of Ages

For many viral pathogens, there are crucial periods during life where a person encounters their first exposure. Often the initial exposure of a pathogen occurs during childhood, outside of the neonatal/infantile period, with relatively mild symptoms that are

self-limiting [128]. However, when exposure occurs in crucial periods of immune development or decline, there are often severe symptoms that often lead to hospitalization and sometimes death [127]. Even then, for some viruses, such as EBV [129] and VZV [12], the age period of initial (youth or adulthood) exposure determines the severity of the infection. Infection in late adolescence and adulthood often leads to severe and chronic symptoms. Likewise, for SARS-CoV-2, mortality rates are seen to be higher in the elderly than youth [130].

1.4.1.1 HHVs

While infection with HHVs is ubiquitous and mostly mild, certain age groups are more at risk for long term sequelae due to infection. Several herpesviruses have disproportional effects in different certain age groups, including CMV, HSV-1, HSV-2, EBV and VZV. For HSV-1, and HSV-1, infection during childhood and youth often yields mild symptom, while CMV is only usually a problem when infection occurs *in utero.* [23]. However, during infancy and at birth, infections can often disseminate into the CNS and other organs, like the liver, causing long term neurological deficiencies and other problems, such as herpetic hepatitis [21,35]. However, VZV and EBV have the inverse effect, causing severe symptoms in those whose first infection with the virus occurs during adolescence and early adulthood. While VZV infection in childhood usually results in self-limiting illness causing rashes and lesions, rarely leading to hospitalization, infection during adulthood can be disastrous [12]. Complications from infection during adulthood can often include pneumonia, encephalitis, and secondary infections, which often

require antiviral treatment; this, however, has also been described and can occur in children as well, although infrequent.

Perhaps the best-known age-dependent HHV infection is EBV, the causative agent of infectious mononucleosis. While initial infection for a large portion of the population occurs during childhood, infection during young adulthood can lead to symptoms that last for weeks to months [107]. However, the symptoms are caused by the body's response to EBV, rather than the virus itself. A majority of the circulating CD8 T-cells during the infection are viral antigen specific, causing a large immune response that leads to debilitating symptoms [107]. How this occurs is poorly understood, but studies have suggested heterologous immunity plays a large role, as EBV cross-reactive CD8 T-cells dominate during the infection [131,132].

1.4.1.2 SARS-CoV-2

During the current COVID-19 pandemic, morbidity and mortality has had a hugely disproportionate affected on the elderly, while youth and children have had mild or asymptomatic illness [133,134]. Overall, children have been less often found to be infected by SARS-CoV-2, contributing overall to 1-2% of cases during the first 6 months of the pandemic. Although this partly reflects biases related to symptom-based testing, some data indicate that children are less likely to acquire SARS-CoV-2 infection after contact with a positive individual [134]. This phenomenon has also been reported in other emergent CoVs, including SARS-CoV-1 and MERS-CoV, which is contrast to the

pattern observed with other respiratory pathogens, such as RSV, influenza, parainfluenza, etc., which tend to have disproportionately high incidence and severity in children [134]. Several mechanisms have been suggested for this, including differences in endothelium and clotting function, lower ACE2 expression at younger ages, prior exposure to endemic CoVs, and immunosenescence related inflammaging and CMV reactivation in the elderly [135]. However, studies exploring these mechanisms have yet to find a definitive cause for the reason children are protected from severe COVID-19 beyond associations [135].

1.4.2 Ontogeny of Innate and Adaptive Responses

The ontogeny of the immune system describes the stages of development that occur in utero, throughout childhood and into adulthood, each preparing the body for, and responding to, specific exposures [107]. In the fetus, the immune system is primed for tolerance to prevent immune activation and harmful inflammation due to maternal alloantigens and, perhaps, the intense exposure to myriad microorganisms immediately upon the transition from the sterile amniotic environment, through the birth canal and into life outside the womb. Then, exposure to external infectious threats dictates the development of proinflammatory immune responses [128]. In the newborn, many aspects of innate immunity are relatively weak, with neutrophils, monocytes, and DCs less abundant and responsive than in adults, and with lower expression of TLRs and human leukocyte antigen (HLA) (Fig. 2) [128,136]. Other innate cells, such as pDCs and NK cells also show an inability to secrete type I IFNs in response to stimuli and are less responsive to activation by cytokines [107,128]. Likewise, with respect to adaptive

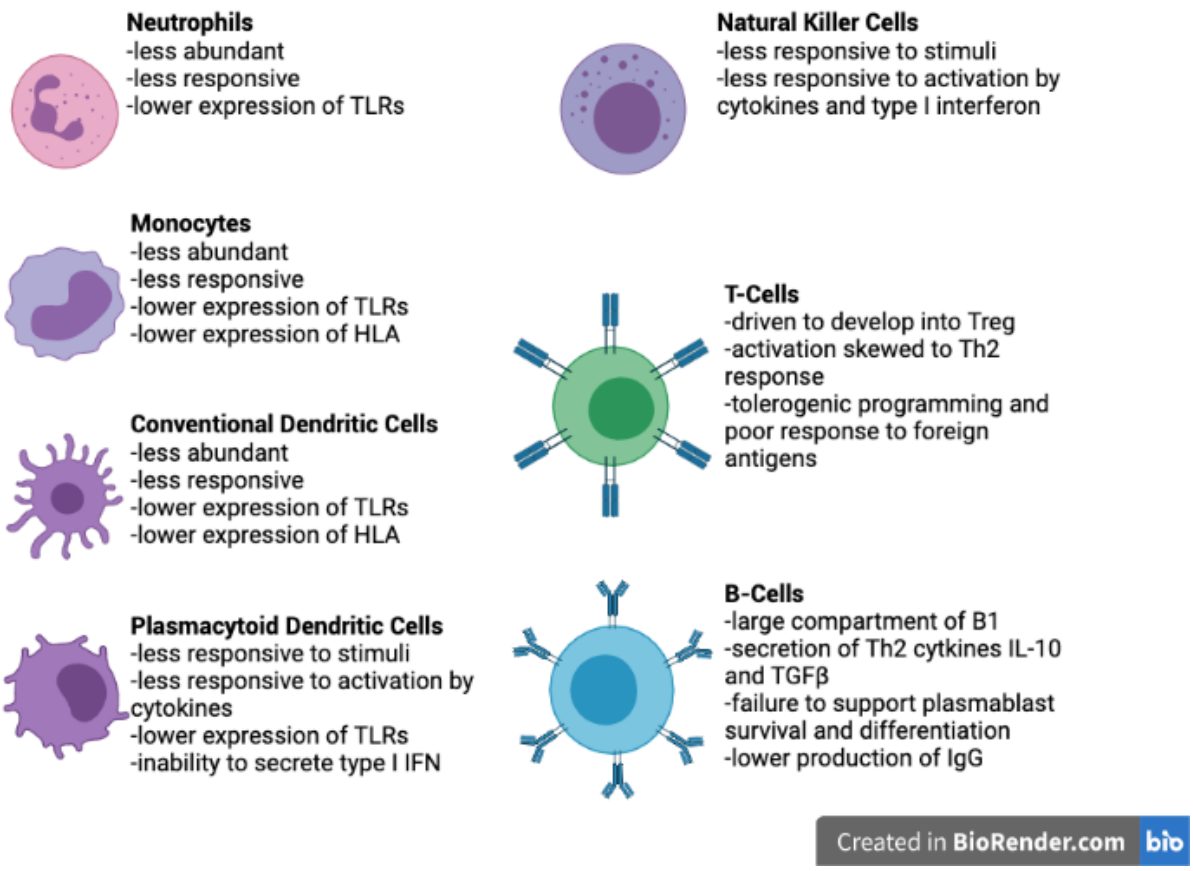


Figure 2 - Functional Differences in Neonatal Immune Cells

immunity, neonatal T-cells are driven strongly to develop into regulatory T-cells by maternal alloantigens, and activation of naïve T-cells typically is skewed towards a Th2 response [107]; this is characterized by tolerogenic programming and poor responses to foreign antigens [128,137]. In general, B1 B-cells in the neonate are programmed towards this Th2 response by secreting IL-10 and tissue growth factor beta (TGFβ) [107,127]. Furthermore, failure of bone marrow to support plasmablast survival and differentiation leads to reduced production of IgG [107]. As such, this period represents a window of extreme vulnerability to severe infection and neonatal death.

During infancy, various facets of the immune system become more robustly reactive at different rates, and susceptibility to infection by various pathogens gradually declines accordingly. Maternal antibodies wane, and children develop active responses to incident infections and vaccinations [107]. T-cell memory and B-cell memory are supported by accessory cells and individuals are able to produce long lived IgG responses [107,138]. Many asymptomatic infections also occur at this time, including HHVs, such as CMV and EBV, which produce strong T-cell responses [107]. At this time, B-cells, can also build more diverse and better binding antibodies through somatic hypermutation (SHM) [101]. As such, the memory repertoire in adults expands considerably due to the different encounters with unique pathogens—rendering in individuals, a unique immune system that responds distinctly to pathogenic encounters.

Immune responsiveness and the efficacy of some vaccines begin to decline in old age, leading to increased susceptibility to acute infection [139]. As immune function declines, tolerance to self-antigens also becomes impaired and autoimmune diseases become more common [140]; cancer risk also increases as inflammation is promoted and anti-tumour activity is replaced by self-reactivity [107]. T-cell and B-cell compartments also shrink at this time, reducing the diversity and pool of cells available to recall past infections [107,138]. Senescence is also seen in the innate immune system as well, with neutrophils [141] and macrophages becoming less reactive [142]. DCs are also less able to present antigens and the removal of apoptotic cells is impaired, leading to inflammatory state [143]. Overall, in old age, the immune system begins to function more like a newborn, with reduced reactivity of innate immune effectors and narrow functional recall of adaptive immunity.

1.5 Thesis Structure and Hypotheses

Due to the lack of continuity between supervisors and projects, the scope of this thesis is broad, with each chapter exploring a unique aspect of viral immunology and/or epidemiology. Therefore, the lack of cohesiveness in the research studies and the investigations conducted are acknowledged: Chapter 1 focuses on exploring the differences between neonatal innate immune cells and how their responses to stimulation may be different compared to adult cells; Chapter 2 investigates the role that latent HHV reactivation may play on the development of neurodegenerative diseases such as Alzheimer's Disease; and Chapter 3 investigates the acquisition of endemic

CoV infections and how cross-reactive antibodies may contribute to the disproportionate COVID-19 related morbity and mortality seen elderly age groups and in males.

As such, the overall hypotheses are as follows: (i) Given the “immaturity” of the adaptive immune system in neonates and their reliance on innate immunity in early life, the innate immune populations and their resting gene expression will be different compared to adults. (ii) Neurotropic HHVs, such as HSV-1, HHV-6, or HHV-7, if implicated the initiation, development, or progression of neurodegenerative diseases will be found more abundantly and frequently in diseased brains compared to brains of healthy individuals. (iii) If antibody dependent enhancement or “immune imprinting” is involved in developing severe COVID-19, past exposures and antibody titres to endemic CoVs will be higher and more frequent in elderly age groups and males.

Chapter 2 – A Comprehensive Comparison of Neonatal vs Adult Innate Antigen Presenting Cell Populations

2.1 Introduction

Antigen presenting cells provide the interface between innate and adaptive immune systems in response to infection. These cells include dendritic cells, monocytes, and B-cells that express MHC class II and are considered “professional” antigen presenting cells [144]. Antigen presenting cells take up antigens, process them, and present them on their surface to engage and activate T-cell help, with the help of co-stimulation and cytokine secretion [145]. However, during gestation, the fetus is exposed to a unique environment surrounded by maternal alloantigens, thus creating the risk of rejection [146]. At birth, this new environment that the neonate is exposed to requires a shift in immune function, changing from tolerogenic programming to pathogen identification and protective responses [128]. During this crucial transition, the neonate is susceptible to severe infection by a host of pathogens.

2.1.1 Monocytes, Dendritic Cells and B-Cells as Professional Antigen Presenting Cells

Monocytes and DCs are myeloid-derived cells crucial to linking the innate immune response to the adaptive immune system [147]. Circulating monocytes are also part of a heterogeneous population that can be divided into two main subsets based on CD14 and CD16 expression [148]. CD14+ monocytes represent 90% of circulating monocytes

and are considered the largest population, while CD16+ monocytes are upregulated during infection and produce large amounts of TNFα, resulting in them being called inflammatory monocytes [149,150]. Likewise, considered the prototypical antigen presenting cells, DCs can not only process and present antigens, but efficiently express co-stimulatory molecules needed to mediate fully activate adaptive T-cell responses against pathogens [151]. DCs are themselves a heterogeneous group of cells that can be separated into two main subgroups, conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [150,151]. For antigen presentation, cDCs are the most potent cells for activating naïve T-cells, while pDCs are potent secretors of type I IFNs during viral infection that can also present antigens [84,152].

After encountering an antigen and becoming stimulated via PAMPS, patrolling DCs and monocytes migrate towards lymph nodes to mature, present, and promote the activation of naïve T-cells through CD40L-dependent and -independent pathways [93]. This directly involves trafficking antigen molecules to the surface of the DC and presenting them on MHC. Concurrently during activation, surface expression of T-cell co-stimulators increases. Furthermore, as DCs mature and express CCR7, they migrate towards secondary lymphoid organs [145,153]. DCs can express three classes of MHC: MHC class I, MHC class II, and CD1. Classically, the MHC class I antigens, present proteasomal degraded products of the cell [145]. MHC class II antigens are processed by the DC after internalization, via phagocytosis, before being presented on the surface of the cell [145,154]. CD1 antigens are unique, being glycolipidic antigens, rather than being protein derived [155,156]. Depending on the PAMPs encountered by the DC,

polarizing signals will promote the development Th1 and Th2 T-cell responses [93,97,145]. Typically, TLR activation results in the maturation of Th1-promoting DCs, however, this is specific to the pathogen being encountered to prime the correct immune response [145].

While DCs and monocytes are considered the prototypical antigen presenting cells, B-cells are also able to present MHC class II peptide complexes in addition to their role as secretors of antibodies. Around 45% of naïve B-cells migrate to the spleen to sample antigens in the blood, while the remainder reside in lymph nodes and the mucosa-associated lymph tissue [157]. Using their BCR, B-cells are able to capture and internalize antigens to be presented on MHC class II, where they can activate CD4 T-cell responses in germinal centres or promote T-cell tolerance [157,158]. Their ability to recognize high affinity antigens through interactions with their BCR also allows B-cells to promote strong antigen presentation via enhanced MHC class II expression [157]. However, in this chapter, the main focus will be on the innate antigen presenting cells, monocytes and DCs.

2.1.2 Fetal Environment Vs. External Environment

There are critical adaptations to the immune system that must take place for the developing fetus and its mother. This is driven by the presence of paternal non-self antigens expressed by the fetus that can result in harmful inflammation [159]. Thus, during pregnancy, the developing fetus and mother are protected against rejection by a

more tolerogenic immune programme [146]. Linked to the mother by the placenta, cells at the interface of mother and fetus are crucial to promote and protect the pregnancy [160]. Trophoblast cells and NK cells in the placenta have been found to promote anti-viral activity through expression of TLRs and secretion of beta defensins, antileukoproteinase, and IFNβ [159]. In the event of viral infection, production of inflammatory cytokines that activate the maternal immune system can result in placental damage, abortion or preterm labour [159,161]. The placenta also acts as a physical barrier between the two immune systems, helping to regulate both environments [159–161].

Upon birth, newborns are instantly exposed to the new external environment containing a myriad of microbes and pathogens [162]. As a result of the tolerogenic programming still present in early life and the naïveté of the immune system, the neonate is at high risk for severe infection and sepsis [163]. In this new environment, the microbiome begins to develop, and commensals begin to colonize the skin, GI, and other mucosal surfaces, helping develop the neonatal immune system [164,165]. Furthermore, as they began to feed, their diet introduces changes in the metabolome, which can influence the colonization of the gut, leading to changes in the microbiome [166]. Thus, during the first few weeks of life, a new steady state environment for the newborn is being established, and sudden changes that the neonate cannot respond to properly render it susceptible to infection and exaggerated host response [128].

2.1.3 Functional Differences

Neonatal antigen presenting cells are functionally distinct from those found in adults. They are relatively hyporesponsive to stimuli and have reduced antigen presenting abilities during the first few weeks of life. Monocytes have been found to express decreased levels of HLA-DR [167] and CD80 on their surfaces [168], leading to impaired presentation of antigens in neonates [169]. Furthermore, reduced expression of adhesins, such as L-selectin, result in reduced localized recruitment into inflamed tissues [170]. NF-κB signalling is also affected, as TLR-4 stimulation by LPS results in expression of TNFa, IL-6 and IL-10, but downstream phosphorylation of p65 and p38 are reduced [171,172]. However, neonates express higher levels of both anti-apoptotic BCL-2 [173] and pro-inflammatory MIF [174], which may lead to excessive and sustained cytokine release and inflammation during sepsis.

Similarly, neonatal DCs appear functionally impaired compared to adults, secreting less Th1 polarizing cytokines upon stimulation. Due to epigenetic remodelling and diminished activity of its co-activator, IRF3, IL-12 secretion following TLR-4 and TLR-2 stimulation is low during the neonatal period but increases with age [137,175]. As a result, cord blood-derived cDCs produce lower levels of Th1 polarizing cytokines, but more of IL-10 and Th17 polarizing cytokines [175]. However, signaling through the anti-viral TLRs, TLR-7, TLR-8 and TLR-9, seems unaffected in cord blood pDCs, as stimulation by HIV, influenza A virus, and HSV produce a potent type 1 IFN response [176]. In contrast to monocytes, DCs from cord blood express TLRs and HLA-DR

comparable to adults, although endogenous immunosuppressive/tolerogenic factors may prevent full function of cDC maturation and T-cell activation [177].

2.1.4 Susceptibility to infection

The early life immune system's tolerogenic bias brings with it a high risk of severe infection [137]. Congenital infection typically occurs by transplacental transmission to the fetus [178]. The so-called "TORCH" group of congenital infections, (T)oxoplasma, (O)ther agents, (R)ubella, (C)MV, and (H)erpes Simplex, are the most common [178]. Symptoms and long-term sequelae are somewhat pathogen-specific, but extensive pathologic overlap is seen. For example, common findings with any of these pathogens include enlargement of the liver and spleen, jaundice, thrombocytopenia, rash, hearing loss, and intracranial calcifications [178]. CMV is the most common congenital infection, and is among the most common causes of childhood deafness and intellectual disability [21,23]. In contrast, postnatal infection with CMV rarely causes significant disease. HSV can cause severe congenital infection, but more often affects newborns due to genital transfer of virus during birth, which, if not treated, typically progresses to disseminated infection and death [36]. Again, a marked age-dependence is seen, with HSV infections before a month of age being life-threatening, while those acquired later resulting in symptoms similar to those in adults (primarily self-limited mucosal lesions) [36].

Neonates are also at risk for sepsis from a large number of other perinatally-acquired pathogens, which cause high rates of mortality and morbidity [179,180]. Neonatal sepsis

can be separated as either early onset sepsis (EOS) or late onset sepsis (LOS), depending on the diagnosis of cultures before or after 7 days of life in infants [180]. While EOS is caused by intrapartum maternal transmission, LOS is more often the result of nosocomial or community acquisition of pathogens [181]. Group B *Streptococcus* and *E. coli* are the most common pathogen for EOS [182,183], accounting for 70% of cases, while coagulase-negative Staphylococcus, occurring primarily in premature infants with central catheters, accounts for 48% of all cases in LOS [180] . As common with sepsis, symptoms can be quite variable, but can progress quickly to CNS infection and death; therefore, prompt identification and treatment of neonatal infections is critical [180,184].

2.1.5 cGAS-STING Pathway and Intracellular Infection

While endosomal sensing of viral nucleic acids occurs via TLRs 7, 8, and 9, cytosolic DNA sensing occurs through the cGAS-STING pathway [185]. Able to detect cytosolic DNA, cGAS senses not only viral nucleic acids, but bacterial and self (genomic and mitochondrial DNA), initiating formation of cGAMP as a second messenger [185,186]. Once cGAMP binds to endoplasmic reticulum localized STING, several signalling cascades can occur. STING can translocate into the Golgi, recruiting TANK-binding kinase 1 and IκB kinases to phosphorylate IRF3 and IκBα, activating type I IFN gene transcription [185] and secretion of type I IFNs, IFNα and IFNβ. In addition, activating NF-κB pathways also lead to the transcription of other proinflammatory cytokines, TNFa

and IL-6. Activation of cGAS-STING signalling can also activate other cellular processes, including autophagy, apoptosis and necroptosis [185].

As neonates are particularly susceptible to intracellular infections by viruses and bacteria, age-dependent signalling by cytosolic PRRs and the resulting type I IFN response are of interest. STING is a direct sensor of cyclic dinucleotides, and bacterial derived cGAMP, from pathogens like *Listeria monocytogenes* can activate STING directly inducing type I IFN production and IL-6 [187]. Moreover, several TORCH pathogens are known to interfere with cGAS-STING signalling, including HSV, CMV, and ZIKV [186]. HSV-1 relies on several accessory proteins to block IRF3 activation via steps in the cGAS-STING signalling cascade, disrupting processes such as limiting cGAMP production, phosphorylation of proteins, and dimerization of STING[186]; CMV relies on directly binding to cGAS to inhibit STING signalling [188]. ZIKV instead relies on cleaving the cytoplasmic loop of STING and recruiting deubiquitinases to target cGAS for caspase-1 mediated cleavage [189].

2.2 Rationale

As neonates and adults live in unique environments, each with different immunological requirements for survival, resting state immune programming of antigen presenting cells should vary between the two groups. However, as traditional assays have investigated these heterogeneous populations on a bulk level, the granularity of the data may be hidden due to their lack of sensitivity. Therefore, using fluorescent activated cell sorting

(FACS), single-cell RNA sequencing (scRNAseq), and bioinformatics, resting monocytes and DCs from umbilical cord and adult peripheral blood were interrogated and profiled. In addition, to understand the difference in responses to stimulations, cells were also incubated with 3'3' cGAMP, a STING ligand, and transcriptome investigated after FACS enrichment.

Because of the functional differences in immune systems between adults and neonates, we hypothesize (i) At rest, adult and neonate monocytes and DCs will have unique transcriptomes; (ii) Similarly, monocytes and DCs will have different gene expression signatures after stimulation by the STING ligand 3'3' cGAMP.

2.3 Methods

2.3.1 Collection of Adult Whole Blood and Umbilical Cord Blood

Whole blood was collected from healthy consenting adults (ages 18-55) and from the placentas of babies delivered via elective caesarian section with consent from the mothers. Briefly, blood was collected via either cubital vein or umbilical cord puncture using a 18G butterfly needle into heprinated vacutainers (BD), and used within 2 hours of collection.

2.3.2 Stimulating Whole and Cord Blood with STING Agonist 3'3 cGAMP

For samples to be stimulated, initially 1 mg 3'3' cGAMP (Invivogen) was resuspend to 1 mg/mL in endotoxin-free LAL water (Invivogen). Concurrently, Lyovec (Invivogen) was also resuspended with 2 mL LAL water and both were left to hydrate at room temperature for 15 minutes. Lyovec was then incubated at a 4:1 ratio with either 3'3' cGAMP or LAL water as a vehicle control and left to equilibrate at room temperature for another 15 minutes. The mixtures were then diluted 50x in 1x RPMI-1640 to a final concentration of 20ug/mL 3'3' cGAMP for a 2x working concentration. Equal parts whole blood and stimulants were mixed, and cells were incubated at 37 °C for 6 hours before isolation.

2.3.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Cord Blood Monoclear Cells (CBMCs)

Cells were isolated from whole-blood samples using density-gradient centrifugation. Briefly, adult whole blood was diluted 1:1 and umbilical cord blood was diluted 1:5 with 1× DPBS, layered on top of Ficoll-Paque Plus (GE Healthcare), and centrifuged at *900g* for 20 min with the brake disengaged. The PBMC layer was resuspended in 50 ml 1xDPBS and centrifuged again at *250g* for 15 min to deplete platelets. The cells resuspended and counted, then 10^6 cells were kept for sorting. Remaining cells from unstimulated samples were resuspended in RPMI-1640 and diluted in 2x freezing media (80% Fetal Bovine Serum; 20% DMSO) and aliquoted into 2.0mL cryopreservation

tubes at concentration of 2×10^6 cells per milliliter. The tubes were kept at −80 °C overnight, then transferred to liquid nitrogen for long-term storage.

2.3.4 Fluorescence Activated Cell Sorting for scRNAseq and Low Input RNAseq

Unstimulated cells to be used for scRNAseq were first resuspended with 100uL of an antibody mastermix (Table 2**Error! Reference source not found.**) in 1xDPBS and i ncubated at 4 °C for 30 minutes. Cells were washed twice after staining with 1xDPBS and resuspended in 1xDPBS for FACS on the Becton Dickenson FACS Aria IIu. For scRNAseq on the 10X genomics platform, ~50,000 cells from each population were bulk sorted into 500uL of receiving buffer (2% BSA; 1xDPBS) and pelleted by centrifugation.

For stimulated samples and vehicle controls, PBMCs and CBMCs were initially lineage depleted (CD19, CD3, CD56, CD235a) using labelled magnetic beads as per manufacturers recommendations (Miltenyi). Briefly, cells were incubated with antibody conjugated magnetic beads for 15 minutes at 4 °C, then separated through magnetic columns. The flow through containing lineage depleted cells were then stained using the protocol mentioned above. ~100 cells were sorted directly into 12.5uL of lysis buffer containing RNAse inhibitor and cDNA primer (Takara) in a 96 well plate (Eppendorf) as per manufacturers recommendations for low input bulk RNAseq. Plates were sealed with foil, snap frozen on dry ice and stored immediately at -80 °C prior to library preparation.

Table 2 - Antibodies and Dyes Used in this Study

Antigen	Fluorochrome	Clone	Manufacturer
scRNAseq Monocyte/Dendritic Cell Bulk Enrichment			
CD3	PE-CF594	UCHT1	BD
CD19	PE-CF594	HIB19	BD
CD235a	APC-Vio770	REA175	Miltenyi
HLA-DR	PE	LN3	eBioscience
CD14	BV480	M5E2	BD
CD11c	APC	Bu15	eBioscience
CD123	PE-Cy7	6N6	eBioscience
CD16	PerCP-Cy5.5	3G8	BD
CD34	BV421	561	Biolegend
Viability Dye	APC-eFluor780		eBioscience
Bulk RNAseq Monocyte/Dendritic Cell Sorting			
CD3	PE-CF594	UCHT1	BD
CD19	PE-CF594	HIB19	BD
CD235a	APC-Vio770	REA175	Miltenyi
HLA-DR	PE	LN3	eBioscience
CD14	BV480	M5E2	BD
CD11c	APC	Bu15	eBioscience
CD123	PE-Cy7	6N6	eBioscience
CD141	BB515	1A4	BD
CD34	BV421	561	Biolegend
CD16	PerCP-Cy5.5	3G8	BD
CD1C	BV605	F10/21A3	BD
Viability Dye	APC-eFluor780		eBioscience

2.3.5 Preparing 10X Genomics Libraries for Sequencing

Cells were initially counted using the Cellometer auto2000 and resuspended to ~1000cells/uL in 1x DPBS and kept on ice until ready for encapsulation and lysis. Briefly cellular suspensions were loaded on a single cell chip and processed on the 10X Genomics Chromium instrument (10X Genomics) to generate single-cell GEMs. (targeting 6000 cells per sample). ScRNAseq libraries were then prepared using the 10X Genomics 5' Gene Expression v2 kit. After GEM generation, cells were subjected to lysis and reverse transcription, resulting in barcoded bead specific single stranded cDNA to identify single cells. Following reverse transcription, cDNA was isolated using Dynabeads MyOne Silane beads (ThermoFisher) and pre-amplified by 12 cycles of PCR. Samples were analyzed by the Qubit DNA HS kit (ThermoFisher) and a Bioanalyzer 2100 using the DNA High Sensitivity Kit (Agilent) to ensure size and quantity. Amplified cDNA was size selected using SPRISelect Beads (Beckman Coulter) and undergone enzymatic fragmentation, end repair and A-tailing. Adapters were then ligated, and sample indices were added for each sample by PCR. Samples were size selected again by SPRISelect and reanalyzed via the Bioanalyzer DNA High Sensitivity kit to ensure proper size, then quantified by KAPA Illumina Quantification (Roche). This process resulted in libraries containing P5 and P7 Illumina sequencings adapters, a 16 bp cell barcode, 10 bp UMI, a ~350bp 5' gene insert and an 8bp i7 sample index for multiplex sequencing. Libraries were stored at -20 °C until sequencing.

2.3.6 Preparing Low Input RNAseq Libraries Using SMARTseq HT Protocol

Low input bulk RNAseq was performed using the SMARTseq HT v4 kit (Takara). Briefly, 96 well plates containing lysed cells were thawed and centrifuged to collect contents at the bottom of the plates. Plates from multiple sorts were then consolidated into a new 96 well plate for the remaining library preparation to minimize batch effects. Samples were then incubated at 72 °C in a preheated thermocycler for 3 minutes, then immediately placed on ice for 2 minutes. One-Step PCR Master mix was added to all the samples, plates sealed, and mixed by vortexing. Samples were PCR amplified a total of 20 cycles. The resulting cDNA was then isolated using the AMPure XP kit (Beckman Coulter), washed twice in 80% ethanol, and eluted in elution buffer. Samples were then size verified, and quality controlled by using the Bioanalyzer DNA High Sensitivity kit, before proceeding to library preparation. Amplicon concentrations were normalized to 0.1ng/mL and sequencing libraries were constructed using the Nextera XT DNA Library Prep kit (Illumina), following the adapted protocol from Takara. After diluting the samples, tagmentation and amplification was performed before pooling and final purification using the AMPure XP kit. Pooled samples were washed with 80% ethanol and eluted in nuclease-free water and size verified using the Bioanalyzer DNA High Sensitivity kit. This process resulted in libraries containing a P5 and P7 Illumina sequencing adapters, a ~450bp gene insert and dual 8bp i5/i7 indices for multiplex sequencing. Libraries were stored at -20 °C until sequencing.

2.3.7 Sequencing of RNAseq Libraries

Following generating scRNAseq libraries, samples were diluted to 2 nM and pooled into a single superpool. Libraries from both PBMC and CMBC populations were pooled from all subjects. These pooled libraries were further diluted to 2pM, then denatures and loaded on a NovaSeq 6000 at the sequencing core at the J. Craig Venter Institute in La Jolla, USA at a depth of ~50,000 reads per cell. Samples were run using the NovaSeq S4 kit (200 cycles), with the following sequencing run parameters: Read 1: 26 cycles, i7 Read Index: 8 cycles, Read 2: 98 cycles.

Low input bulk RNAseq samples were prepared as above but sequenced using a NextSeq 550 (Illumina) at UBC's Sequencing and Bioinformatics Consortium. RNA-seq libraries were sequenced to a depth of >10 million reads per sample. Samples were sequenced using a NextSeq High Output kit (150 cycles), with the following run parameters: Read 1: 75 cycles, i7 Read Index: 8 cycles, Read 2: 75 cycles.

2.3.8 Demultiplexing, Alignment, and Generation of scRNAseq Gene/Barcode Matrices Using Cell Ranger

After sequencing, scRNASeq runs were processed through the CellRanger (10X Genomics) pipeline to generate gene/barcode matrices. Briefly, samples were demultiplexed using the i7 sample indices present the sequencing libraries, then by converting the raw BCL files into FASTQ. Reads were then aligned using the GRCh38 reference genome and STAR [190], as packaged within CellRanger. After alignment,

key sequencing metrics such as frequency of valid cell barcodes, transcript mapping rate, and Q30 bases were assessed for quality control of sequencing and alignment. Finally, for each age group (adult and cord), cell populations were aggregated into a single gene/barcode matrix, resulting in 4 gene barcode matrices.

2.3.9 Canonical Correlation Analysis and Differential Gene Expression Analysis Using Seurat and MAST

Before aligning adult and cord datasets, cells were filtered by the % expression of mitochondrial genes and number of UMIs per unique gene to remove multiplets and stressed cells from the final data using the *Seurat v2* R package [191]. Highly variable genes for each dataset were then calculated using the *FindVariableGenes* function using *ExpMean* mean function and a *LogVMR* dispersion function. To align the adult and cord blood datasets, canonical correlation alignment was utilized. Initially, the top 1000 highly variable genes in each dataset were selected, then datasets were scaled using a Poisson model, and the number of genes, % mitochondrial genes, number of UMIs, and number of UMIs per unique gene were regressed out of the dataset and merged. Using the unique genes present in both adult and cord datasets, the canonical correlation analysis (CCA) was used calculated on the first 50 components. Using the first 25 components calculated from CCA, the subspace between cord and adult datasets were aligned. After alignment of the subspace using canonical correlation components, B-cells (CD19), T-cells (CD3E), and NK cells (KLRG1 and NKG7) were filtered out using lineage markers. Cells in the aligned dataset were then clustered using the first 15 components of a PCA reduced data and SNN clustering at a resolution of

0.8. The resulting clusters were plotted on dimensionally reduced UMAP plots for visualization.

To determine differentially expressed genes between adult and cord, along with gene markers for clusters, MAST [192] was used to determine the top differentially expressed genes found in more than 12.5% of the cells in each cluster. This was to account for drop our rates common to scRNAseq datasets. Genes with an adjusted p value <0.05 were considered significant.

2.3.10 Analysis of Bulk RNAseq Samples Using DESeq2

Sequencing data was returned as individual FASTQ files and processed initially by assessed quality control metrics using FASTQC [193]. Samples were then aligned using STAR [190] using the UCSC hg19 transcriptome, then post-alignment quality control and 5'-3' bias was checked using Qorts [194]. Gene counts were then calculated using HTSeq-count [195] to generate gene counts for individual samples. Finally, MultiQC [196] was used to summarize QC metrics from all the steps within the pipeline.

Using the *DESeq2* package [197], replicate sequencing runs were collapsed for individual samples, and only genes expressed in >1 samples were kept to reduce the size of the dataset. After initial analysis by DESeq2, fold changes were shrunk using the *lfcShrink* function and *apeglm* package, then genes with an adjusted p value of <0.1

were kept for visualization. Ensembl IDs were matched to gene IDs using the *biomaRt* package [198] and the resulting data was visualized using the *EnhancedVolcano* R package. Genes with an adjusted p value <0.05 were considered significant.

2.4 Results

2.4.1 scRNA Reveals Distinct Populations Within Monocyte and DC Populations

Purified PBMCs and CMBCs were initially FACS enriched into two populations, monocytes (CD3-/CD19-/CD56-/HLA-DR+/CD14+/CD16-or+) and DCs (CD3-/CD19-/CD56-/HLA-DR+/CD14-/CD123+ or CD11c+), for scRNAseq on the 10X Genomics platform (Fig. 3). The resulting aligned datasets between adult and cord blood monocytes revealed similar populations between the ages (Fig. 4a). Upon clustering, 6 distinct monocyte populations were identified in total (Fig. 4b) and found to be represented in both age groups (Fig. 4c). Hierarchical clustering of genes revealed distinct signatures for individual clusters of monocytes, including populations with high expression of S100A (Mono1), HLA subtypes (Mono2), proinflammatory cytokines (Mono3), cytotoxic genes (Mono4), cell cycle genes (Mono5), and interferon-stimulated genes (ISGs) (Mono6) (Fig. 5a). Comparing both adult and cord blood, lineage markers for each population remain conserved in both age groups (Fig. 5b), although levels of expression varied between the age groups in some cell populations.

Likewise, DC populations (Fig. 6a) remained similar between adult and cord blood, with 9 DC populations and a transitional monocyte-derived DC population identified (Fig. 6b).

Again, the clusters identified were similarly found in both adult and cord blood (Fig. 6c). Using canonical lineage makers for DC populations, common DC populations were identified, in addition, a new subdivision of DC3 into DC3(A) and DC3(B) was identified based on different S100A, CD1C and CLEC10A expression patterns (Fig. 7a). Another 2 unique populations were also identified, including one expressing complement C1QA and CIQB (DC7), and another expressing the ISGs IFITM3, IFI6, and ISG15 (DC8). Expression of lineage markers remained consistent between both age groups (Fig. 7b).

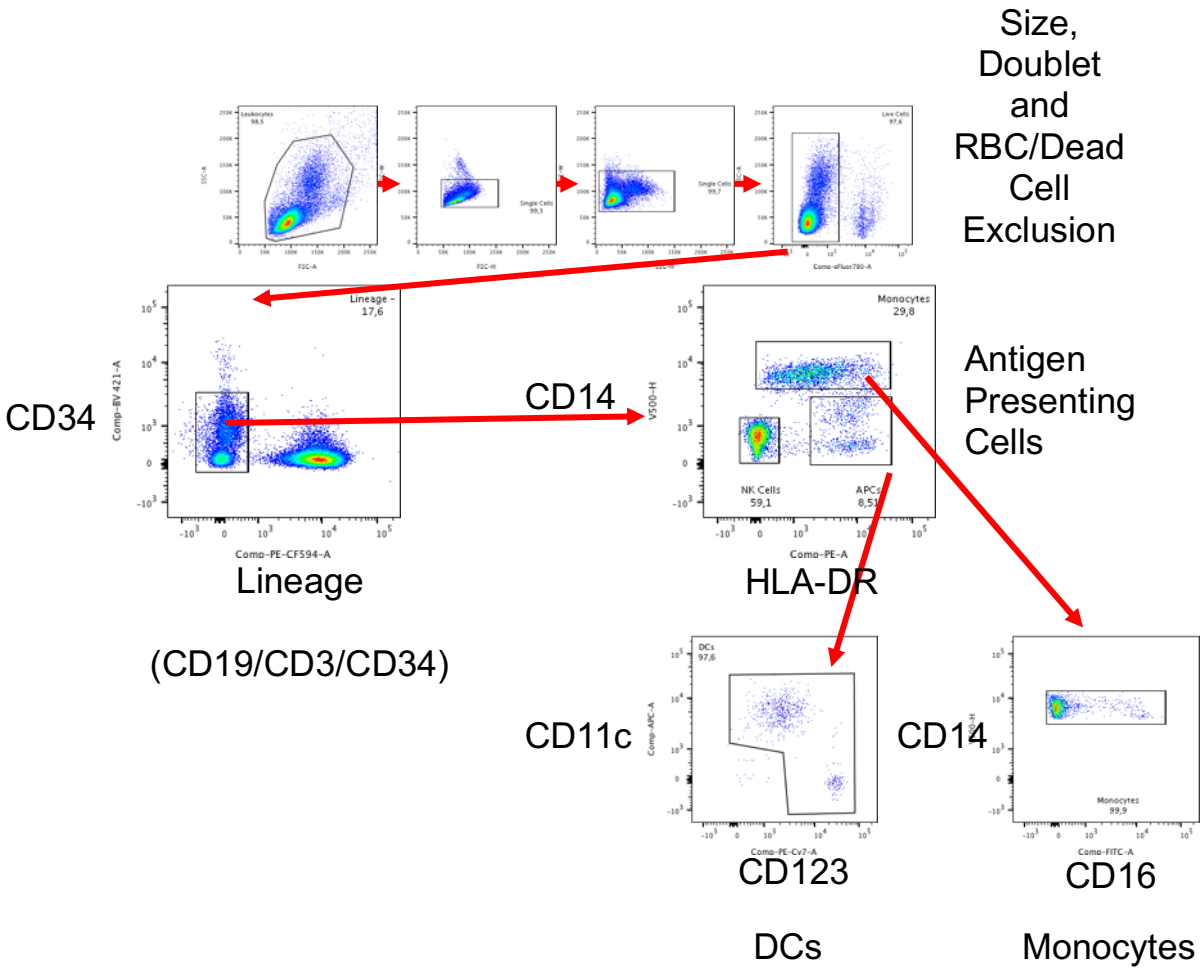


Figure 3 - FACS Panel Used to Enrich Cells for scRNAseq.

CBMC and PBMC were FACS enriched using canonical lineage markers for APCs, CD14 and HLA-DR. For scRNAseq, two populations of APCs were sorted: Monocytes (CD14+/HLA-DR+) and DCs (CD14-/HLA-DR+/CD11c+ or CD123+. To reduce contamination by other cell types, CD3, CD19, and CD34 lineage positive cells were included in ”dump” channels to reduce their chances of being mistakenly included in sorted cell populations.

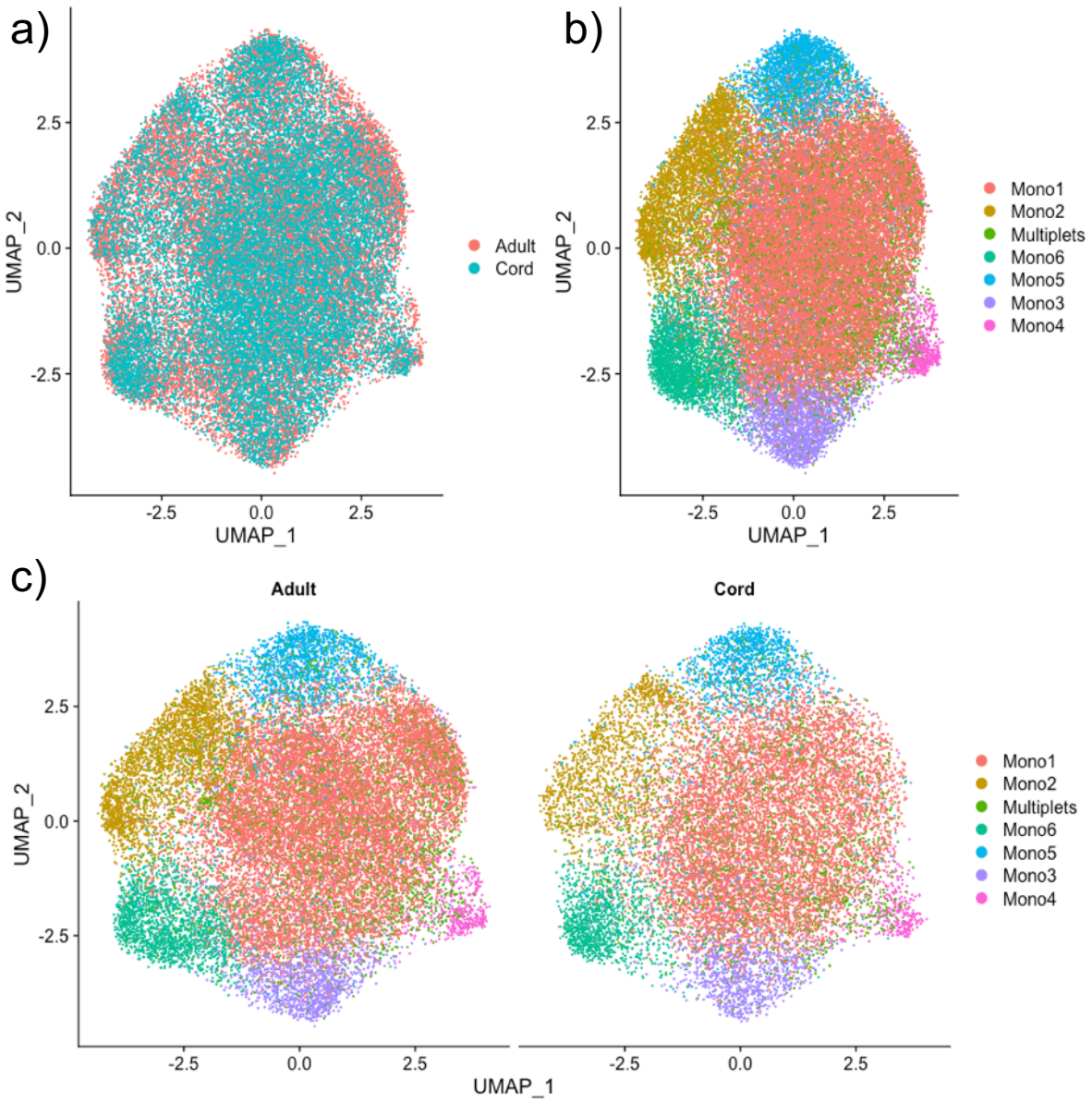


Figure 4 - CCA Aligned and SNN Clustered Monocytes Reveal Populations of Cell Types Found in Both Adult and Cord Blood.

After CCA alignment and UMAP projection, similar cell type populations are found represented in a) Adult and cord blood. b) Six distinct cell type populations were found after clustering the CCA aligned monocyte dataset. c) The six populations are represented in both cord and adult samples.

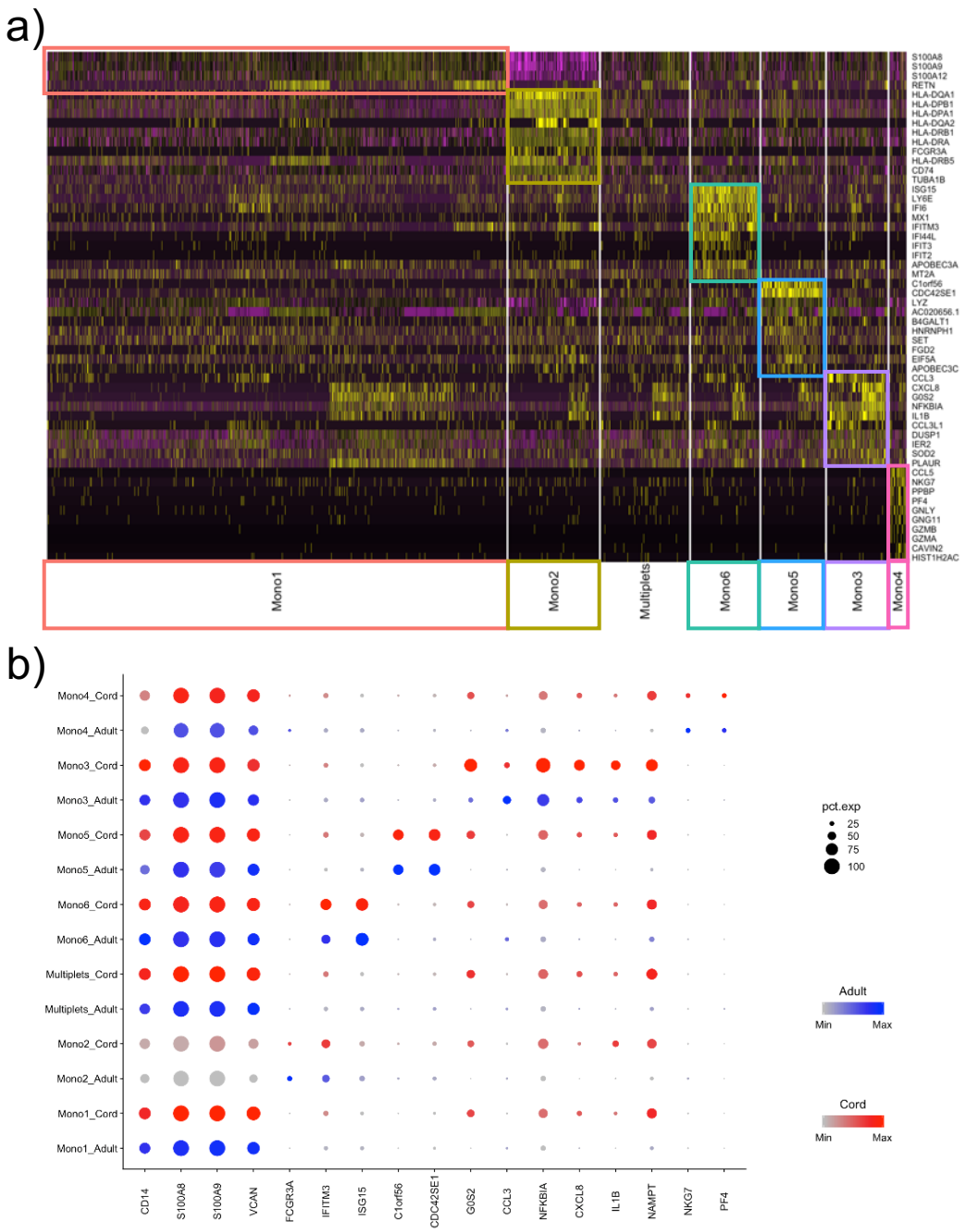


Figure 5 - Heatmap of Marker Genes for Monocyte Cell Type Populations and Conserved Markers Between Cord and Adult Cells.

a) Top 10 genes found differentially expressed between clusters separates cell type populations in sorted monocytes. b) Conserved marker genes are found between cord and adult blood monocyte cell type populations.

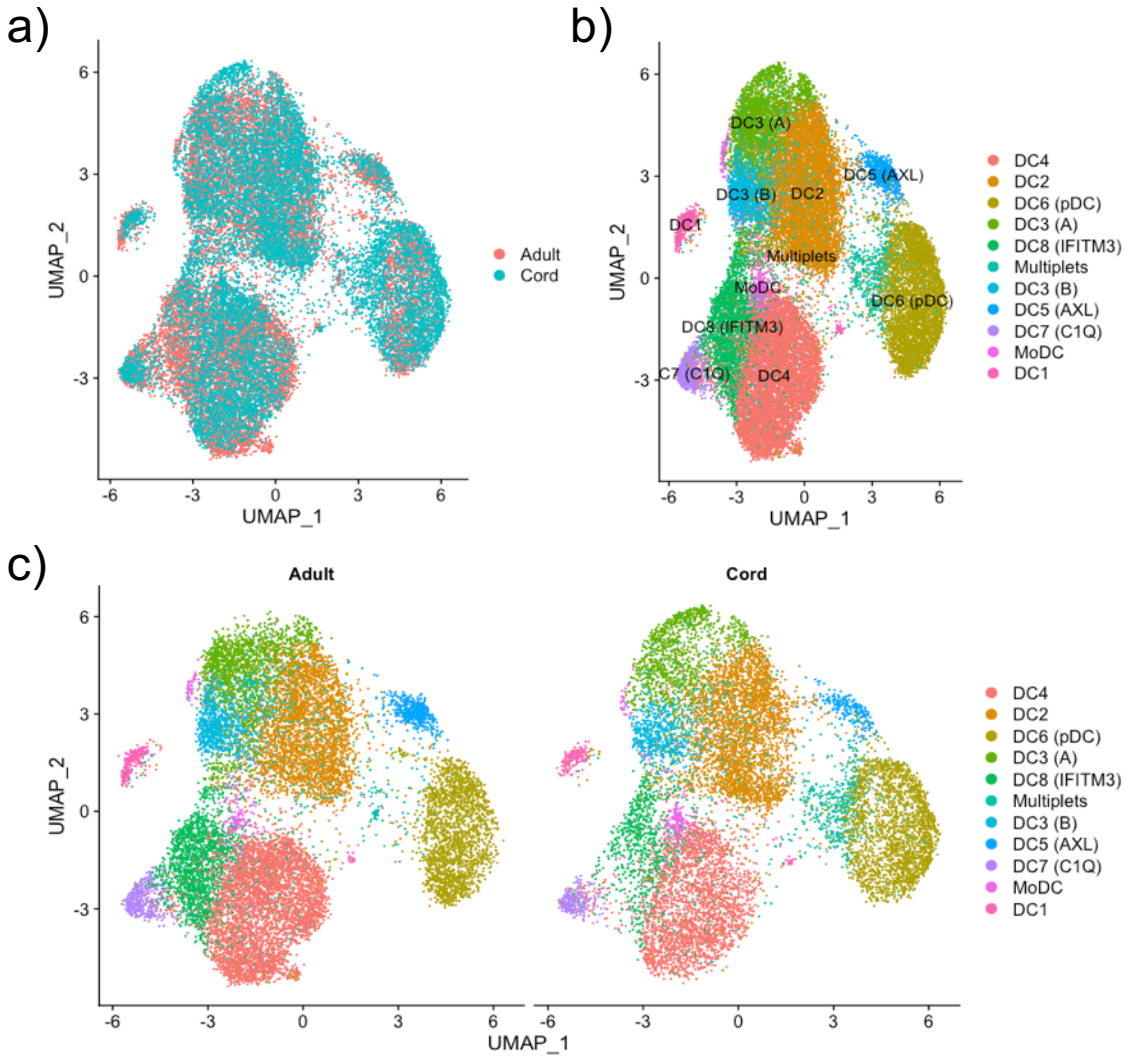


Figure 6 - CCA Aligned and SNN Clustered DCs Reveal Populations of Cell Types Found in Both Adult and Cord Blood.

After CCA alignment and UMAP projection, similar cell type populations are found represented in a) Adult and cord blood. b) Nine distinct cell type populations were found after clustering the CCA aligned monocyte dataset. c) The nine populations are represented in both cord and adult samples.

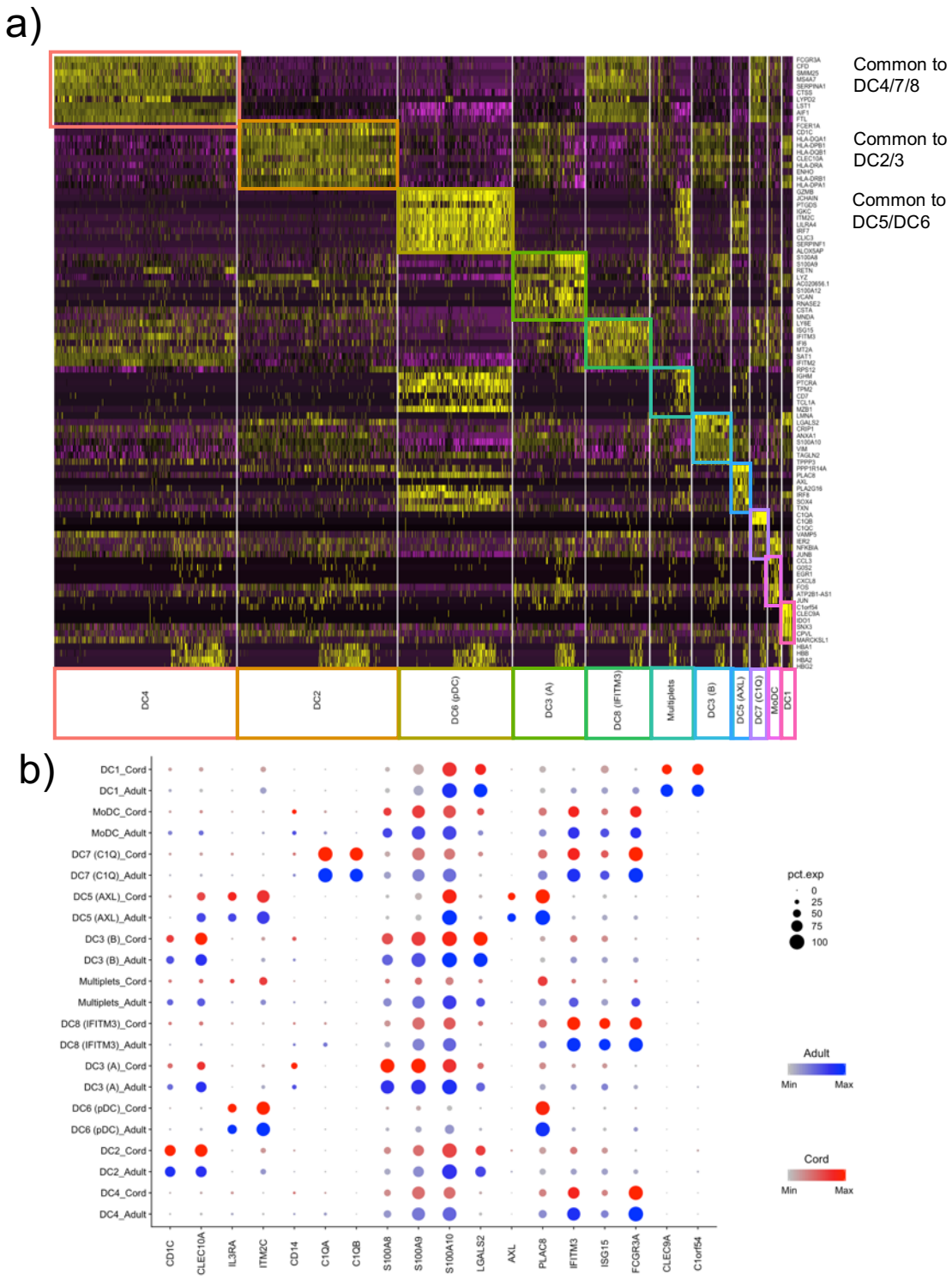


Figure 7 - Heatmap of Marker Genes for DC Cell Type Populations and Conserved Markers Between Cord and Adult Cells.

a) Top 10 genes found differentially expressed between clusters separates cell type populations in sorted DCs. b) Conserved marker genes are found between cord and adult blood DC cell type populations

2.4.2 Cord Blood Monocytes and Dendritic Cells Express Less HLA-DR and CD52 and Have Altered Inflammatory Gene Signatures Between Cord and Adult

Using MAST, differentially expressed genes between clusters and age groups were analyzed. In both monocytes and DCs, there was decreased expression of CD52 (Fig. 8a & Fig. 9a) in cord blood cells versus adults. Furthermore, HLA-DRB5 (Fig. 8a & Fig. 9a) was found to be much more highly expressed in adult cells versus cord. In general, less HLA-DR and HLA-DQ subtypes were expressed in cord blood cells than adult cells overall (data not shown). However, in monocytes, PLAUR (CD87) expression was significantly higher in cord than in adult cells (Fig. 8b).

Similarly, gene signatures relating to inflammation were different between adult and cord cells, including the expression of proinflammatory cytokines IL1B and chemoattractant CXCL8 (Fig. 10a). Moreover, inflammatory gene signatures showed different patterns of activation in cord blood cells versus adults. Both cord blood monocytes and DCs in general had higher expression of proinflammatory genes, RETN and NFKBIA (Fig. 11 & Fig. 13), and in DC3(A)/(B) subpopulations, S100A expression was also found to be higher in cord blood cells compared to adults (Fig. 12). Cord blood DCs also had increased expression of negative regulators of JAK/STAT and mTOR, such as SOCS3 and G0S2 (Fig. 13). However, in some DC populations, there is increased expression of the MAPK kinase kinase MAP3K8, the STAT3 regulator RGS2, and C19orf38 in cord versus adults (Fig. 13).

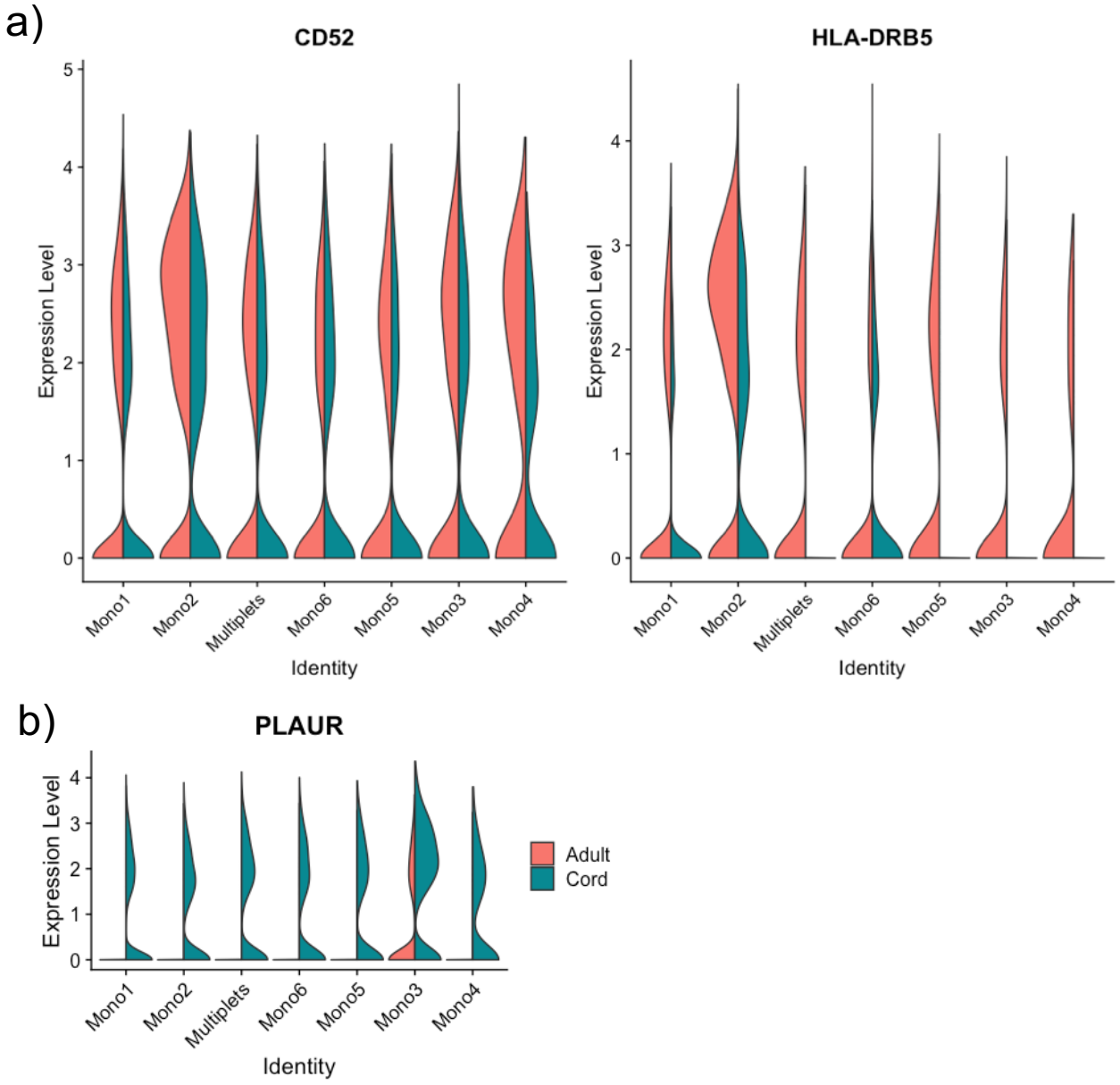


Figure 8 - Cell Surface Proteins Found Differentially Expressed Between Cord and Adult Monocyte Populations.

a) CD52 and HLA-DRB5 expression was found to be higher in adult cells than cord blood cells. b) Cord blood monocytes had elevated expression of PLAUR compared to adult cells.

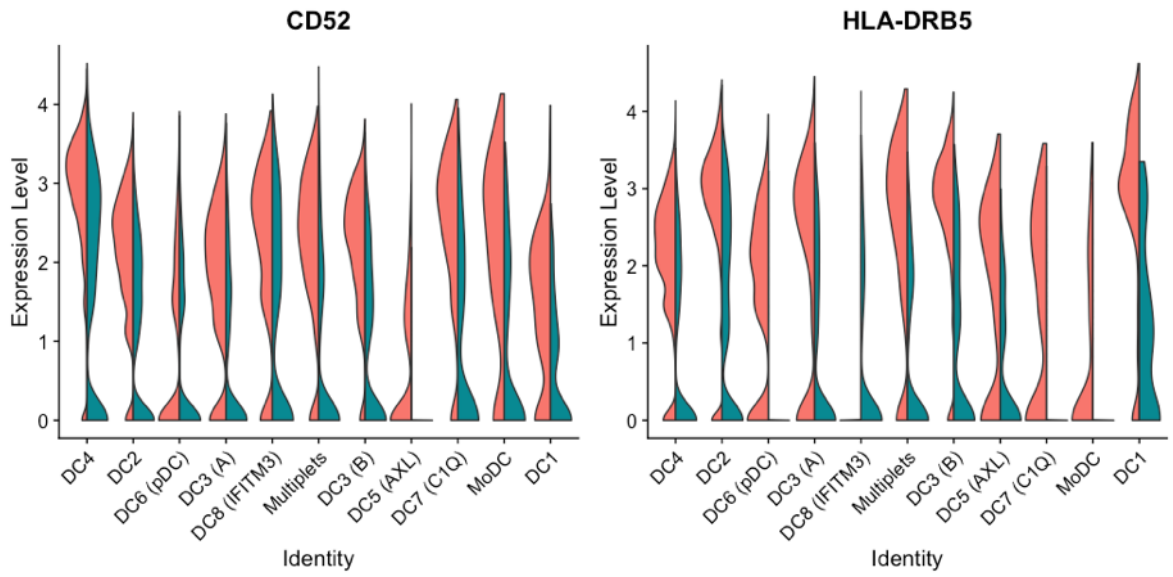


Figure 9 - Cell Surface Proteins Found Differentially Expressed Between Cord and Adult DC Populations.

CD52 and HLA-DRB5 expression was found to be elevated in adult cells than cord blood DCs. Red shading indicates adult blood; teal shading indicates cord blood.

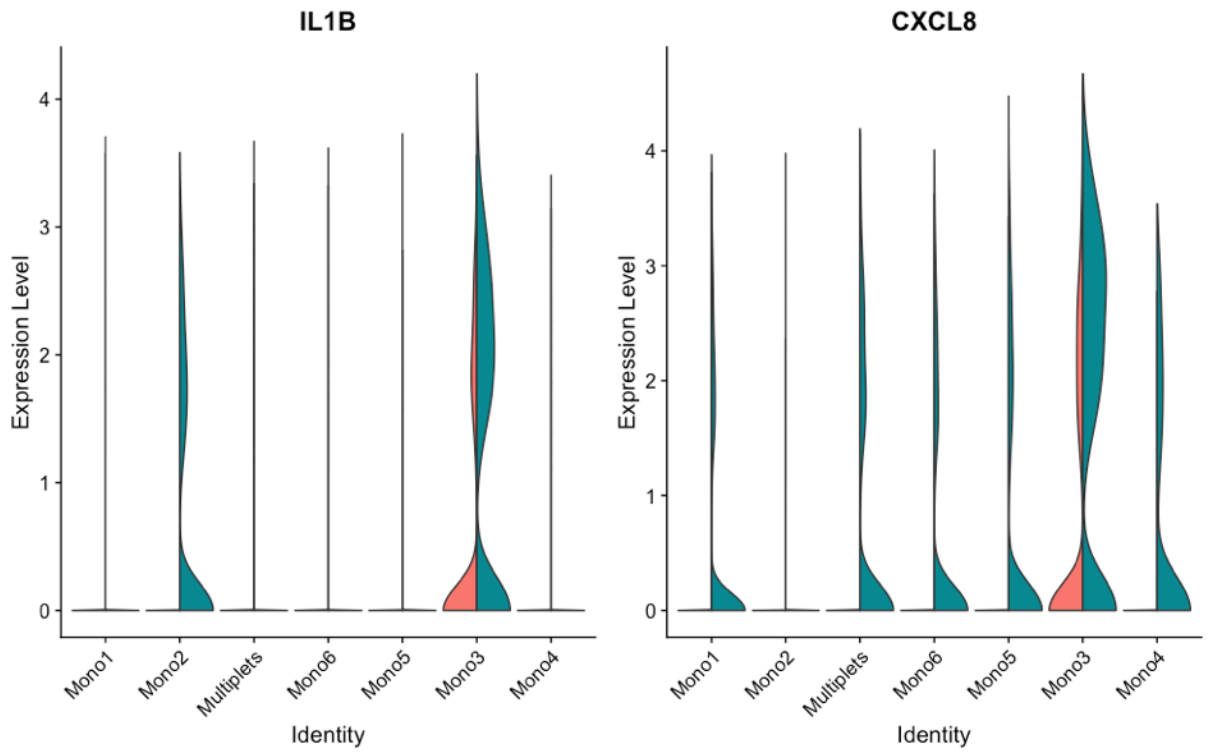


Figure 10 - Higher Expression of Inflammatory Cytokines IL1B and CXCL8 (IL-8) are Found in Cord Blood Monocytes.

Mono2 and Mono3 populations in cord blood samples show elevated IL1B expression. CXCL8 (IL-8) is found expressed in all cord blood monocyte populations except Mono2, while adults only show some expression in Mono3. Red shading indicates adult blood; teal shading indicates cord blood.

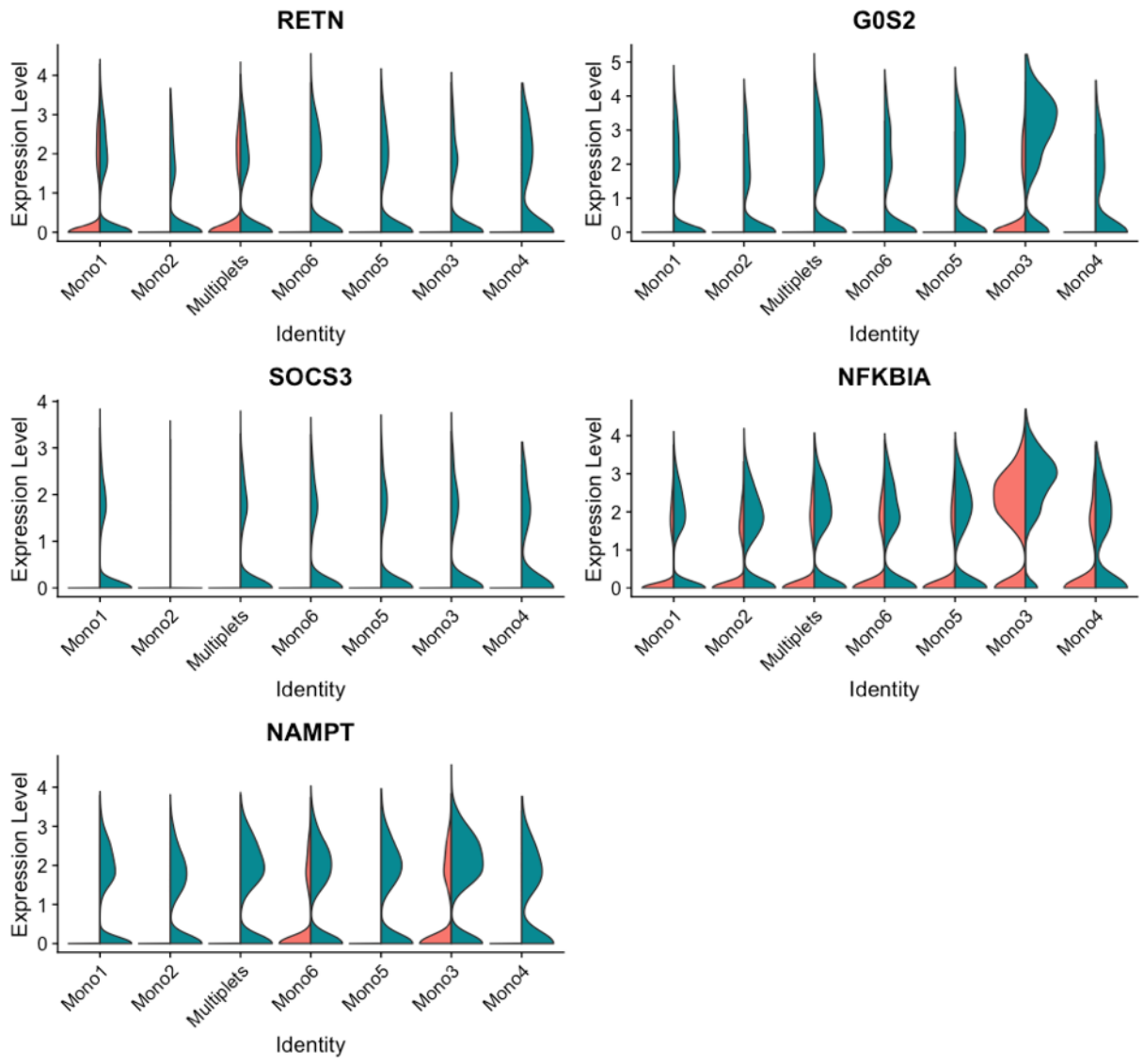


Figure 11 - Higher Expression of Immune Regulatory and Metabolic Genes in Cord Blood Monocytes.

Cord blood monocytes show elevated expression of immune regulatory pathway genes including G0S2, SOCS3, and NFKBIA, compared to adults, while also expressing more RETN, an innate immune modulator. Moreover, cord blood monocytes also expressed more NAMPT, which possibly indicates higher metabolic activity in cord blood monocytes compared to adults. Red shading indicates adult blood; teal shading indicates cord blood.

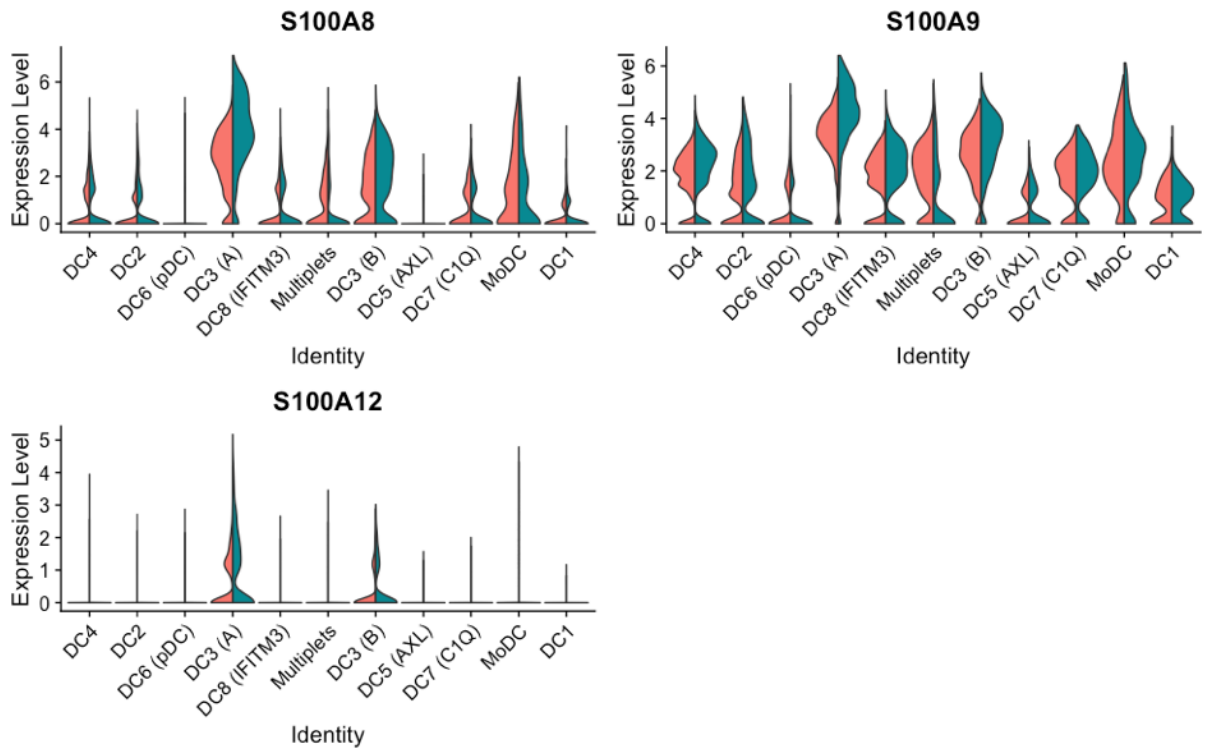


Figure 12 - Higher Expression of S100 Alarmins are Found in Cord Blood DCs.

Cord blood DCs show higher expression of several S100 Alarmins, including S100A8, S100A9, and S100A12 in comparison to adult cells. Red shading indicates adult blood; teal shading indicates cord blood.

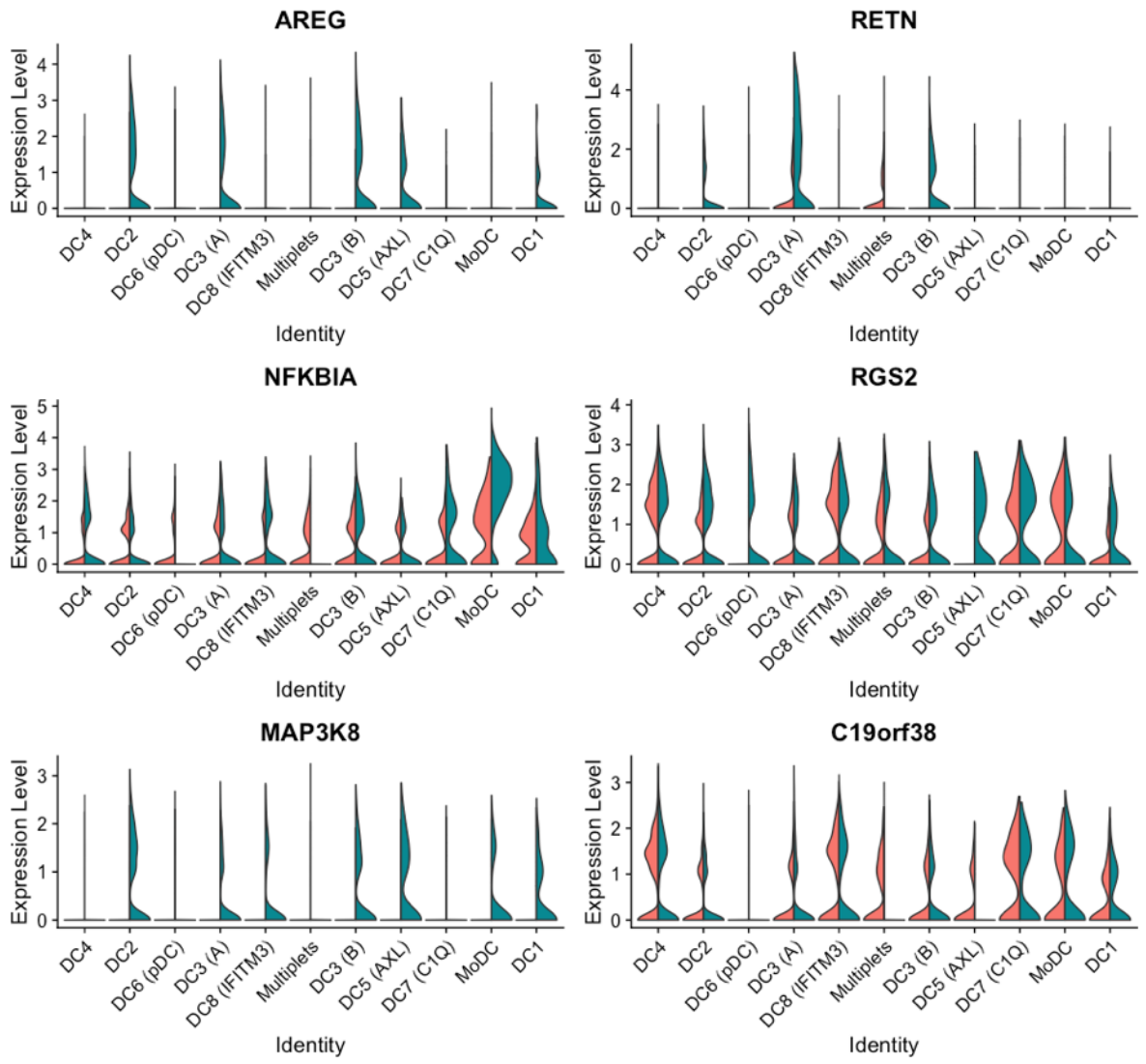


Figure 13 - Higher Expression of Inflammation Related and Immune Regulatory Genes in Cord Blood DCs.

Cord blood DCs showed higher expression of AREG and RETN, which are secreted regulators of inflammation and immunity. Furthermore, immune signaling pathways appear altered in cord blood cells compared to adults, with strong expression of NFKBIA, RGS2, and MAP3K8. Additionally, expression of C19orf38 or HIDE1 precursor, was found in higher levels in cord blood over adult blood. Red shading indicates adult blood; teal shading indicates cord blood.

2.4.3 3’3’ cGAMP Stimulated pDCs and Monocytes Do Not Differ in Type I Interferon Response Between Cord and Adult

3’3’ stimulated and unstimulated PBMCs and CMBCs were initially FACS-enriched into four populations for low input RNAseq: monocytes (Lineage-/HLA-DR+/CD14+/CD16-or+, CD1c DCs (Lineage/HLA-DR+/CD14-/CD11c+/CD1C+), CD141 DCs (Lineage-/HLA-DR+/CD14-/CD11c+/CD141+), pDCs (Lineage-/HLA-DR+/CD14-/ CD123+). Initial principal component analysis revealed sorted monocytes and pDCs formed distinct clusters, while CD1c and CD141 DCs were more in-dispersed (Fig. 14a). Similarly, sample conditions, as well as age groups, also clustered together among the different cell types (Fig. 14b). However, upon further analysis, it was revealed that sorted cord blood CD1c and CD141 DCs were heavily contaminated with CD19 B-cells and were excluded from further analyses. Initial analysis revealed that STING expression in monocytes was overall higher in adult than cord, but similar in pDCs (Fig. 14c)

There was little difference in gene expression between cord and adult monocytes in either the 3’3 cGAMP-stimulated samples or the vehicle controls (Fig. 15a). Both cord and adult monocytes expressed a generic type I IFN response gene signature, including strong expression of IRF, IFIT, IFITM gene families, and ISG15, in response to 3’3’ cGAMP stimulation (Fig. 15b). In monocytes, there was negligible induction of IFNA (Fig. 15a), but some expression of IFNB1 and IFNE (Fig. 15c). Hierarchical clustering of genes revealed samples grouped by IFN response, while cord and adult samples are separated by a cluster of metabolic genes, glycoproteins and proteases (Fig. 16).

In pDCs, a similar gene expression pattern was found between adult and cord samples (Fig. 17a). Similar to monocytes, stimulated and vehicle control pDCs produced a generic type I IFN response signature to 3’3’ cGAMP stimulation (Fig. 17b). Notably, while most type I ISGs, including IFIT1 (Fig. 17c), were found similarly expressed between adult and cord blood cells, SLFN5, the STAT1 co-repressor, was found to be expressed more in cord over adult cells (Fig. 17d). Similarly, pDCs from both age groups showed induction of IFNE, but neither IFNB nor IFNA subtypes were induced in response to 3’3’ cGAMP (Fig. 17e). However, unlike the monocytes, hierarchical clustering of genes in pDCs did not reveal any separation between cord and adult samples (Fig. 18).

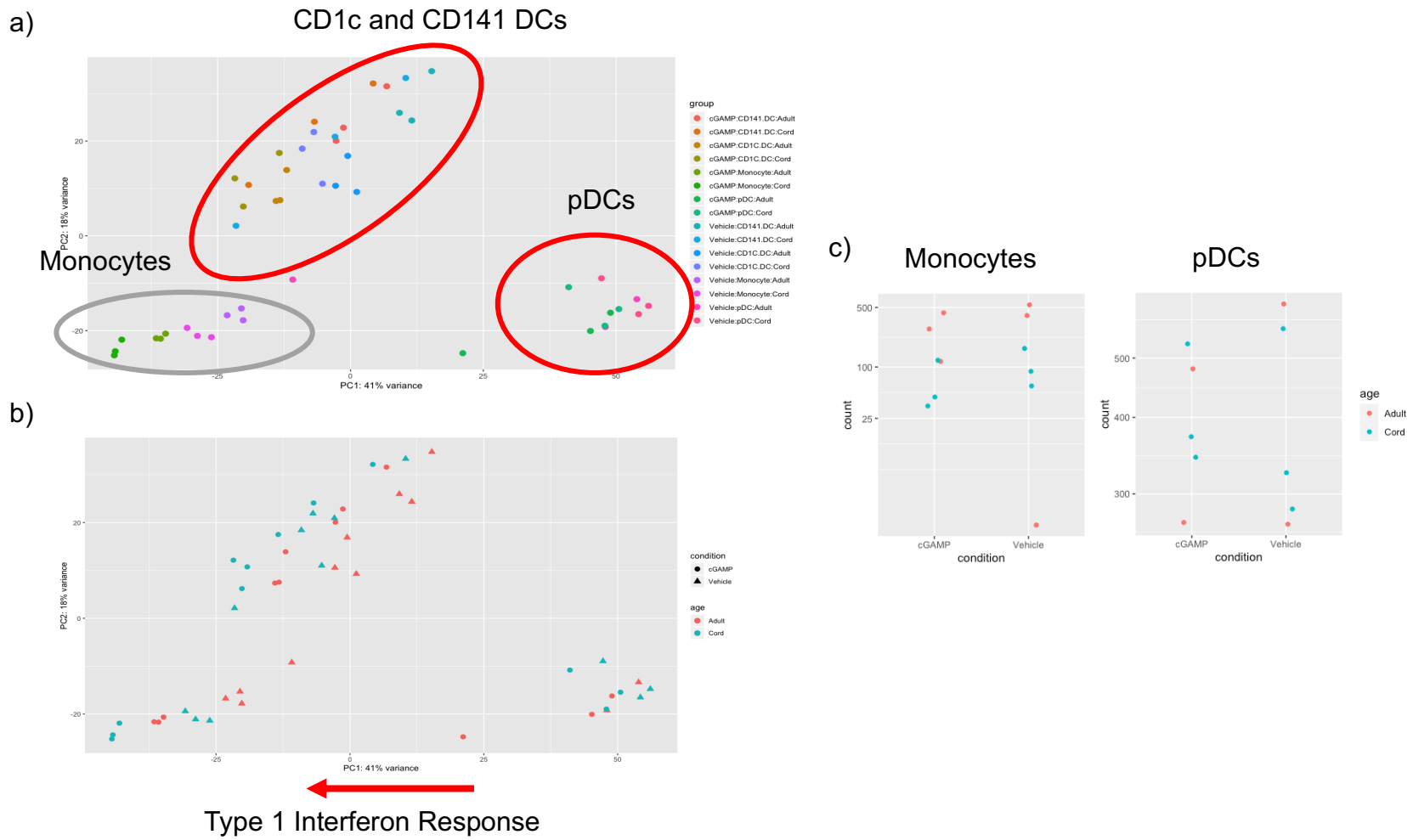


Figure 14 - PCA Reveals Cell-Types and Sample Conditions Cluster Together; Expression of STING in Adult and Cord Monocytes and pDCs.

Samples cluster together by a) Cell-type and by b) condition. Variation in PC1 is driven mainly by interferon response between 3'3' cGAMP stimulated cells and vehicle controls. It was revealed that cord blood CD1c and CD141 DCs contained heavy contamination by B-cells due to strong MS4A1 (CD20) expression, therefore they were excluded from further analysis. c) Cord blood monocytes showed less expression of STING, but pDCs expression was similar.

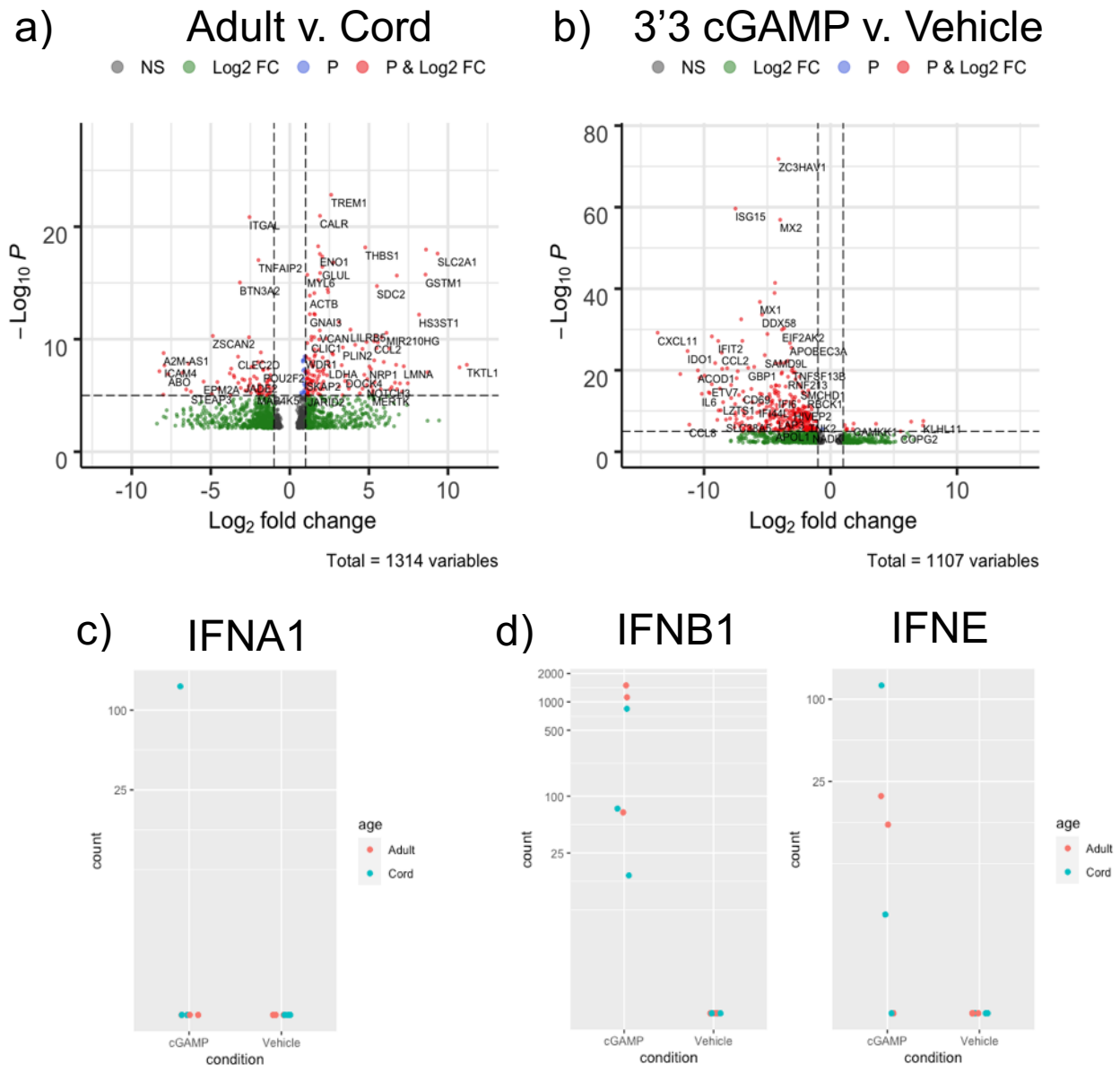


Figure 15 - No Age-Related Differences were Found Between 3'3' cGAMP stimulated Monocytes and Vehicle Controls.

Volcano plots reveal a) cord and adult monocytes gene expression is similar, while a generic type I interferon response is induced in b) 3'3' cGAMP stimulated cells. Induction of c) INFA1 was not commonly expressed in cells after stimulation by 3'3' cGAMP, while d) IFNB1 and IFNE were strongly induced after stimulation.

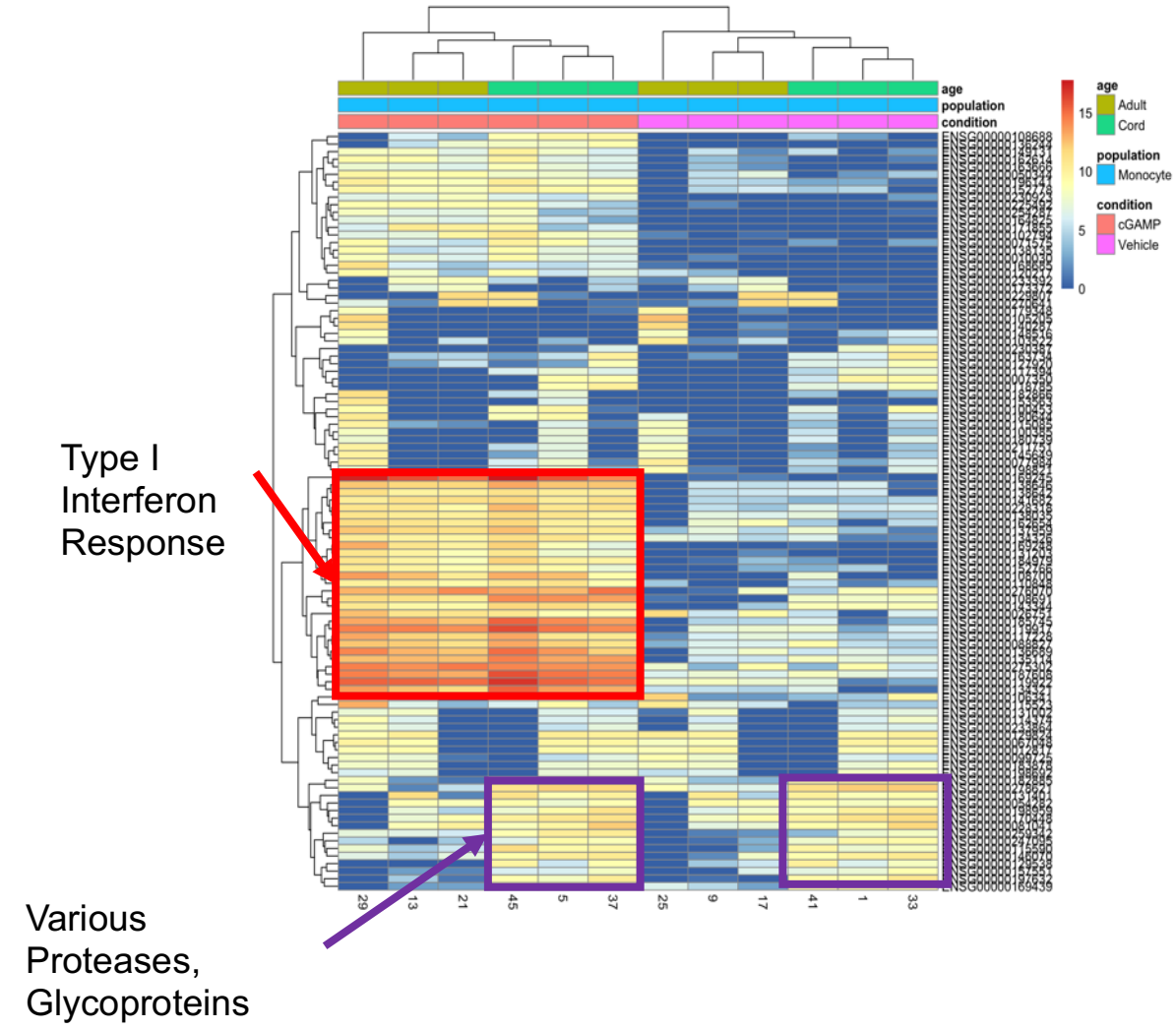


Figure 16 - Heatmap and Clustering of Genes Expressed Reveal Groups of Genes Related to Type I Interferon Response and Genes that Separates Age in Monocytes.

Red box indicates type I IFN response genes; Blue box indicates various proteases and glycoproteins found expressed in cord cells.

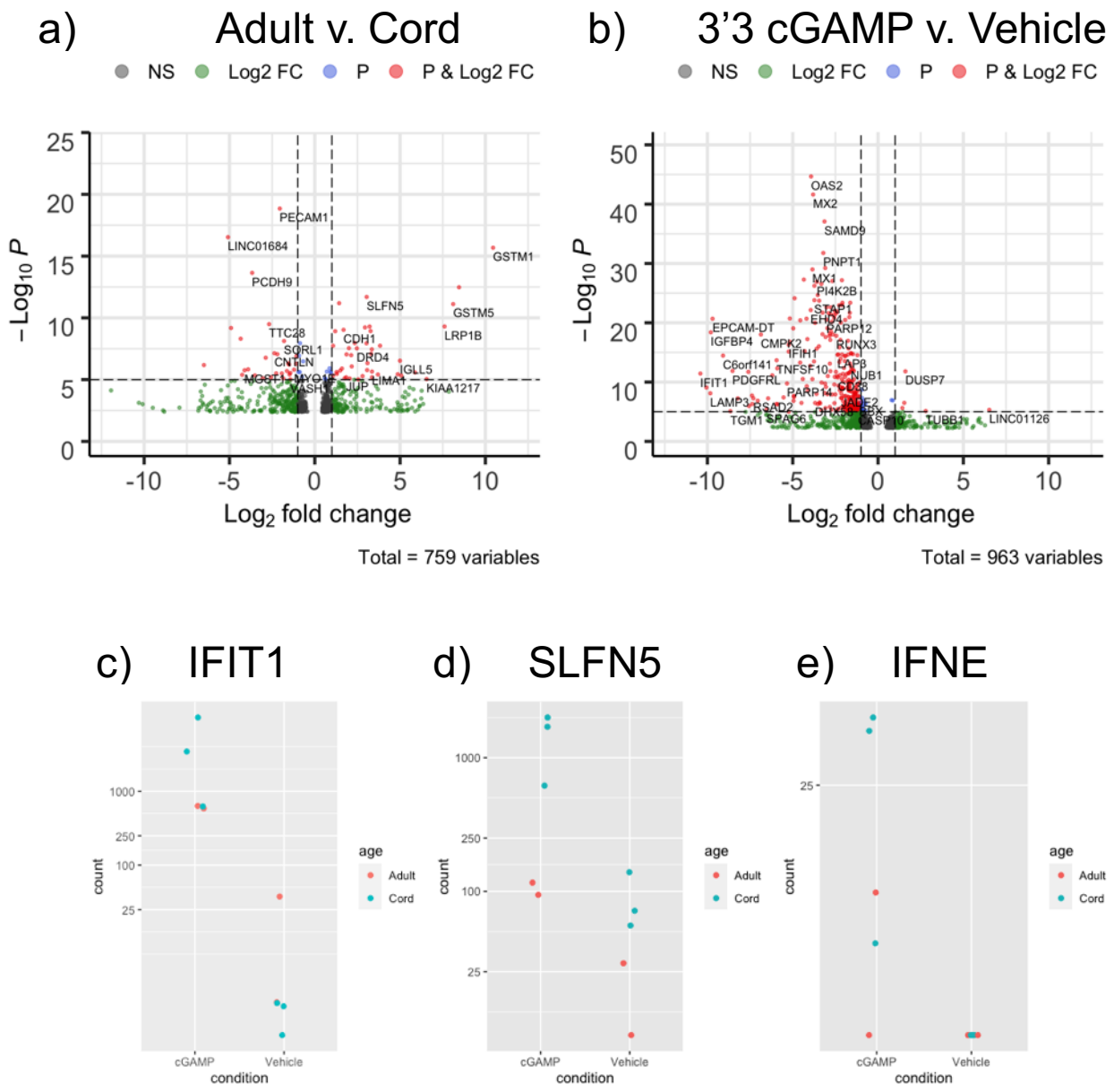


Figure 17 - No Major Age-Related Differences were Found Between 3’3’ cGAMP stimulated pDCs and Vehicle Controls.

Volcano plots reveal a) cord and adult pDC gene expression is similar, while a generic type I interferon response is induced in b) 3’3’ cGAMP stimulated cells. Induction of c) IFIT1 was strongly expressed in cells after stimulation by 3’3’ cGAMP, while d) SLFN5, a transcriptional co-repressor of STAT1 was differentially expressed between cord and adult cells. Only e) IFNE showed any induction after 3’3’ cGAMP stimulation in pDCs

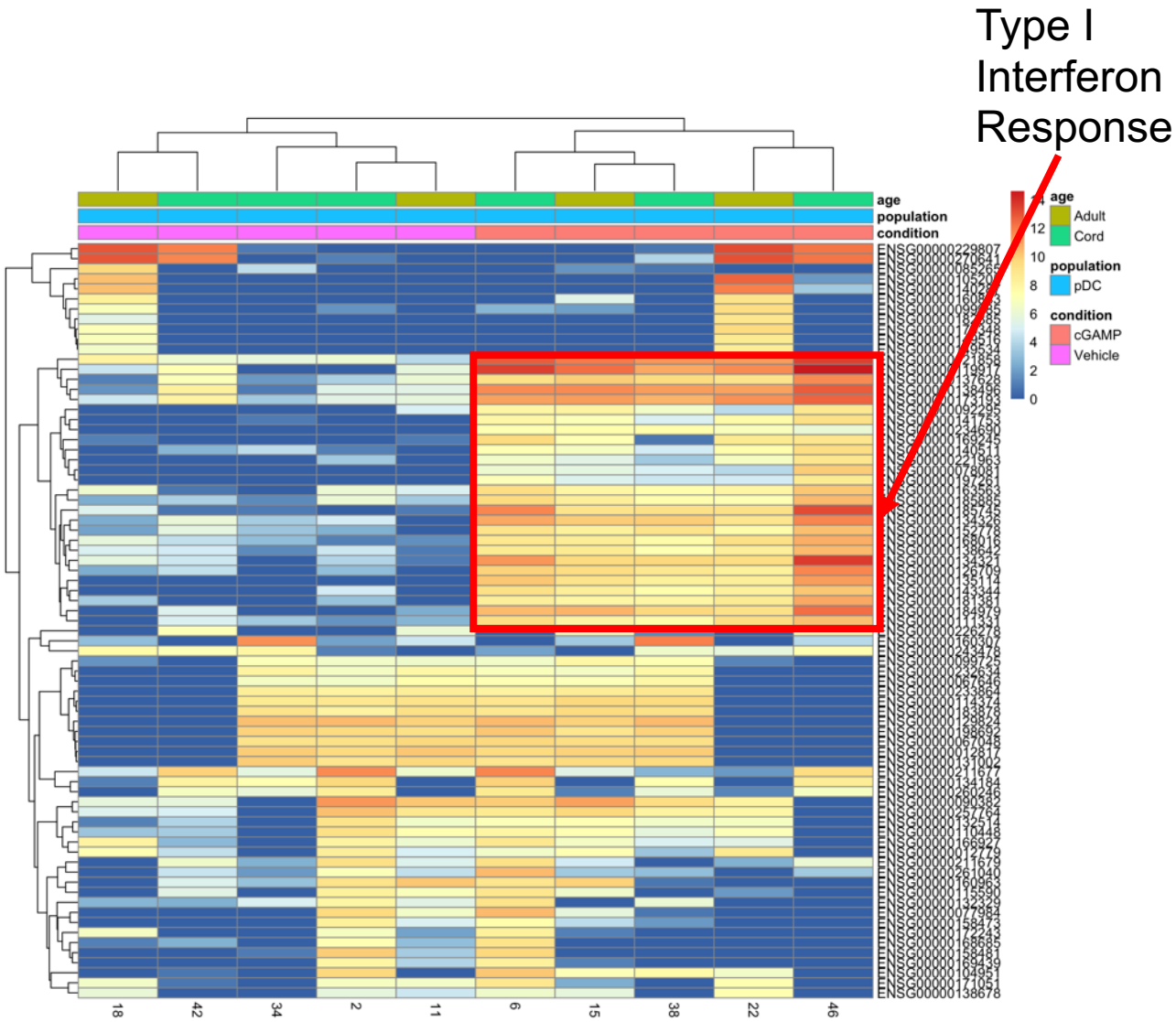


Figure 18 - Heatmap and Clustering of Genes Expressed Reveal Clusters of Genes Related to Type I Interferon Response in pDCs.

Red box indicates type I IFN response genes.

2.5 Discussion

In this study, cord blood and adult peripheral mononuclear cells were studied using FACS enrichment, followed by single-cell or bulk transcriptome sequencing, to uncover differences in cell populations and gene expression between neonates and adults. Using FACS enrichment, antigen presenting cells were purified from heterogeneous PBMCs and CBMCs, to enhance the granularity of scRNAseq data, uncovering rare and unique populations, while also teasing apart phenotypically plastic populations. Furthermore, FACS combined with low-input RNAseq also allowed for targeted transcriptome analysis of rare cell populations under resting as well as 3'3' cGAMP-stimulated conditions. Together, these techniques allowed us to determine that not only do neonates and adults have phenotypically similar populations of antigen presenting cells, but at rest, their transcriptomes are quite similar. Furthermore, upon stimulation by 3'3' cGAMP, adult and cord blood monocytes and pDCs appear to respond similarly.

To examine the different cell populations within monocytes and DCs, FACS-enriched cells were profiled using 10X Genomics scRNAseq platform. In total, 6 distinct populations were found in both cord and adult peripheral blood, 4 of which previously classified and 2 new. CD14 and CD16 (FCGR3A) expression in Mono1 and Mono2 determined that these populations were indeed classical and non-classical monocytes previously classified by scRNAseq by Villani et al. [199]. Mono3 represented a smaller cluster of cells that expresses NAMPT, G0S2 and several cytokines, including IL1B and CXCL8, which are important to monocyte migration, polarization, and differentiation [199]. Interestingly, the Mono4 cluster represented a unique population of monocytes

that expressed both traditional markers of monocytes, but also genes expressed by NK cells. This population has been previously classified as “natural killer dendritic cells” by their expression of NKG7, and cytotoxic genes, GZMA, GZMB, and GNLY [200–202]. However, functional characterization is needed to determine validate these cells as being a unique population, as contaminating NK cells or T-cells could also cause this population as suggested by several groups [203,204].

Of the new monocyte populations found in this study, the Mono5 population displayed high expression of cell-cycle proteins, while Mono6 expressed several ISGs. Expression of CDC42SE1, FDG2, and EIF5A in the Mono5 cluster suggests these could be immature monocytes, as CDC42 activity is crucial to myelopoiesis and development [205]. Several ISGs are found in the Mono6 cluster, including IFITM3, IFIT2 and IFIT3, ISG15 and MX1, suggesting anti-viral activity [206]. Previous studies of patients with systemic lupus erythematosus (SLE) have found this population enhanced compared to healthy subjects, with function unknown [207]. This population is found distributed in both cord and adult samples, suggesting that this population may exist during steady state rather than being induced.

Within the enriched DCs, we identified 8 distinct populations and 1 transitionary monocyte-derived DC population that expressed low levels of CD14. DC1 represented the rare CD141 DC population identified by Villani et al. that is better identified by its expression of CLEC9A; the DC2 population represented the traditional non-

inflammatory CD1C DCs that also express CLEC10A [199]. In contrast to Villani et al, inflammatory CD1C DC3s clustered into two distinct subpopulations, DC3(A) expressed less CD1C, but more S100A8 and RETN, while DC3(B) expressed more CD1C and LGALS2. This suggests that these DC3s may exist as a heterogeneous population that expresses a gradient of inflammatory S100A. DC4, however, represents a population of DCs that are both CD1C- and CD141- while sharing many markers with monocytes. This population of cells lacks strong CD14 expression, but shows elevated expression of CD16 and could potentially be closer to non-classical monocytes, rather than DCs [203]. DC5 expressed both markers of cDCs and pDCs, but also AXL and SIGLEC6, representing a progenitor population previously characterized to become either cDCs or pDCs [199,203]. Finally, DC6 represented pDCs through their strong expression of CD123 (IL3RA) and IRF7.

In addition to the 6 populations previously identified by Villani et al., 2 new populations were found within cord and adult samples in our dataset. This includes DC7, a population of DCs that express IFITM2, IFITM3, and other ISGs, similar to Mono6, and DC8, which strongly expresses complement genes C1QA, C1QB, and C1QC. Expression of C1Q has been previously found to be a marker of mouse regulatory monocyte-derived DCs that support the differentiation of IL-10-secreting CD4 T-cells and DC8 could be the human equivalent [208,209].

Interestingly, within monocyte and DC populations, small numbers of Mono6 and DC7 populations that expressed similar ISG signatures are found in both cord and adult. The

type I IFN induced IFIT and IFITM families of genes were highly expressed in these cells, as well as ISG15 and LY6E. Expression of these genes could suggest a population of anti-viral antigen presenting cells that patrol the blood. Previous studies have found that several viruses including DENV and HIV use LY6E to gain entry into monocytes and DCs [210,211]. However, whether these populations exist in steady state or are induced by viral exposure will have to be further examined.

Although there were some differences between the transcriptomes between adult and cord blood populations, the differences were overall rather subtle. In DCs and monocytes, cord blood samples had reduced surface expression of HLA-DRB5 and CD52, but increased expression of PLAUR. Cord blood monocytes also displayed a more pronounced imbalance between pro- and anti-inflammatory signalling pathways. Specifically, cord blood monocytes in general expressed more pro-inflammatory RETN, IL1B and CXCL8 than adult cells, while also expressing anti-inflammatory genes, including G0S2, SOCS3 and NFKBIA. SOCS3 is especially interesting, as it influences the IL-6/IL-10/STAT3 axis and has found to be important in type 1 IFN responses against CMV [212], HSV-1 [213,214], EBV [215], and RSV [216], and could underlie some of the neonatal susceptibility to these viruses, with the exception of EBV. Furthermore, in cord DCs, there was also enhanced expression of RGS2, NFKBIA, and MAP3K8 in some DC populations including DC1, DC2, and DC6, suggesting imbalanced signalling casc

s compared to adults. These differences seen in DCs have been suggested to be mainly epigenetic, i.e., resulting from age-dependent chromatin modifications in these cells [217]. Functional characterization of these monocyte and DC subsets will help understand the importance of such changes in neonatal cells.

As neonates are susceptible to intracellular infections by pathogens like Listeria monocytogenes, we examined type I IFN responses through the STING pathway, using the canonical sting ligand 3’3’ cGAMP. While FACS-sorted CD141 and CD1c DCs from cord blood contained extensive B-cell contamination and were excluded from the detailed analysis, monocyte and pDC populations were of high purity. Initial PCA analyses revealed that cell types clustered close together, while variance in PC1 were primarily driven by the type I IFN response in 3’3’ cGAMP stimulation. Despite some differences in STING expression in monocytes, gene expression signatures of monocytes and pDCs revealed a similar generic type I IFN response under stimulation. Surprisingly, there was a lack of expression of IFNA in either population, which other studies have shown after stimulation using double stranded DNA cultured *in vitro* assays [218–220] .

Differences between cord and adult monocytes were mainly driven by metabolic proteins, proteases, and other non-immune related genes. However, SLFN5, a type I IFN induced STAT1 co-repressor [221], was found to be expressed higher in cord than in adult pDCs, possibly suggesting biased type I IFN feedback loops in cord blood

pDCs. Of note, SLFN5 was not found to be differentially expressed between cord and adult pDCs (DC6) in the scRNAseq dataset.

Despite the similarity of the two transcriptome methodologies, there was a lack of correlation between the differentially expressed genes in the two datasets. Notably, genes found to be differentially expressed using either method were not confirmed by the other assay in the same cell types. This highlights the variability that can occur in systems biology generally, and specifically when comparing bulk RNAseq and scRNAseq methods. Furthermore, a challenge in confirming these differentiated genes in rare populations is two-fold. First, isolating enough cells to do analyze gene or protein expression requires large sample volumes. Second, some populations, like Mono5, Mono6, DC7, and DC8 that lack identified CD markers cannot be FACS enriched definitively or confirmed via flow cytometry.

Another challenge working with cord blood samples was the presence of contaminating cells during FACS enrichment. Even using “dump” channels to remove T-cells and B-cells, these cells were still evident in our FACS enriched cord blood samples and required computationally “gating out” these populations out during pre-processing. Moreover, in the bulk sorted cells for RNAseq, samples were depleted of cells expressing CD56, CD3, CD19, or CD235a using negative selection, but this still resulted in heavy B-cell contamination in the CD141 and CD1c enriched samples.

Furthermore, a more philosophical question exists about whether the expression of transcripts alone is enough to define a population. Some cell populations that were identified lacked expression of a discrete combination of protein surface markers, making identification difficult, while some genes or proteins were only expressed under certain physiological conditions. Whether this represents a distinct cell type or merely a cell state has been debated [222,223]. For example, the Mono6 and DC7 populations with expression of ISGs, typically induced by type I IFN, may represent a cell state rather than a specific population of cells. Whether a cell state is a distinct population is one challenge of using transcriptomics alone to identify populations, therefore careful characterization is needed to understand any population identified by scRNAseq methods, like here.

In conclusion, using single-cell and bulk transcriptomic methodologies, we were able to characterize and compare antigen presenting cells, specifically monocytes and DCs, from cord and adult peripheral blood. Using scRNAseq, we uncovered several new populations of DCs and monocytes that expressed cell cycle proteins, ISGs, and complement proteins. Although comparisons of differentially expressed genes between age groups revealed only minor differences between cord and adult, signalling biases along the IL-6/IL-10 axis and NF-κB were uncovered. Similarly, under stimulation from 3’3’ cGAMP, cells from cord and adult blood exhibited a similar type I IFN response. Overall, this study suggests that at rest and under stimulation by 3’3’ cGAMP, antigen presenting cells from cord and adult samples have very similar gene expression. Thus, differential antigen presenting cell phenotypes or STING signaling is unlikely to explain

the markedly increased susceptibility of newborns to severe infection with intracellular pathogens.

Chapter 3 – Human Herpesvirus DNA Is Not Present in Significant Quantity in Either Alzheimer's Disease or Normal Control Aged Brain

3.1 Introduction

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disease characterized by neuroinflammation, the widespread accumulation of insoluble forms of amyloid-beta (AB) and tau, structural brain damage, and ultimately cognitive decline [224,225]. Models of AD primarily focus on genetic factors [226], AB [227] and tau pathology [228], with additional interest in potential environmental risk factors, such as infectious agents [229,230]. Herpes simplex virus 1 (HSV-1) and other human herpesviruses (HHVs), have long been hypothesized to be involved in AD pathogenesis [231].

Hippocampal sclerosis (HS) is characterized by marked neuronal loss and gliosis of the hippocampal formation, and is associated with pathologic TDP-43 (a hyper-phosphorylated, ubiquitinated and cleaved form of TDP-43) in persons with Alzheimer's dementia as well as in those with AD pathology. While HHVs have not been studied in the context of age-related HS, they are clearly related to younger-onset HS associated with epilepsy. The extent of convergence between epilepsy associated HS and age-related HS is unclear [232].

3.1.1 HHVs Associated with AD

HSV-1, HHV-6, and HHV-7 are ubiquitous herpesviruses showing varying levels of neurotropism [233]. Encephalitis caused by HSV-1 and HHV-6, and to a lesser extent HHV-7, is classically limited to the hippocampus and surrounding limbic structures, areas that are also commonly affected in AD, and by definition affected in HS [234]. Preclinical work demonstrate that infection with HSV-1 causes accumulation of pathological tau and AB, and colocalization of HSV-1 with AD pathology, most notably within neuritic plaques [235], which may sequester the virus in a protective response [236]. In organotypic models, HSV-1 infection recapitulates an AD phenotype (tau, AB, upregulation of PSEN1 and 2, and inflammation) while acyclovir mitigates this effect [237]. In mice, repeated HSV-1 activation causes progressive accumulation of AD pathology and cognitive deficits that eventually become irreversible [238].

3.1.2 Review of Data Supporting a Causal Relationship Between HHVs and AD

Human case control studies employing PCR testing of post-mortem tissue report elevated levels of HHV-6,7 and HSV-1 DNA in AD brains compared to non-AD controls [235,239]. Convergent serological data suggests that HSV-1 IgM levels, a putative surrogate marker of the chronic level of HSV-1 reactivation, are correlated with AD development [240,241]. These studies, along with many others that show an association between APOE4 and HHV infectivity and clinical expression [242], highlight a plausible connection between HHVs and AD. There are, however, many negative or contradictory studies in the literature [243,244].

3.2 Rationale

Recent high profile studies have re-ignited the interest in HHV as a causative factor of AD [236,243,245,246]. In particular, the results of a recent paper [245], which assessed levels of HHV RNA and DNA in brain samples from multiple well described aging cohorts, reported an association of multiple HHV and AD. These results have, however, been controversial, and multiple reanalyses of the same data not replicating this finding [247–250]. Given the discordant results, we sought to quantify the HHV DNA in a subset of brain samples from one of these same cohorts, from the Rush Memory and Aging Project (MAP) [251]. As this was an exploratory study meant to inform the utility of analyzing the larger MAP cohort, we selected cases with clear and advanced AD or HS pathology from the larger sample.

Our hypotheses were as follows. (i) Samples of hippocampus from cases with a neuropathological diagnosis of Alzheimer pathology or HS would have more HHV than those without these diagnosis; (ii) Brain regions more severely affected by Alzheimer pathology would demonstrate more HHV than a less severely affected region, the primary visual cortex; (iii) As cognitive impairment increased from none, to diagnoses of mild cognitive impairment (MCI) and Alzheimer's dementia (DEM), HHV detection would increase accordingly.

3.3 Material and Methods

3.3.1 Ethics Statement

This study was conducted under the approval and review by an Institutional Review Board of Rush University Medical Center, and the Clinical Research Ethics Boards of the University of British Columbia, Vancouver General Hospital and BC Children's Hospital Research Institute. Written informed consent was obtained from all participants. All participants signed an Anatomic Gift Act for donation of brain, spinal cord, nerve and muscle, and a repository consent that allowed their data and biospecimens to be shared.

3.3.2 Clinical Specimens

Homogenized human brain specimens were obtained from 62 autopsied brains from a large community-based aging study, the Rush Memory and Aging Project, as described by Bennett et al. [252], Samples from up to four different regions per brain were obtained: hippocampus, middle frontal cortex gyrus, inferior temporal cortex, and primary visual cortex. Brains were selected to represent two of the major age-related pathologies affecting the hippocampus – AD (plaques and tangles), and HS, as well as brains without a pathologic diagnosis of either. HS in the elderly is commonly associated with AD pathology; brains meeting both pathological criteria were grouped as HS. Pathological diagnoses were made by a Board-certified neuropathologist. The presence of AD pathology was defined by NIA-Reagan criteria as high or moderate likelihood. Participants were classified as either having no cognitive impairment (NCI),

mild cognitive impairment (MCI), or Alzheimer's dementia (DEM) based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria [252,253]. If cognitive impairment was present but not severe enough to merit a diagnosis of dementia then mild cognitive impairment (MCI) was diagnosed as described [254].

3.3.3 DNA Extraction from Brain Tissue

DNA was extracted from up to 100mg of brain homogenates using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), with slight modifications to the manufacturer's instructions. Briefly, brain homogenates were incubated with lysis buffer and proteinase K for 5 hours at 55C to lyse cells, then proceeded to be extracted as recommended. To ensure maximum elution of DNA, a second DNA elution step was performed to extract the remaining DNA after the initial elution. Final DNA concentration was quantified using the dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA) on a Qubit 3 Fluorimeter (Thermo Fisher Scientific, Waltham, MA). DNA was then stored at -20 °C until use.

3.3.4 Quantitative Polymerase Chain Reaction

Duplexed quantitative PCR (qPCR) was carried out using the CFX96 Real Time System (Bio-Rad, Hercules, CA). The primers and probes used for HSV-1 [255], HHV-6 (conserved between HHV-6A and B) [255], and HHV-7 [256] amplification are provided in Supp. Table 1. Human glyceraldehyde 3-phosphate dehydrogenase (*hGAPDH*),

which is present at two copies/cell, was used as a reference housekeeping gene. Control amplicons ("gBlocks") were designed for each target on Serial Cloner and synthesized by IDT (Integrated DNA Technologies, Coralville, IA) to create a standard curve (Sup. Table 2). DNA quantitation was performed on 96-well plates at a final reaction volume of 20uL, which consisted of 10uL of 2X PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA), 0.5uL of 40X PrimeTime Assay of each of the primers and probes for *GAPDH* and the relevant virus (Integrated DNA Technologies, Coralville, IA), and 5uL of specimen DNA. Negative (no-template) controls, in which water was used in place of sample DNA, were included in each plate. The standard curves consisted of 5uL of duplexed *hGAPDH* gBlock plus HSV-1, HHV-6, or HHV-7 gBlock as DNA template, using serial 10-fold dilutions from $2x10^6$ copies/uL to $2x10^0$ copies/uL. Each plate also included biological controls consisting of $1\text{-}5x10^4$ copies of HSV-1, HHV-6, or HHV-7 gBlock spiked into 50ng of extracted peripheral blood mononuclear cell (PBMC) DNA. Two technical replicates were performed for each sample, when necessary, samples were prioritized for HSV-1, HHV-6, then HHV-7. Plates were sealed with film (Bio-Rad, Hercules, CA) prior to thermocycling. qPCR amplification cycling parameters were as follows: 95 °C for 3 minutes, followed by 45 cycles at 95 °C for 15 seconds, and 60 °C for 30 seconds. A positive reaction for any HHV was defined as any qPCR reaction that amplified both viral DNA and *hGAPDH*.

3.3.5 Data Analysis

All analyses were carried out with JMP v14 software. Following qPCR amplification, viral and *hGAPDH* DNA copy numbers in each reaction were determined using Bio-Rad CFX Manager (Version 3.0). Virus copy numbers were normalized to the number of cells in each reaction based on *hGAPDH* copy number and expressed as virus copy numbers per $1x10^6$ cells. Samples without *hGAPDH* amplification in replicate samples were excluded from the final analysis. A series of repeated measures analyses of variance, co-varying for age, were planned to evaluate differences in virus copy number between brain regions, in relation to pathologies, with the alpha level set to .05.

3.4 Results

The demographic, pathologic, and cognitive characteristics of participants appear in Table 3. A total of 241 specimens, representing up to four brain regions per individual, were tested by qPCR. *hGAPDH* was amplified in both reactions for all specimens but 1, at a median of $3.99x10^5$ cells per reaction (IQR = $2.06x10^5$).

The frequency of HHV DNA detection in all samples was low, independent of pathological or clinical classification, or brain region (Table 4). DNA from more than one virus was not detected in any individual and at most one of the four anatomical regions in any individual was positive for HHV. Among the few positive specimens, viral loads were close to the limit of detection (<10 copies/reaction); the highest observed (175

copies/10^6 cells or 64 copies/reaction) was HHV-6 in the inferior temporal cortex of one participant with AD pathology and MCI.

The small cell sizes and the negative results precluded any meaningful statistical analysis of regional tropism, or of the distribution of individual viruses as a function of pathological or cognitive diagnosis.

Table 3 - Participant Demographics, Pathologic and Cognitive Diagnoses.

	No Pathological Diagnosis	**Alzheimer Pathology**	**Hippocampal sclerosis**
n	22	24	16
Age – mean (SD)	85.5 (7.6)	88.8 (5.8)	90.1 (6.6)
Female	12/22 54.6%	18/24 75.0%	8/16 50.0%
NIA Regan high or intermediate Alzheimer Disease pathology	0/22 0.0%	24/24 100%	13/16 81.3%
No Cognitive Impairment / Mild Cognitive Impairment / Dementia	11 / 7 / 4	2 / 2 / 20	0 / 3 / 13

Table 4 - Detection of HHV DNA in Brain Specimens by Anatomical Region and Pathological Diagnosis.

	HSV1			HHV6			HHV7		
	NPD	**AD**	**HS**	**NPD**	**AD**	**HS**	**NPD**	**AD**	**HS**
HIPP	0/20	0/23	0/15	0/21	1/23	0/15	0/21	0/23	0/15
n/n, %	0.0%	0.0%	0.0%	0.0%	4.4%	0.0%	0.0%	0.0%	0.0%
ITC	0/22	0/23	0/16	0/21	1/24	1/16	1/21	0/23	0/16
n/n, %	0.0%	0.0%	0.0%	0.0%	4.2%	6.3%	4.8%	0.0%	0.0%
MFC	0/21	0/24	1/15	0/21	0/24	0/16	1/21	1/24	0/16
n/n, %	0.0%	0.0%	6.7%	0.0%	0.0%	0.0%	4.8%	4.2%	0.0%
OCC	0/21	0/24	0/14	1/21	0/24	0/14	0/21	1/24	0/14
n/n, %	0.0%	0.0%	0.0%	4.8%	0.0%	0.0%	0.0%	4.2%	0.0%

NPD: No pathological diagnosis AD: Alzheimer's disease pathology; HS: hippocampal sclerosis with or without AD pathology)

HIPP: hippocampus, ITG: inferior temporal cortex, MTG: middle temporal cortex, OCC: primary visual cortex

3.5 Discussion

In this well-characterized cohort of aged individuals from the Rush Memory and Aging project, HHV DNA was detectable in brain samples at such low frequencies that inferential statistics were abandoned. Our a priori analytic plan was to analyze HSV-1, HHV-6, and HHV-7 as a function of brain location as the extant literature suggests, especially for HSV-1 but also for the other viruses, that the hippocampus and surrounding structures may be more prone to infection. We detected HSV-1 in zero of 58 hippocampal samples, 23 of which were from individuals with pathologically confirmed AD and 15 of which had HS. HHV-7 was also not detected, and HHV-6 detected once, in these same samples. Frequencies were similarly low for all other regions, precluding any meaningful statistical analysis of anatomical tropism.

As no individual harboured more than one virus, and that virus, if present, was never present in more than one of the four anatomical locations, our results also do not support a theory of general immune senescence or deficiency creating vulnerability for CNS invasion by HHVs. If this were the case, we would have expected multiple viruses to be found in certain individuals, or some consistency in viral DNA recovery across regions in a single individual.

While some previous studies utilizing PCR to detect HHVs in autopsied brain samples suggest that HHV DNA may be frequently present, these relied on nested PCR or other non-quantitative methods, which can be prone to environmental contamination or

amplification biases if no prior optimization is performed [239,257–259]. Furthermore, studies that reported elevated expression of viral and host response genes relied on using transcriptomic and metagenomic approaches [236,245] that can potentially introduce amplification biases during the library generating process [260]. The most highly cited of these [245], drew from three well described aging cohorts; (i) the Rush Memory and Aging Project, the Religious Orders Study (ROS), and the Mayo Temporal Cortex cohort (MAYO TCX). They described frequent detection of HHV DNA and RNA in these brains, associations between the level of HHVs and AD clinical diagnosis, dementia rating scales, and AD neuropathology through analyses of transcriptome and whole exome sequence data derived from the samples [245]. However, these analyses have been questioned, with three separate publications arguing that the statistical methods were inappropriate [247,248], and two groups failing to show an association analyzing the same dataset with similar although not identical techniques [249,250]. Our study sought to circumvent these limitations by including biological controls in each qPCR assay, which showed limited inter-assay variability and quantitatively established the lower level of detection. We were unable to verify the presence of HHVs using targeted amplification in the vast majority of these samples, with a rate of less than 2% for any of HSV-1, HHV-6, or HHV-7, irrespective of clinical groups. Recently Allnutt et al., studying the ROS cohort (very similar to ours and one of the three analysed by Readhead et al.) using digital droplet PCR (ddPCR), a method with even greater sensitivity that our qPCR assay, detected HHV DNA in brain samples at a rate that approximates our present analysis of the MAP cohort [243].

The strengths of this study include the well described cohort, and the use of a highly sensitive and specific qPCR method. Furthermore, we analyzed different neuroanatomic regions, which increase the likelihood of detecting specific herpesvirus with varying regional neurotropism [261–264], and the inclusion of MCI as well as AD patients allowed assess the potential correlation between level of cognitive impairment and viral detection.

Our findings do not necessarily preclude a direct role for HHVs in the pathogenesis of AD. Firstly, we cannot rule out a higher frequency of latent virus or low levels of viral replication in the brain that could be below the limit of detection. Furthermore, a causal effect of HHV infection or replication in the brain on the development of AD could have been remote, if the cohort subjects were infected long before death. Such a “hit and run” model, wherein HHVs trigger an inflammatory event early in the pathology of neurodegenerative diseases such as AD and multiple sclerosis, has been proposed previously [265]. Indeed, it is conceivable that HHV infections could trigger CNS pathology without direct neuroinvasion, as has been described for numerous other viral infections [266,267].

In summary, our study examined the role of HHVs in the pathogenesis of AD by assays that directly amplify viral DNA in brain tissue. Although we found the prevalence of HHV to be low, our data do not preclude the possibility that HHV replication may have occurred earlier, triggering pathology that then independently sustains a

neurodegenerative process. More studies, particularly using large prospective cohorts, perhaps including the use of antiviral therapy [235] are indicated to definitively determine whether and how HHV infections may influence the risk of AD.

Chapter 4 – Age Distributions of Endemic Coronavirus Serology Reveals Initial Infections Occur in Early Childhood

4.1 Introduction

Coronaviruses (CoVs) belong in the family of Coronaviridae, consisting of 4 genera: Alpha, Beta, Gamma, and Delta. The emergent human CoVs, Middle East Respiratory Syndrome (MERS)-CoV, Severe Acute Respiratory Syndrome (SARS)-CoV-1, and SARS-CoV-2, are highly pathogenic members of the Beta genus. SARS-CoV-1 caused an epidemic affecting ~8100 cases and ~774 deaths worldwide in 2002-2004, and several MERS-CoV outbreaks in the Middle East and Asia resulted in ~2500 cases and ~886 deaths in 2012 [65]. In December of 2019, SARS-CoV-2 emerged as the cause of a highly transmissible and severe disease (COVID-19), causing over 141 million cases and 3 million deaths globally as of April 2021.

In contrast, the endemic CoVs that infect humans, the Alpha-CoVs HCoV-NL63 and HCoV-229E, and the Beta-CoVs HCoV-HKU1 and HCoV-OC43, all typically cause only mild illness in immunocompetent hosts, and account for 10-30% of upper respiratory tract infections in adults [44].

4.1.1 Age Distributions of Endemic Coronavirus Exposures

The age distributions of sero-reactivity to CoVs are relatively unstudied and unknown as endemic CoVs have not been traditionally included in routine diagnostic testing.

Therefore, the exposure characteristics of humans and CoVs is not well characterized. Long term longitudinal surveillance studies have presented as a challenge, as repeated viral detection measurements require frequent sampling to measure transient infections and may lack any data on immunity [268]. Previous studies have suggested initial exposure occurs as a young child [269], with repeated infections common among the population [61,270].

Alternatively, using serology to measure antibodies in the blood can help uncover age distributed exposure as the durability of antibody responses to CoVs lasts up to 12 months after infection [271]. Past serosurveys conducted that looked age-dependent patterns of both IgM and IgG, showed that IgM declined to undetectable levels after 14 years of age, suggesting that initial infections likely occurred during adolescence [272,273]

4.1.2 COVID-19 Immunopathology is Age-Dependent

Interestingly, the age gradient found with COVID-19 cases suggests that children could be at lower risk for complications, or susceptibility to infection. Why that occurs is not known but could be due to lower risk for inflammatory response, or reduced risk for infection due to heterologous immunity by recent endemic CoV infection [135]. COVID-19 also disproportionately affects the elderly [130], with morbidity and mortality directly proportional to age at infection [274]. While some of the risk for complications and death are related to co-morbidities [135] and possibly angiotensin-converting enzyme 2

(ACE2) expression [275], these do not explain all of the risk involved in developing severe COVID-19. Furthermore, between the sexes, there is a significant elevation in risk for complications and death in males over females [276,277]. Thus, understanding the underlying immunopathology and its relationship with past CoV exposures may reveal more information on the risk of developing severe symptoms.

4.1.3 Antigen Cross-Reactivity Between CoVs and the Role of Previous Infection with Endemic CoVs

Several studies have found cross-reactive responses to SARS-CoV-2 antigen in previously unexposed subjects, including T-cell, B-cell, and serological responses. Studies characterizing helper T-cell responses have found that cells isolated from pre-COVID-19 pandemic years still have some reactivity against SARS-CoV-2 [278,279]. In B-cells, others have found strong cross-reactive activation against SARS-CoV-2 antigens, while serological antibody cross-reactivity is weak in pre-COVID-19 donors [280]. Furthermore, neutralizing antibody responses against spike in serum have also been found to wane rapidly, while memory B-cells remained stable or increased over time [281]. Whether these cross-reactive memory responses contribute to the variability and severity of immunopathology seen in patients is yet to be determined.

Many questions remain about the role that past infections with endemic CoVs play on the immunopathology of COVID-19. There are no recent studies that have looked at the age when initial exposures to endemic CoVs occur, nor the relative stability of

antibodies within the population between respiratory virus seasons. These are crucial pieces of information that can help understand why children and the elderly show such varied responses to SARS-CoV-2 infection. Higher titres against cross-reactive endemic CoVs in children could protect against developing COVID-19, while conversely, higher titres in the elderly could be harmful if they cause antibody-dependent enhancement or prevent highly protective COVID-19 responses due to immune imprinting.

4.2 Rationale

To understand the seroprevalence of endemic CoVs and emergent CoVs SARS-CoV-1 and SARS-CoV-2 across ages, an age-stratified cross-sectional sero-survey was conducted on residual deidentified serum samples from spring 2013 and spring 2020 in British Columbia. Using a multiplex chemiluminescent immunoassay, snapshots of the reactivity of antibodies to the spike proteins of endemic CoVs, HCoV-OC43, HCoV-NL63, HCoV-HKU1, HCoV-229E, along with SARS-CoV-1 and SARS-CoV-2 were performed on winter respiratory virus seasons prior to, and at the start of, the COVID-19 pandemic.

Our hypotheses were as follows. (i) Sero-reactivity and seroprevalence to endemic CoVs in the population should increase from childhood into adulthood due to exposures in the community; (ii) Elderly individuals and males where COVID-19 mortality and morbidity are the highest should display differences serology compared to adults and children; (iii) Due to the strong immunological response to SARS-CoV-2 infection, those

previously infected with SARS-CoV-2 should show strong production of cross-reactive antibodies against endemic CoV antigens.

4.3 Material and Methods

4.3.1 Sampling

For each season, at least 500μL of anonymized residual sera were obtained from patients attending one of ~80 diagnostic service centres of the only outpatient laboratory network in the Lower Mainland, BC (LifeLabs) in 2013 and 2020. Consistent with the WHO Unity protocol [19], sera were obtained from between 20-100 patients within each of the following age groups (male and female): <5, 5-9, 10-19, 20-29, 30-39, 40-49, 50-59, 60-69, 70-79, ≥ 80 years. Accompanying detail included age in years and sex. Specimens were included only if at least 200μL of residual sera were to remain after testing. In addition, 40 samples that tested serologically reactive for SARS-CoV-2 on at least one of two commercial serology platforms were included in the study.

4.3.2 Mesoscale Diagnostics (MSD) Multiplexed Coronavirus Immunoassay (MIA)

MSD's MIA (V-PLEX Coronavirus Panel 2 and Coronavirus Panel 6 Kits) was performed per manufacturer's instructions as previously described [76]. Panel 2 measures detection of antibodies to trimeric SARS-CoV-2 spike, SARS-CoV-2 S1 RBD, SARS-CoV-2 S1 NTD, SARS-CoV-2 nucleocapsid, and spike proteins for HCoV-HKU1, HCoV-NL63, HCoV-OC43, and HCoV-229E. Panel 6 detects antibodies to nucleocapsid for

HCoV-HKU1, HCoV-NL63, HCoV-OC43, HCoV-229E, SARS-CoV-1, SARS-CoV-2, and MERS-CoV, in addition to SARS-COV-2 spike, and S1 RBD. Both coronavirus panel kits used were specific to detecting all IgG subtypes.

Assays plates were initially blocked with Blocker A (MSD); meanwhile sera were diluted 1:5000 in Diluent 100. Samples, including controls and standards, were then incubated in assay plates for 2 hours at room temperature shaking. After incubation, plates were washed 3x with wash buffer (MSD) before final incubation with detection antibody. After a final 3x wash with wash buffer, Read Buffer A (MSD) was added to the wells and immediately measured on the MSD QuickPlex SQ 120.

Raw data was processed using MSD's Discovery Workbench software and imported into R to interpret signal cut-off values. Spike values above 1500 (except for NL63, which required values above 750), Nucleocapsid values above 2000, and S1 RBD values above 500 were considered as positive based on previous validation data. To be considered serologically positive for SARS-CoV-2 using this assay, samples must be positive for SARS-CoV-2 spike, S1 RBD, and nucleocapsid. SARS-CoV-2 signal-cut-off values were previously validated on PCR and serologically confirmed SARS-CoV-2 positive and pre-COVID-19 reference serum samples (Data not shown). Endemic CoV signal cut-off values were determined by assaying a previously confirmed negative serum sample from a premature infant [282].

4.3.3 Statistical Analysis

Processed data and available clinical metadata were imported into R and cleaned using the R package *tidyverse* and visualized using *ggplot2* and *ggpubr.* To compare groups, a non-parametric Kruskal-Wallis test was used and when comparing multiple groups, p values were adjusted as per the Benjamini-Hochberg procedure. P values less than 0.05 were considered significant.

4.4 Results

Study participants. In total, there were 936 sera included in this study; 407 specimens were from the 2013 season, while 489 were from the 2020 season (Table 5). For 2020 samples, all were screened negative for antibodies to SARS-CoV-2 S1 and nucleocapsid previously. Of 40 samples in 2020 that tested positive for either SARS-CoV-2 S1 and nucleocapsid antigens, 20 tested positive for both; these 40 samples were excluded from the initial age-stratified analyses. In total, females represented 62.3% of samples from 2013 and 50.9% of samples from 2020, resulting in a representation of 56.1% in total.

Table 5 - Summary of Age and Sex Distributions of 2013, 2020 Seasons, and 2020 Serologically Screened Positive Subjects

Seasons:	**2013**		**2020**		**2020 CLIA Positive**
Age Group	**Median Age**	**n [M(F)]**	**Median Age**	**n [M(F)]**	**n [M(F)]**
<1			1	3(1)	
2-4	3.0	2(5)	3.0	12(22)	1(1)
5-9	6.5	6(4)	7.5	25(25)	
10-19	16.0	16(32)	17.0	25(25)	1
20-29	27.0	14(34)	25.5	25(25)	1(2)
30-39	34.0	4(45)	35.5	25(25)	4(3)
40-49	46.0	19(30)	45.0	25(26)	3
50-59	56.0	24(25)	54.0	25(25)	5(4)
60-69	65.5	21(28)	64.0	25(25)	1(2)
70-79	75.0	29(21)	73.0	25(25)	3(3)
80+	89.0	19(30)	84.0	25(25)	3(3)
Total		153(254)		240(249)	22(18)
% Female		62.3%		50.9%	45.0%

4.4.1 Initial infections with Endemic CoVs Occur During Adolescence.

Overall, infection with endemic CoVs increased during adolescence (Fig. 19a). For the alpha CoVs (HCoV-229E and HCoV-NL63), sero-reactivity rapidly increased during early childhood, reaching close 100% in the 5-9 year age bands, and remained sero-reactive through old age. For the beta-CoVs, sero-reactivity increased through the 5-9 age bands for HCoV-HKU1, while HCoV-OC43 increased earlier through the 2-4 age bands. For adults (20+ years) sampled in 2013 and 2020, sero-reactivity for endemic CoVs remained stable into old age (80+ years) (Fig. 19b). When sero-reactivity is plotted against age in young adults (<20 years), an overall trend increasing until age 14 is seen where antibody titres remain stable overall (Fig. 19c). Seroprevalence is also seen to stabilize into adulthood for endemic CoVs, with HCoV-NL63 and HCoV-OC43 reaching ~100% by age 10, while HCoV-HKU1 and HCoV-229E seroprevalence is ~100 by age 20 (Fig. 20).

4.4.2 Natural Cross-Reactivity to SARS-CoV-1 and SARS-CoV-2 Increases in Adolescence.

In SARS-CoV-2 naïve samples from the pre-COVID-19 2013 and 2020 seasons, sero-reactivity to SARS-CoV1 and SARS-CoV-2 antigens are seen increasing through adolescence into adulthood (Fig. 21a). In total, of samples screened serologically negative for previous infection by SARS-CoV-2, 86/896 (9.59%) were reactive for SARS-CoV-2 nucleocapsid, 450/896 (50.2%) were reactive to S1 RBD and 6/896 (0.67%) were reactive to spike protein above MSD signal cut-off thresholds. In addition,

sero-reactivity is seen increasing through adolescence and stabilizing through adulthood and old age (Fig. 21b).

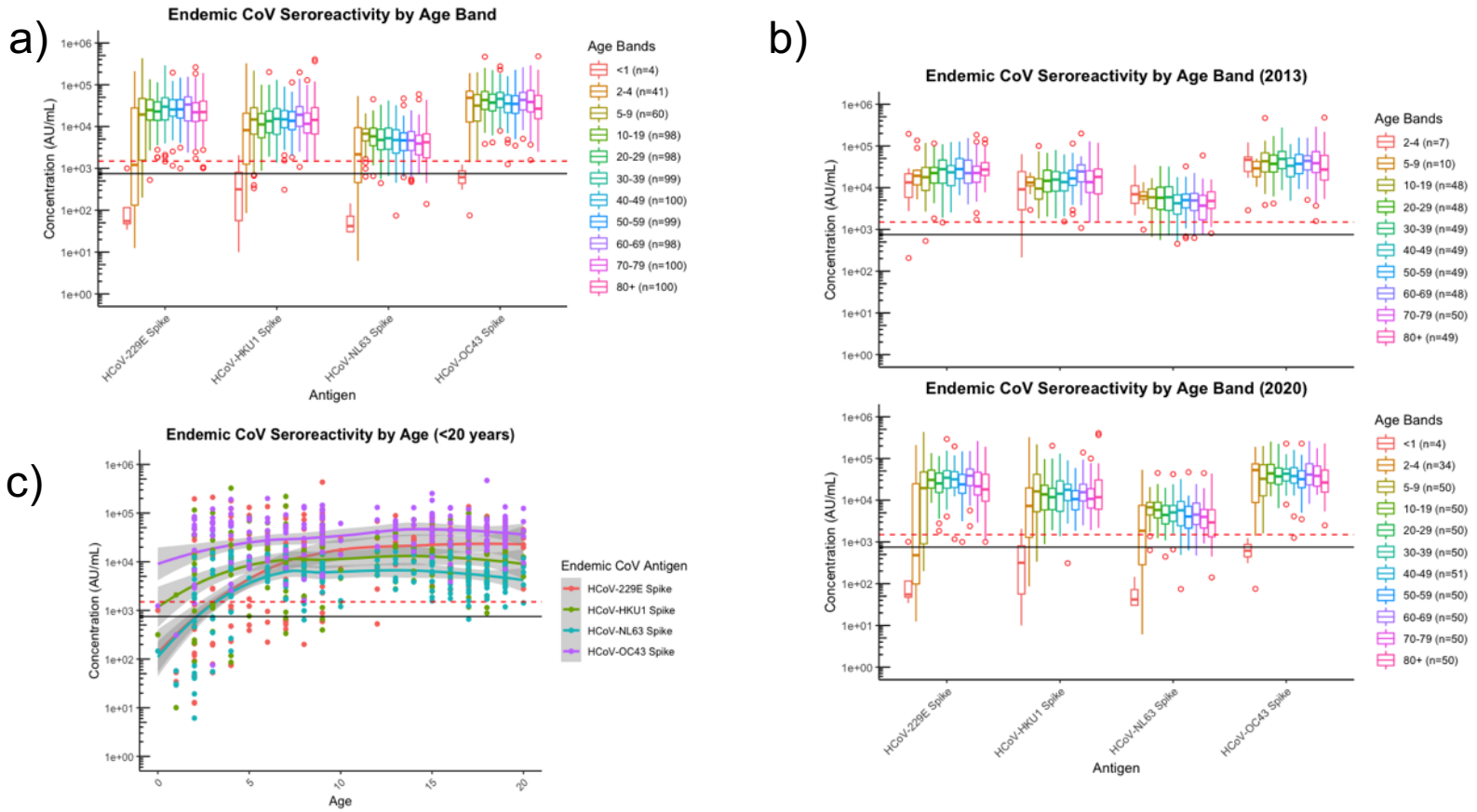


Figure 19 - Initial Infections by CoVs Occur During Youth; Sero-Reactivity to Spike Protein Increases with Age and Stabilizes into Adulthood and Old Age.

a) Age-stratified sero-reactivity from 2013 and 2020 seasons b) 2013 and 2020 seasons separated c) Mean 0 – 20 year sero-reactivity plotted by age reveal initial infections with CoVs occur during young childhood and into youth, with most persons by 20 years having been infected by all 4 endemic CoVs. Red dotted line (HCoV-229E, HCoV-HKU1, & HCoV-OC43) and black line (HCoV-NL63) represents positive signal-cutoff values for interpretation. Grey shading represents 95% confidence interval.

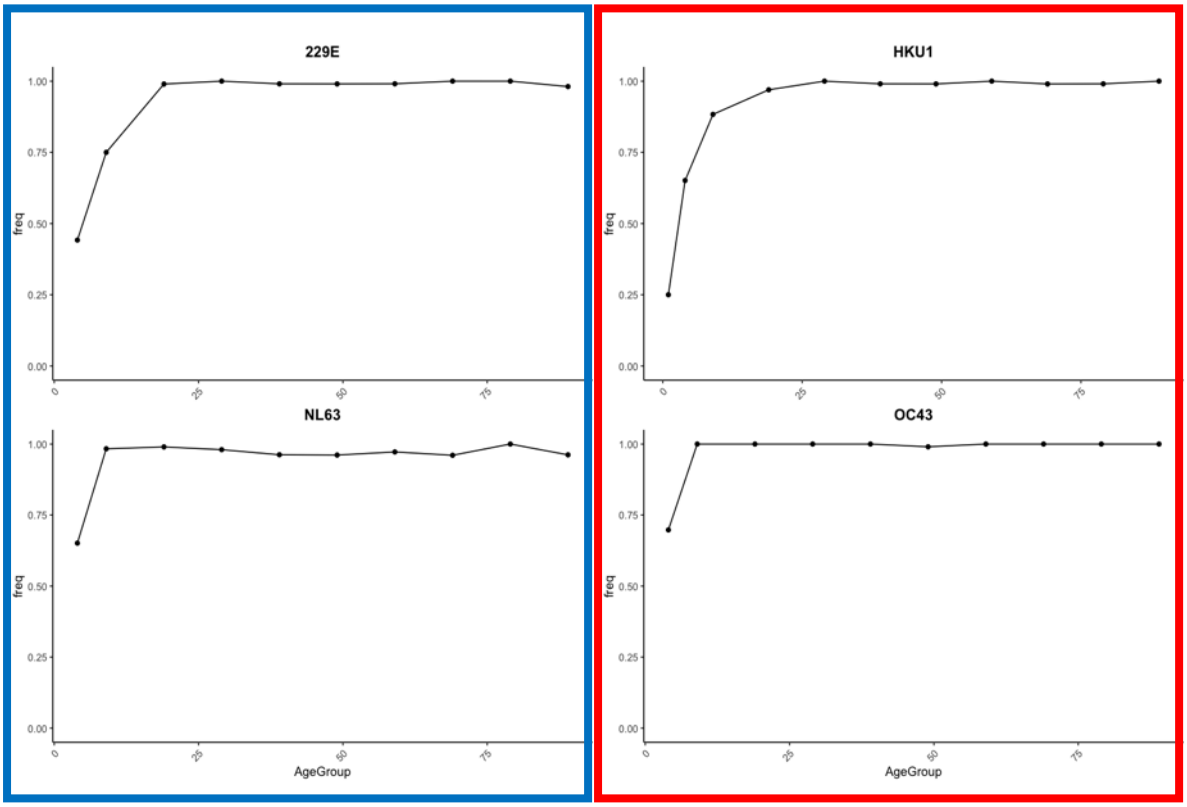


Figure 20 - Population Seroprevelance Against Endemic CoVs Increases Until 20 Years and Stabilizes into Adulthood and Old Age.

Blue box represents alpha-CoVs; Red box represents beta-CoVs.

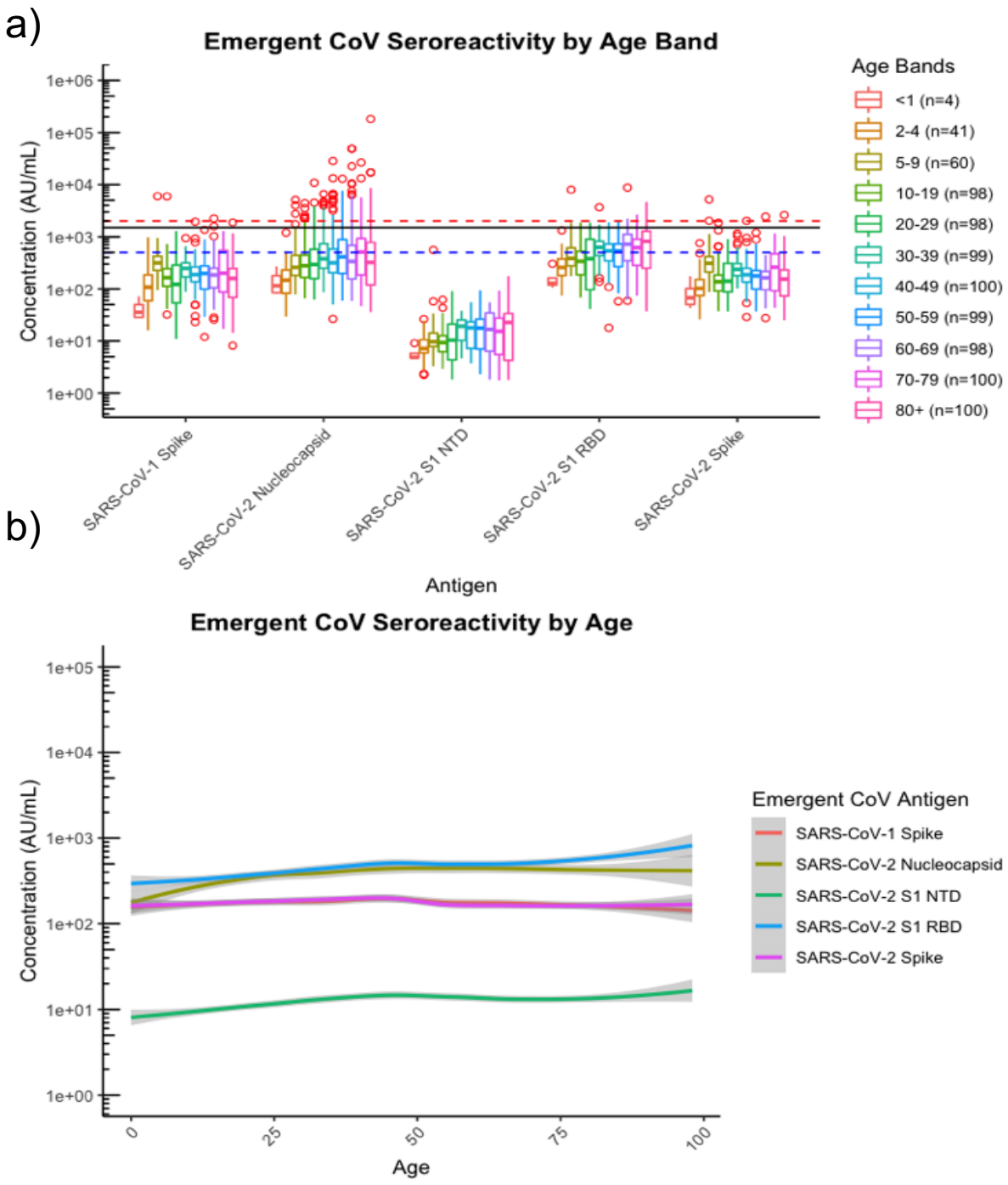


Figure 21 - Sero-reactivity Against Emergent CoVs SARS-CoV-1 and SARS-CoV-2 Increases with the Acquisition of Natural Infections with Endemic CoVs in Youth.

a) Sero-reactivity by age band b) mean sero-reactivity by age. Cross-reactive antibodies produced by natural infections with endemic CoVs lead to increased sero-reactivity against SARS-CoV-1 and SARS-Cov-2 antigens. Blue dotted line represents positive signal-cut off for S1 RBD; Red dotted line represents positive signal-cut off for nucleocapsid; Black line represents positive signal-cut off for spike protein. Grey shading represents 95% confidence interval.

4.4.3 Subtle Seasonal and Sex Differences Noted in Antibody Reactivity

Overall, there is a lack of seasonal difference between the sero-reactivity of adults (20-69 years) (Fig. 22a) and elderly (70+ years) (Fig. 22b) to endemic CoVs in 2013 and 2020. However, against SARS-CoV-1 and SARS-CoV-2 antigens, there is a statistically significant increase cross-reactivity to these antigens in 2013 over 2020. Most notably, 350/407 (85.9%) samples in 2013 were positive for S1 RBD. This difference holds true between adults and the elderly.

Among the males and females in adults (20-69 years), there are differences between the sero-reactivity to endemic CoVs HCoV-NL63 and HCoV-OC43 (Fig. 23a), however, this is not seen within the elderly (70+ years) age groups for any of the endemic CoVs (Fig. 23b). Similarly, this is also seen with SARS-CoV-1 and SARS-CoV-2 spike and S1 RBD proteins in adults (Fig. 23a), however, is also not found within the elderly group (Fig. 23b). Upon down sampling data to balance the sexes, only HCoV-NL63 spike protein ($p<0.05$), and SARS-CoV-2 spike ($p<0.05$), S1 RBD ($p<0.05$), and S1 NTD ($p<0.05$) remain significantly different (Fig. 24).

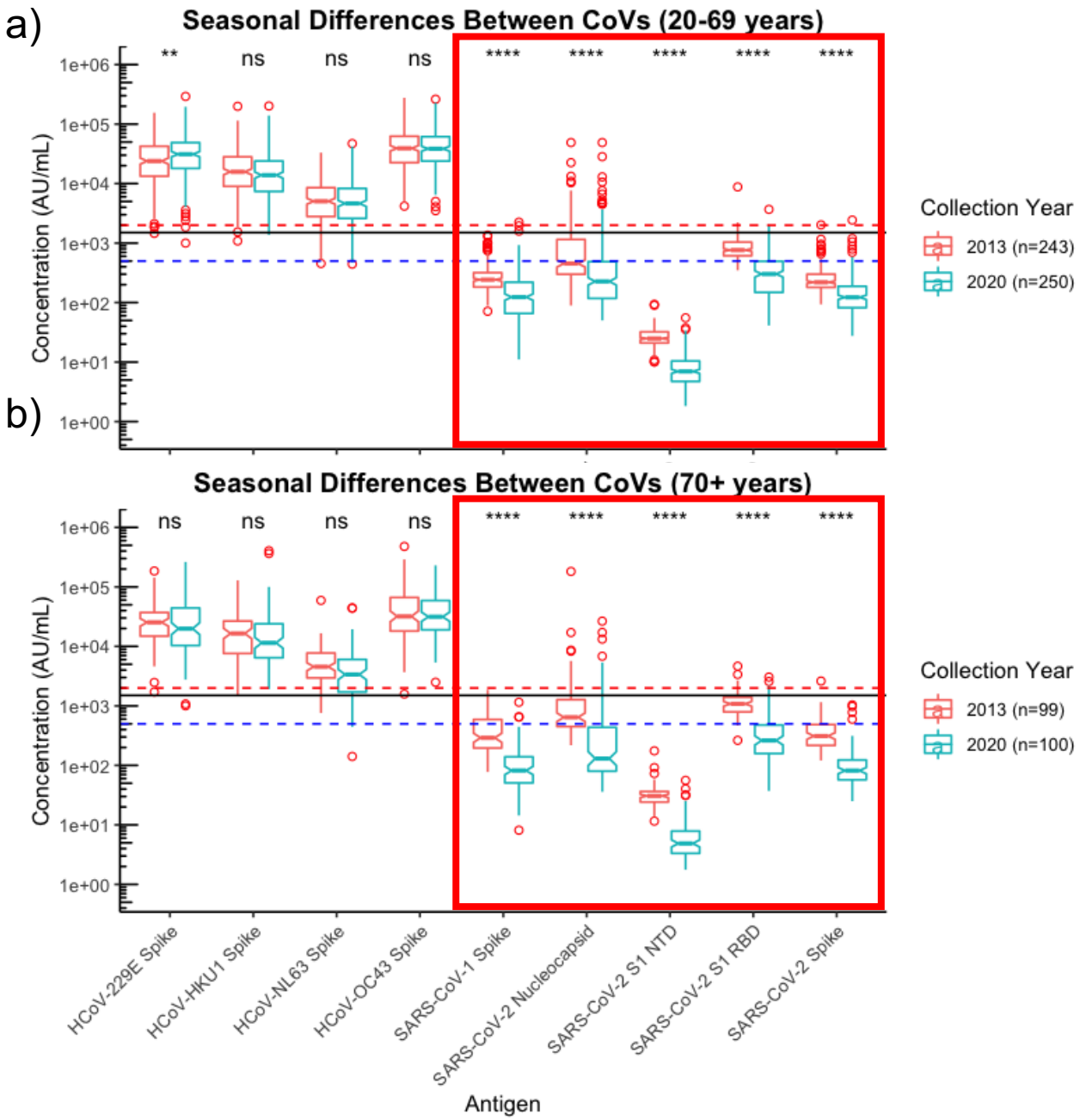


Figure 22 - Seasonal Differences in Cross-Reactivity are seen with SARS-CoV-1 and SARS-CoV-2 Antigens in all Ages, but only HCoV-229E in 20-69 years.

Both a) 20 - 69 years and b) 70 + years showed higher median levels of cross-reactive antibodies against SARS-CoV-1 and SARS-CoV-2 antigens in 2013 over 2020. In only HCoV-229E and 20-69 years, is there a difference between median sero-reactivity in 2013 and 2020. Blue dotted line represents positive signal-cut off for S1 RBD; Red dotted line represents positive signal-cut off for nucleocapsid; Black line represents positive signal-cut off for spike protein. Notches on boxplots represents 95% confidence interval. ** p<0.01; **** p<0.0001

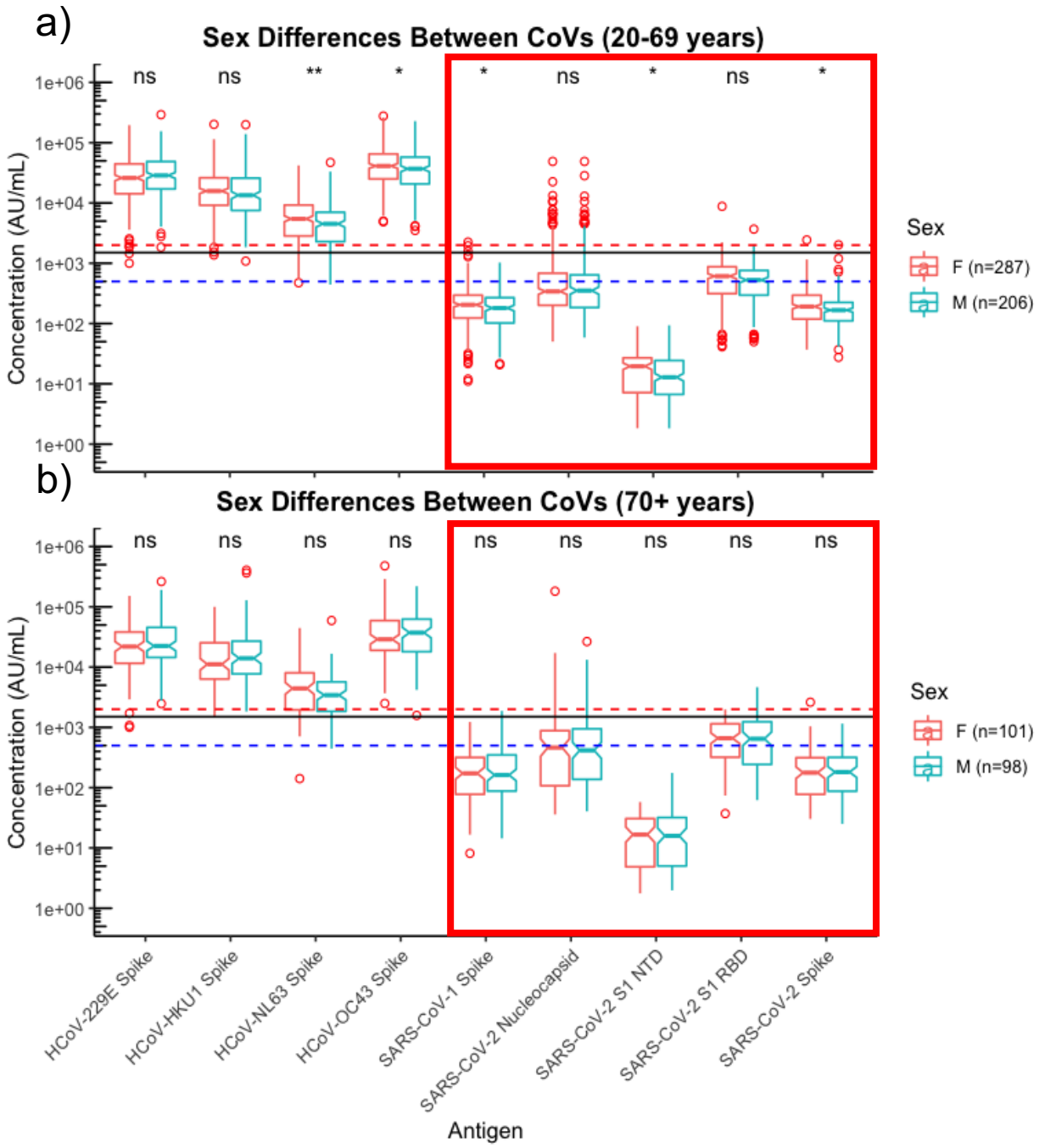


Figure 23 - Sex Differences in Cross-Reactivity are Seen with SARS-CoV-1 and Some SARS-CoV-2 Spike antigens, but Only HCoV-NL63 and HCoV-OC43 in 20-69 Years.

Only a) 20-69 years old females showed higher median levels of cross-reactive antibodies against SARS-CoV-1 spike and SARS-CoV-2 spike and S1 NTD antigens compared to b) 70+ years. Females between a) 20-69 years also displayed elevated median HCoV-NL63 and HCoV-OC43 values versus those who were b) 70+ years. Blue dotted line represents positive signal-cut off for S1 RBD; Red dotted line represents positive signal-cut off for nucleocapsid; Black line represents positive signal-cut off for spike protein. Notches on boxplots represents 95% confidence interval. *p<0.05; ** p<0.01

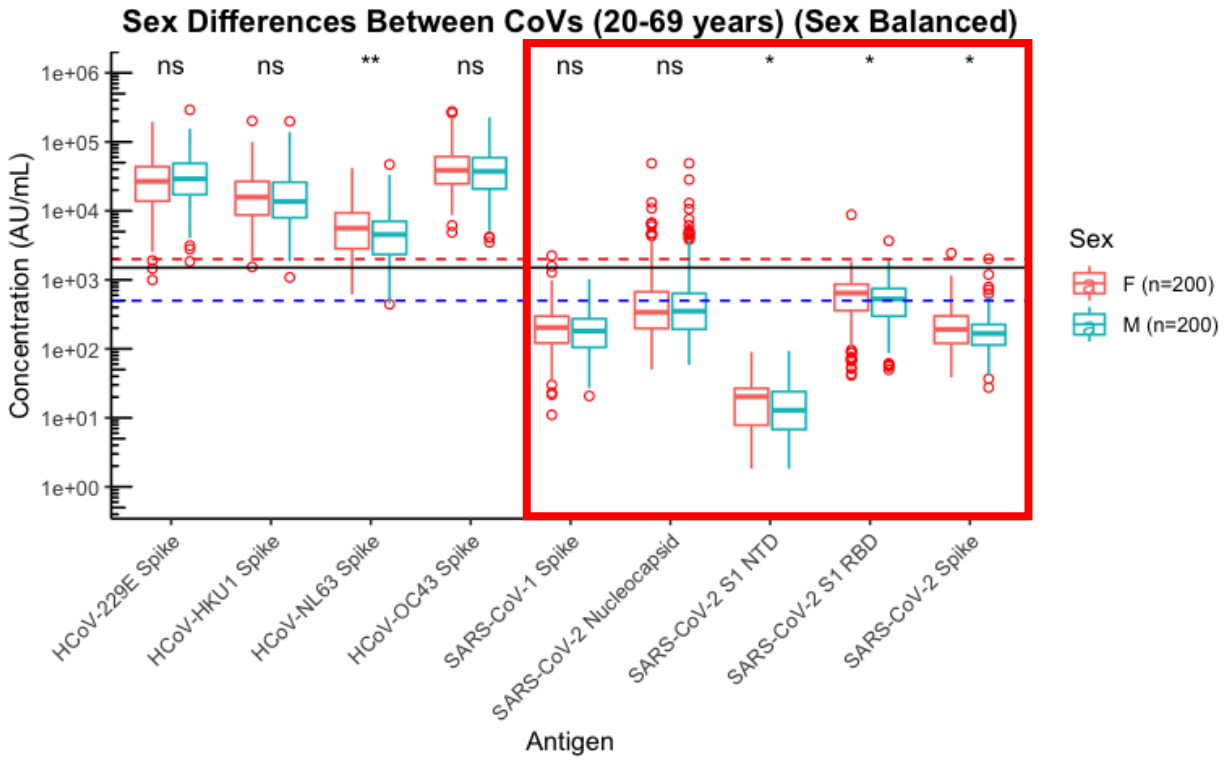


Figure 24 - Sex Differences in Cross-Reactivity are Still Seen SARS-CoV-2 spike, but only HCoV-NL63 in 20-69 Years After Balancing for Sex.

After down sampling data to balance sexes, 20-69 years old females still showed higher median levels of cross-reactive antibodies against SARS-CoV-2 spike and HCoV-NL63 spike. Unexpectedly, differences were lost for SARS-CoV-1 spike but found for SARS-CoV-2 S1 RBD. Blue dotted line represents positive signal-cut off for S1 RBD; Red dotted line represents positive signal-cut off for nucleocapsid; Black line represents positive signal-cut off for spike protein. *p<0.05; ** p<0.01

4.4.4 Increased Sero-Reactivity to Endemic CoV After SARS-CoV-2 Infection

Among the 896 samples that were previously screened negative or were pre-COVID-19 (2013) 892/896 (99.5%) were interpreted by MSD as being non-reactive against SARS-CoV-2 (Table 6); of the 20 that previously screened positive for both S1 RBD and nucleocapsid previously, 16/20 (80%) were interpreted as positive and 1/20 (5%) inconclusive by MSD. In addition, those interpreted positive via MSD produced values 2 log values higher than those considered negative by screening. Overall, there was 20/20 (100%) agreement with samples that previously screened inconclusive and were interpreted negative via MSD.

For the 20 samples previously screened positive for SARS-CoV-2, median values for SARS-CoV-1 and SARS-CoV-2 antigens were elevated compared to negative adult (20+ years) samples ($p<0.0001$) (Fig. 25a). Furthermore, increases in serum reactivity to HCoV-229E ($p<0.01$), HCoV-HKU1 ($p<0.05$), and HCoV-OC43 ($p<0.001$) spike protein are also seen. After excluding the 20 samples that screened inconclusive previously, samples interpreted as positive via MSD also showed elevated reactivity to SARS-CoV-1 and SARS-CoV-2 antigens ($p<0.0001$) (Fig. 25b). Likewise, those interpreted as positive via MSD also showed elevated reactivity to HCoV-229E ($p<0.05$), HCoV-HKU1 ($p<0.05$), and HCoV-OC43 ($p<0.0001$) spike proteins.

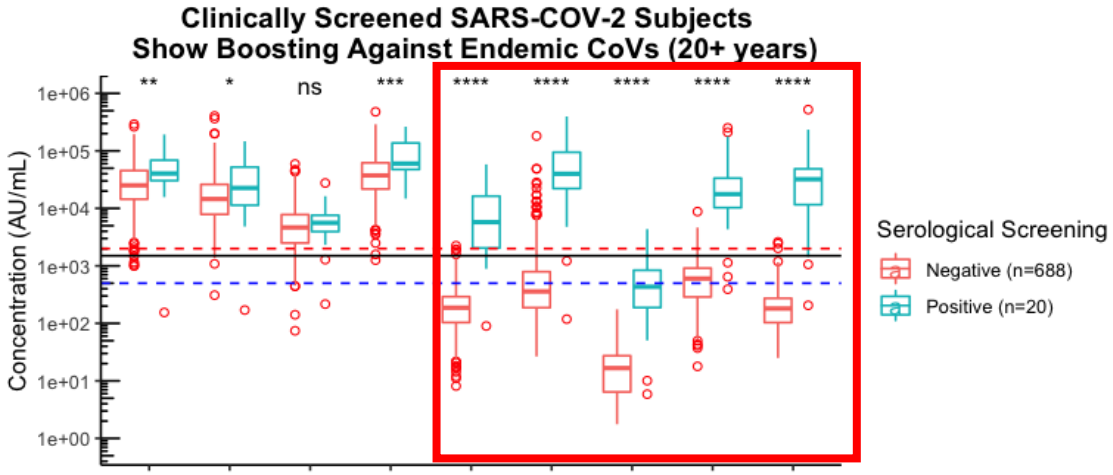
108 samples from the 2013 and 2020 seasons and the 20 confirmed SARS-CoV-2 controls were further tested on an additional panel containing nucleocapsid for all CoVs.

In those that were confirmed SARS-CoV-2 positive, median values were significantly lower for nucleocapsids of alpha-CoVs, HCoV-229E ($p < 0.05$), HCoV-NL63 ($p<0.01$), and MERS-CoV ($p<0.05$) (Fig. 26a). Notably, there was no difference between median values for endemic beta-CoVs, but nucleocapsid for SARS-CoV-1 was found elevated after SARS-CoV-2 infection ($p<0.0001$). Furthermore, between 2013 and 2020 seasons, there was a difference between the sero-reactivity for HCoV-OC43 nucleocapsid ($p<0.05$) and cross reactivity for SARS-CoV-1 ($p<0.05$) and SARS-CoV-2 ($p<0.05$) (Fig. 26b) in those greater than 20. However, no differences in nucleocapsid reactivity were seen between the different sexes (Fig. 26c).

Table 6 - Summary of Serological Status Via Commercial Platforms Compared to MSD Interpretations

				SARS CoV-2 Antigen					
				Spike		S1 RBD		Nucleocapsid	
Serological Status	**MSD Interpretation**	**n**	**Median Age**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Negative	Negative	892	45	1.76E+02	1.73E+02	5.02E+02	5.60E+02	3.29E+02	4.77E+02
Negative	Positive	3	49	2.61E+03	1.58E+03	1.40E+03	3.49E+03	3.51E+03	1.43E+03
Negative	Inconclusive	1	12	1.85E+03		7.27E+02		1.20E+02	
Positive	Negative	3	51	1.06E+03	6.15E+02	6.46E+02	3.72E+02	4.77E+03	3.46E+03
Positive	Positive	16	35.5	3.47E+04	3.39E+04	2.12E+04	4.05E+04	6.18E+04	7.48E+04
Positive	Inconclusive	1	28	5.65E+03		4.32E+03		1.22E+03	
Inconclusive	Negative	20	70	1.26E+02	9.40E+01	3.95E+02	3.67E+02	2.78E+02	1.48E+03

a)



b)

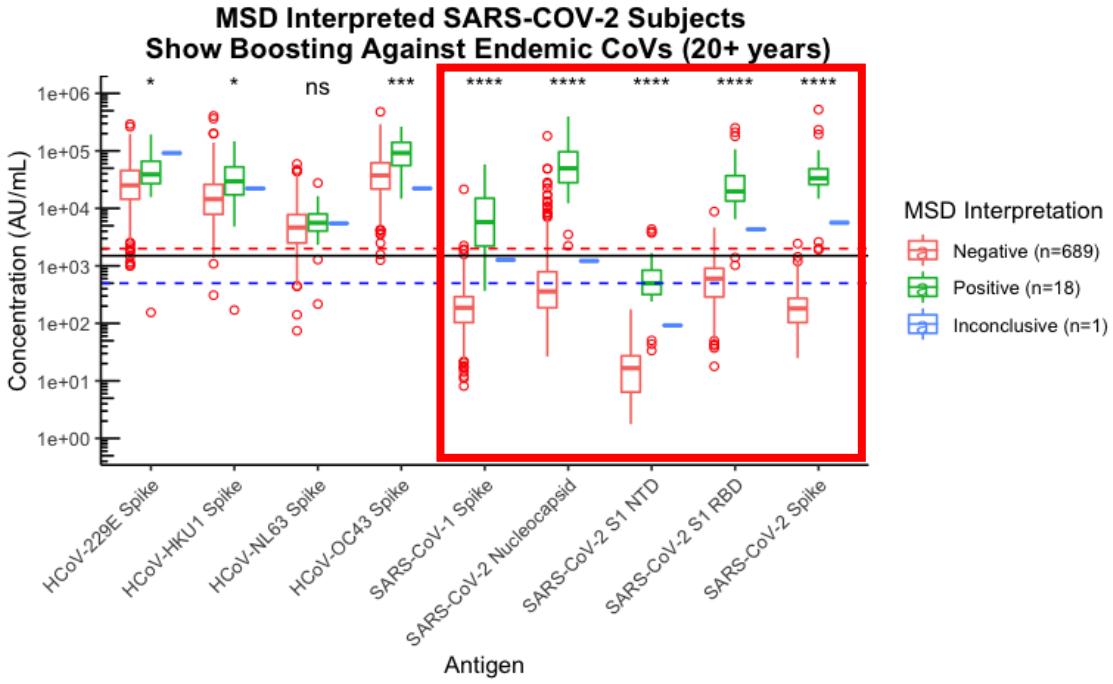


Figure 25 - Positive SARS-CoV-2 Screened Samples Show Evidence of Increased Beta-CoV Antibody Reactivity, while MSD MIA Platform Confirms Clinical Screening Platforms and Differentiates Confirmed SARS-CoV-2 Subjects from Negative Screened and Pre-COVID-19 (2013) Samples.

Samples confirmed as a) serologically positive or b) interpreted as positive via MSD for SARS-CoV-2 showed evidence of increased endemic CoV antibody responses compared to sero-negative adults 20+ years. Blue dotted line represents positive signal-cut off for S1 RBD; Red dotted line represents positive signal-cut off for nucleocapsid; Black line represents positive signal-cut off for spike protein. *p<0.05; ** p<0.01; ***p<0.001; ****p<0.0001

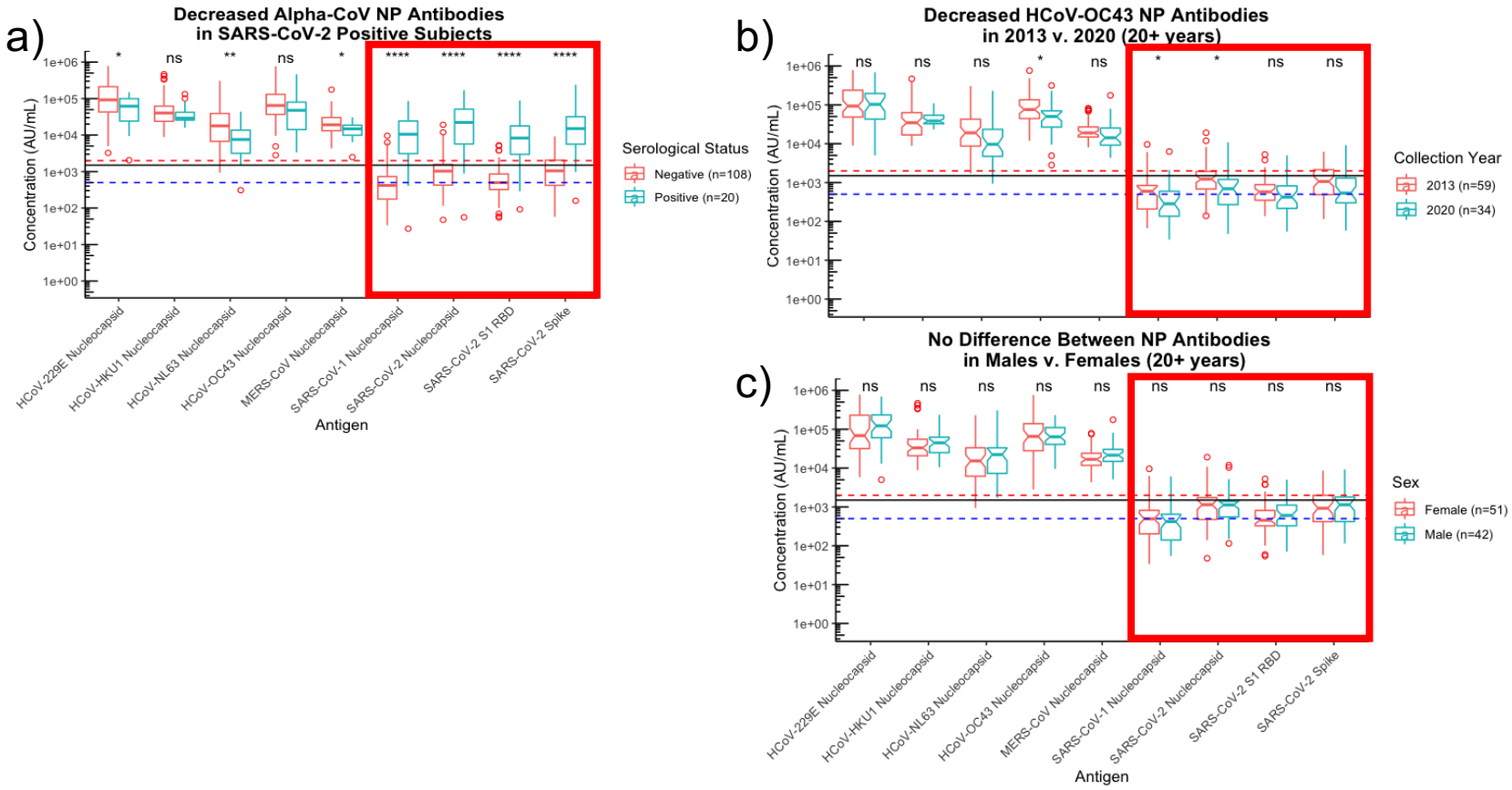


Figure 26 - Alpha-CoV Nucleocapsid Antibodies are Found to be Decreased After SARS-CoV-2 Infection.

A subset of serologically negative and all serologically positive subjects were run additionally on a pan-CoV nucleocapsid assay. Subjects serologically screened as a) SARS-CoV-2 positive were found to have decreased antibodies directed against alpha-CoV nucleocapsids. Between b) 2013 and 2020 seasons, there was also decreased cross-reactivity between HCoV-OC43, SARS-CoV-1, and SARS-CoV-2 nucleocapsids. However, no c) sex related differences were seen between sero-reactivity to any CoV nucleocapsid. Blue dotted line represents positive signal-cut off for S1 RBD; Red dotted line represents positive signal-cut off for nucleocapsid; Black line represents positive signal-cut off for spike protein. Notches on boxplots represents 95% confidence interval. *p<0.05; ** p<0.01; ***p<0.001; ****p<0.0001

4.5 Discussion

We report in this study, age-stratified seroprevalence to endemic CoVs, HCoV-229E, HCoV-NL63, HCoV-HKU1, and HCoV-OC43 in British Columbia, Canada. Our estimates of seroprevalence come from anonymized residual sera obtained from an outpatient laboratory in British Columbia. Retroactive sampling from pre-COVID-19 (2013) and in pre-screened samples after the first pandemic wave (2020) in British Columbia were used to mitigate the possible effects of SARS-CoV-2 infection on interpreting seroprevalence due to possible production of cross-reactive antibodies. Using a high-throughput MIA and age-stratified sampling, we are able to determine initial exposures to endemic CoVs occur during adolescence and seroprevalence remains stable into adulthood and old age.

To examine antibodies to endemic CoVs and emergent beta-CoVs, SARS-CoV-1 and SARS-CoV-2, we used a multiplex chemiluminescent immunoassay from MSD, applying validated cut-offs and an algorithm to determine positivity for each virus. Using SARS-CoV-2 antigens, spike, S1 RBD, and nucleocapsid, we were able to separate confirmed SARS-CoV-2 positive subjects from those presumed negative or previously screened negative. Overall, sero-reactivity and seroprevalence was found to increase over adolescence for all endemic CoVs, reaching 100% in young adulthood (20+ years), with sero-reactivity typically increasing until 14 years and stabilizing. Several studies have also found that initial exposure to endemic CoVs occurs quite early in youth and in adults sero-incidence remains steady into old age [273]. Other studies have also reported the same trend in endemic CoV seroprevalence, with many previous studies

using on ELISA, neutralization and HA inhibition, estimating 70-100% seroprevalence in the adult population [273].

Between sampling 2013 and 2020 years, we also found that the seroprevalence and sero-reactivity remains consistent in adulthood (20+ years), suggesting that absent an emergent strain of CoV, antibody tires and prevalence are relatively stable within the population. To determine the recency of exposures and/or reinfections, measuring both acute-IgM and longer-lived IgG could help understand the kinetics better, as previously done by Zhou et al. [272]. Likewise, with the emergent CoVs, we generally see an increase in sero-reactivity from childhood, but seroprevalence remains negative in pre-COVID-19 and negatively screened sera. This suggests that initial exposures to endemic CoVs likely produce some level of cross-reactive antibodies to SARS-CoV-1 and SARS-CoV-2 antigens due to the similarity of the spike proteins [280]. However, these antibodies are likely not to be protective as suggested by recent studies [280,281].

Many samples showed some level of cross reactivity to the SARS-CoV-2 nucleocapsid. To understand this, 108 samples were assayed alongside 20 SARS-CoV-2 confirmed samples on a nucleocapsid specific CoV panel. We noted that in SARS-CoV-2 seropositive samples that there were elevated antibodies against SARS-CoV-1 nucleocapsid, while unexpectedly found decreased antibodies against Alpha-CoVs. Moreover, in those over 20 years, there was also some seasonality observed between

2013 and 2020 samples, resulting in reduced sero-reactivity against HCoV-OC43, SARS-CoV-1, and SARS-CoV-2 nucleocapsids.

When seasonality was compared to sero-reactivity, there was no observed difference between antibody tires for endemic CoVs, however, for SARS-CoV-1 and SARS-CoV-2 antigens, there were significant differences reported between 2013 and 2020. This is particularly interesting as S1 RBD is used in our algorithm for first pass screening; a majority of 2013 samples would have met signal/cut-off thresholds if using a single antigen alone. This could possibly have been due to inter-assay variability. However, as median sero-reactivity remained similar endemic CoVs, this is unlikely. Another possibility is that during the 2013 and 2020 respiratory virus season, different CoVs could have been circulating, which could affect the production of cross-reactive antibodies. To confirm this, qPCR data and IgM would be important to delineate any predominant strain and exposures during those seasons. Nonetheless, understanding seasonal cross reactivity to SARS-CoV-2 antigens is important for many studies and these results support using multiple antigens to interpret seroprevalence to reduce potential false positives.

There were differences noted between the sexes in adults (20-69 years) for both endemic and emergent CoV sero-reactivity. Notably, in the adult group, there was an imbalance between the sexes (287 females v. 206 males), mostly driven by 2013 sampling, that could have been a result of the difference in n. However, when the data

was down sampled to balance the sexes (200 females v. 200 males), most of the differences still held, suggesting that there may be some albeit slight differences in antibody tires between the sexes. Notably, while antibodies against SARS-CoV-2 spike showed differences between sex, nucleocapsid antibodies exhibited no differences between the sexes in those over 20 years. Likely if there were any true sex differences, this would appear in the elderly as well, since mortality rates for COVID-19 are disproportionately higher for males than females [130]. Thus, the observed differences in mortality rates seen between females and males are likely due to other factors implicated in the immunopathology of COVID-19.

Boosting of antibodies against endemic CoVs has been reported in several studies after SARS-CoV-2 infection, though they do not appear to be cross-protective [283]. In our samples from confirmed SARS-CoV-2, we found evidence of increased reactivity to the Beta-CoVs, HCoV-HKU1 and HCoV-OC43, as well as SARS-CoV-1 and Alpha-CoV HCoV-229E. This is similar to previous studies that described strong cross-reactive antibody responses following infection with SARS-CoV-1 [284]. Moreover, antibodies to S1 and RBD domains are typically highly subtype-specific. Therefore, cross-reactive antibodies produced during any emergent CoV infection, are likely directed to the more highly conserved S2 domain region and nucleocapsid [285,286]. However, in our data we were only able to see increased reactivity of nucleocapsid antibodies towards SARS-CoV-1, but none towards the endemic Beta-CoVs. Moreover, when we examined antibodies directed to other CoVs, more specifically Alpha-CoVs, we paradoxically

found decreased antibodies against HCoV-229E, HCoV-NL63, and MERS-CoV nucleocapsid.

There are limitations to this study. Delays in generating memory antibody responses and timing of the blood draws could affect the detection of CoV specific antibodies [273,287], or the durability of the antibody responses in the serum could lead to under-estimation of seroprevalence [288]. Furthermore, in the absence of clinical data such as viral nucleic acid testing, measuring acute phase antibodies, such as IgM or IgA subtypes, could provide indication of recent exposure to the CoVs. Furthermore, these anonymized outpatient samples lacked any indication for clinical testing, thus possibly any underlying details for prior testing—samples could have come from unhealthy patients or those with underlying comorbidities. However, given the consistency of the samples sampled, this likely represents the over-estimation of seroprevalence within the population. In addition, our study lacks interrogation of the quality of the antibody response, while prevalence is estimated, the protectiveness of the antibody response is unknown. Gold standard neutralization assays could provide insights into the strength and quality of the antibodies within ages groups [289]. Other limitations include limited residual sera available in 2013 to confidently gather seroprevalence for adolescents, and the uneven representation of sexes, which could have led to the suggestion of sex-based differences in adults, but not the elderly.

In summary, using residual anonymized sera collected from an outpatient laboratory in British Columbia and a high throughput multiplex immunoassay, we are able to present an age-stratified sero-survey for CoV seroprevalence. Our data suggests that initial exposure to endemic CoVs occurs during childhood and into adolescence, with seroprevalence remaining stable into old age. The stability of endemic CoV-specific and SARS-CoV-2 cross-reactive antibody levels from childhood to old age does not provide a straightforward explanation of the strong age- or sex-dependent risk of severe or fatal COVID-19. We cannot, however, rule out the possibility that other characteristics of the humoral response to endemic CoVs (i.e., neutralizing or non-neutralizing functions) may differ by age and contribute to COVID-19 pathogenesis. This study also highlights the importance of cross-reactive antibodies to SARS-CoV-2 antigens in pre-COVID-19 sera that can affect clinical serologic tests for COVID-19, and the need for multiple SARS-CoV-2 antigens to reduce potential false positive results. Further investigation into the durability and quality of the antibody response will be helpful in understanding the role of cross-reactive antibodies for natural and vaccine-induced protection against SARS-CoV-2 infection and severe COVID-19.

Chapter 5 – Conclusions

The work in this thesis represents a collection of different projects completed during my PhD that focus on the immunology and epidemiology of viral infectious disease, specifically HHVs and CoVs. This includes research on exploring the immune ontogeny of antigen presenting cells using systems biology techniques; the role of HHVs on the development of AD; and antibody responses to CoVs at different ages. Although seemingly unrelated, these projects are all crucial to understanding the role that viruses play in human health and disease.

Using scRNAseq to interrogate innate antigen presenting cells in both adult peripheral and umbilical cord blood, we revealed that monocytes and DCs in adult and cord blood are transcriptionally very similar at rest. However, there was also some evidence of biased immune signalling pathways in cord blood cells due to the expression of inhibitory genes that affect downstream NF-kB and STAT3 signalling, namely expression of SOCS3 and NFKBIA. In addition, bulk RNAseq revealed that under stimulation by 3’3’ cGAMP, monocytes and pDCs have similar type I IFN responses in both adult and cord blood. Although this suggests that at rest and under strong exogenous stimulation, adult and cord blood cells behave similarly, it does not explain the difference in susceptibility of neonates to infection by intracellular pathogens. Understanding this will likely require investigations that use neonate relevant pathogens like HSV and CMV to uncover differences in immune response in relevant cells and the contributions of SOCS3 and NFKBIA expression.

To understand the role of HHVs on the development of AD, qPCR targeting neurotropic HHVs in diseased and healthy brains was performed. In general, there was detection of low amounts of HHV DNA in some brains, but neither increased detection frequency nor elevated amounts of HHV DNA were observed in those with AD. While previous studies using whole genome sequencing and ddPCR were able to detect HHV DNA at higher frequency, these results confirm other recent studies that have found a lack of difference between the detection and frequency of HHVs in AD brains over healthy controls. Future studies that examine serological responses longitudinally or vaccinations against HHVs could provide valuable insight into the possible mechanisms of developing AD or other HHV-associated progressive disease.

Finally, antibody responses to CoVs over different ages represent a knowledge gap in public health. The results of our study examining the age structure of CoV infections in the population revealed that infections are more common than previously thought. This seroprevalence study also revealed that the first infection by CoVs typically occurs during adolescence and almost 100% of the adult population 20+ years has been infected by all 4 endemic CoVs. Furthermore, recent infection by SARS-CoV-2 resulted in the strong production of cross-reactive spike antibodies against some endemic CoVs and closely related SARS-CoV-1. While seroprevalence studies like this can inform on the exposure history and timing of infections in the population, they lack information on the durability and quality of the antibody response. Examining the temporal dynamics of

CoV antibodies in addition to their acquisition will help better inform vaccination strategies and public health policies.

5.1 The Importance of Understanding Age-Related Immune Responses to Infection

Many viral infections, such as HSV and CoVs studied in this thesis, often show age-related pathology and immune responses. For example, untreated HSV infection typically causes severe disease or death within the first month of life, but thereafter results in mild, self-limited lesions like those seen in immunocompetent adults. These differences, though complex to study, present an opportunity to better understand immune ontogeny as well as the basis of pathogen-specific immune control, with far-reaching implications for novel treatments and vaccination strategies in early life. Understanding first the physiological and immunological response, including the cell populations involved, helps focus and narrow the research questions that are asked. Only then, can more exploratory questions be posed, and relevant systems biology techniques used to uncover new mechanisms.

Young children and elderly typically form much of the morbidity and mortality due to viral infection, thus studying age-related immune responses are incredibly important and valuable. Despite their similar susceptibility, the underlying reason for their more severe symptoms is likely due to very different mechanisms. Understanding the physiological and cellular determinants that contribute to their lack of control infection or enhanced

tissue damage can help inform strategies and interventions to mitigate severe effects and improve outcomes. For viruses that disproportionately affect newborns, such as HSV, studying immune ontogeny and age-dependent immune responses helps understand their disproportionate risks for morbidity and mortality. Likewise, examining the underlying mechanisms of elevated morbidity and mortality in the elderly by SARS-CoV-2 infection can provide crucial information on not only immunopathology of COVID-19, but the effects of aging, immunosenescence, and immune imprinting. Together, understanding not only how the immune system responds to infection, but also how that response changes with age helps illustrate a more complete idea of how and why certain populations are more susceptible to certain viral diseases and compromised outcomes.

5.2 Complexity of Investigating the Immune System and Response to Viral Infections at Different Ages

Understanding the development of the human immune system is a challenge due to the heterogeneous nature of immune cell populations and vast number of different stimuli that they encounter. This is further complicated by the logistic constraints of sample availability and high cost of assays, which limit the ability to comprehensively compare large numbers of individuals and types of responses. To determine age-dependent differences in specific anti-viral responses, a major challenge I faced was to determine the most relevant conditions to study. In our simplest experiment, we used cutting-edge scRNAseq to interrogate the differences between antigen presenting cell populations in

both neonates and adults, uncovering new populations of cells with functions that are poorly characterized or not yet studied. However, exploring whether the cell populations we uncovered are distinct populations of cells or reflective of a cell state are part of the complexities of studying the immune system.

In addition, using direct stimulating ligands, like 3'3' cGAMP, *in vitro* also present their own set of challenges, as this may not accurately represent how cells sense a given pathogen nor the physiological milieu in which those cells exist. *In vitro* time course experiments involving relevant neonatal pathogens and immune cells could provide some insight into the cellular processes of pathogenesis during infection. The use of 3D organoid infection models could also provide some insight into the dynamics of infection and immune response as well. However, the most relevant information may come from ideally using clinical samples from tissue biopsies or blood draws during acute infection. These would provide important systemic information, along with more relevant tissue level local immune response and could leverage the granularity of scRNAseq data.

Furthermore, one cannot ignore that although bulk RNAseq and scRNAseq can provide immense amounts of data, they are prone to substantial variability. This can greatly affect the way that data is analyzed and interpreted from one study to another or even within samples in the same study. Despite the plentiful data that originates from a single sample, having very focused comparisons and large samples sizes are still needed to draw proper conclusions. Ideally, in studies involving using systems biology techniques,

the metadata that accompanies a clinical sample should be just as rich as the transcriptome data. Careful focus on the comparisons being made and samples being assayed, can result in data that can be better used for follow-up studies characterizing relevant genes, proteins, pathways, etc. that may be key in understanding differences in potential disease outcomes.

5.3 The Difficulties of Attributing Causality of Diseases to HHVs, and How This Might Be Accomplished

Attributing diseases to chronic HHV infections are challenging for several reasons, including the ubiquity of these infections, the early age at which initial infection occurs, and the ability for the virus to stay latent until reactivated. Most HHV infections typically occur during childhood, therefore much of the population has been infected by the time they have reached adulthood. Because of this, correlating HHV infection with progressive diseases, such as AD, multiple sclerosis, and SLE, which tend to occur in later life is difficult as the initial viral triggering event may have occurred much earlier. Furthermore, the complex biology of HHVs and their persistence in the human body also makes being infected or uninfected not strong evidence alone to implicate causality to HHVs causing disease. HHVs also initiate strong immune response during primary infection and remain hidden in latent reservoirs until reactivated, possibly multiple times in one's life, making measuring viral load over decades and capturing the exact occurrences of these reactivations a challenge.

Previous studies investigating the association between HHVs and progressive disease have relied traditionally on using indirect serological data to draw these correlations; they lack the direct temporal connection between HHVs and pathology. Even of studies that have directly interrogated for HHV DNA, they have relied on interrogating post-mortem tissue, as ours did, which can only account for active replication at the time of death. Although these methods can capture the pathology, they provide no longitudinal data on whether or not HHVs are directly implicated in the initial development of disease, immunopathological or not. Direct association of disease by HHVs has only been found in several cases, mostly in malignancies caused by oncogenic HHVs. EBV DNA and gene expression has been directly found in the cancerous tissues of gastric carcinomas, Burkitt's lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma; KSHV has been found in Kaposi sarcoma. Even then, infection or presence of virus alone is not enough to implicate HHV causality directly, as interactions between other genetic, physiological, and/or environmental factors may be more important in the initiation of disease than the presence of virus alone.

Longitudinal studies that investigate the age of seroconversion, or that involve vaccination against HHVs could potentially help provide key information on the contribution of HHVs and the development of disease. Having temporal data of age-dependent infection by HHVs would likely strengthen their associations with developing progressive diseases like AD in later life. Likewise, vaccinations that produce partial or sterilizing immunity, could also be very valuable to better attribute causality of disease to HHVs. If sterilizing immunity against HHVs is possible through vaccination, there

should be some observable differences between groups of those that develop disease. Even in the case of partial immunity through vaccination, preventing chronic HHV reactivations or severe symptomatic infections, age-dependent or not, could still be enough to observe differences in developing HHV-associated diseases.

5.4 SARS-CoV-2 and COVID-19 Demonstrate the Need to Understand the Mechanisms of Immune-Mediated Protection and Pathology of Emerging Pathogens

The COVID-19 pandemic is a prime example of how even within a group of viruses that typically cause mild illness, an emergent strain with higher morbidity and mortality can potentially cause a global healthcare crisis. Although CoVs are not new pathogens in humans, there has been a relative paucity of clinical research on their prevalence in different populations. During the initial stages of the pandemic, there was rather little information on the potential role of infections with endemic CoVs or cross-reactive immune responses that could explain the age-dependent severity of COVID-19. Investigating these cross-reactive antibody responses was undertaken to assess their potential association with risk of severe COVID-19 or vaccine induced disease, given the ubiquity of infection with endemic CoVs. Our study revealed that endemic CoVs infections are extremely common in British Columbia, and that initial infection occurs during childhood. This suggests that many or most of these infections go undetected due to their mild and non-specific symptoms in children. Furthermore, the relatedness of endemic CoVs to their emergent counterparts revealed that past antibody responses

towards endemic CoVs are cross-reactive with emergent CoVs. Despite the evidence of cross-reactive antibodies being present in those previously infected by endemic CoVs, there was a lack of evidence that endemic CoVs produced strong cross-reactive antibody responses that could contribute to protection or disease. However, antibodies are not the only components of the adaptive immune system that can show potential cross-reactivity; T-cell responses, such as those from T-helper or CTLs, likely would provide different information on the associations between past endemic CoV infections and developing severe COVID-19 immunopathology.

Investigating the underlying mechanisms of immune-mediated protection and pathology is important for SARS-CoV-2, as well as other potentially emergent CoVs that have the potential of becoming endemic and part of the respiratory virus season. Due to the repeated exposures that likely occur when a virus becomes endemic, immune imprinting can produce potentially beneficial or harmful effects upon heterologous exposure. While there has been no evidence of this occurring during vaccination, the interactions between immune responses endemic CoVs and emergent CoVs is not well understood. Influenza is considered the prototypical model for imprinting, as previous studies have found that one's initial exposure to a certain strain of virus influences not only vaccine responses, but morbidity and mortality during pandemic flu seasons. If there is any element of imprinting between Alpha- or Beta-CoVs, like influenza, there could be the potential for designing universal vaccines in childhood that can mitigate the potential morbidity in later ages.

What is apparent, however, is the importance of understanding the seroprevalence of viral infections, especially respiratory viruses within the population. Endemic CoVs have been present in the human repertoire for over 40 years, but due to their mild symptoms, they have been under studied and reported. SARS-CoV-1, SARS-CoV-2, and MERS-CoV have shown that even innocuous pathogens that typically result in mild illness, have the potential of becoming serious public health threats that result in disproportionate negative outcomes in the elderly. In the current situation, one must also consider the possibility that a child's first exposure to a CoV might be SARS-CoV-2 rather than an endemic CoV and potentially what consequences could result with future exposures to other CoVs. Thus, understanding the immune related protection and pathology of emergent CoVs is important and begins with investigating the age-related responses to the pathogen compared to their endemic counterparts.

5.5 Valuable Insights as a Future Science Educator

Although the chapters in this thesis are loosely thematic, they represent the broad research in infectious disease and immunology utilizing the scientific method. These experiences are incredibly valuable for a future educator, as I have been given the opportunity to approach medical research from both a systems biology approach and a more traditional reductionist approach. Having done this, I have a deeper understanding and appreciation for hypothesis generating and hypothesis confirming studies. Using unbiased and unsupervised "big data" approaches to understand neonatal immunology, countless more questions have been unearthed, while simple qPCR and serological assays have allowed me to answer more discrete questions focused on HHVs and

CoVs. These contrasting approaches to research used during my PhD have allowed me to explore complex questions in different perspectives.

Furthermore, the techniques and applications of research that I have been exposed to have proved to be incredibly valuable. From using some of the most basic and traditional techniques in health research, PCR and ELISA, to cutting edge single-cell transcriptome sequencing, I have broadly explored the approaches needed to answer important questions. Having a background in so many of techniques used in research gives me an opportunity to explain and explore biology in different ways in the classroom, whether in labs or other activities. All these experiences have ultimately given me plenty of ideas of how to explore complex topics with students and to encourage their exploration.

Importantly, these insights and technical skills will allow me to educate students and future scientists creatively. Having students understand that the different approaches in sciences are complementary, rather than competing, will encourage them to have a more flexible understanding of relationships with biology to other disciplines. This includes focusing on different subjects they are potentially passionate about and how they can use these interests to explore topics and problems they are genuinely interested in solving and understanding. More thrilling is the concept of developing interactive activities to connect science's application in society: biology and math in public health, or computer science and biology in bioinformatics. Encouraging students

to have a more flexible view of biology could instead inspire students to combine interests and passions towards finding a uniquely fruitful career.

Perhaps more significantly, all the insights and questions these experiences in research have raised are not exhaustive—highlighting the continuous evolution of science and encouraging a future educator's continual development.

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