$CD4^+$ T CELLS IN INFANT IMMUNITY

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ABSTRACT

Myra Grace A. dela Peña: $CD4^+$ T cells in Infant Immunity
(Under the direction of Kristina Abel, PhD)

Age and immune maturation are interdependent and dynamic processes. The infant immune system undergoes many developmental changes after birth, and compared to adults, cellular and molecular differences have been observed in infant immune responses. This dissertation is focused on the functional features of $CD4^+$ T helper cells, as these cells are critical players in adaptive immunity. In general, infants show a bias toward $CD4^+$ T helper 2 (Th2) responses, whereas adults generate Th1 responses against infectious diseases caused by intracellular pathogens. The infant's Th2 bias is reflected in increased susceptibility to infectious pathogens, and higher morbidity and mortality rates. We hypothesized that reduced functionality of infant $CD4^+$ T cells would be due to intrinsic differences in the $CD4^+$ T cell signaling that alters responsiveness of these cells, and thereby influences maturation, function and the generation of protective immunity. Specifically, we sought to i) characterize the immune development of $CD4^+$ T cells in infants, (ii) determine infant $CD4^+$ T cell response to exogenous cytokine stimulations, and (iii) assess the effectiveness of infant immunity in the control of a viral infection.

First, we used rhesus macaques raised as specific pathogen free (SPF) and conventional status (non-SPF) to characterize basic immunological changes in peripheral blood cell populations with age and how they are affected by chronic cytomegalovirus infection (RhCMV). Our findings indicated that some age-related changes in major blood cell populations from birth to adulthood were common to both SPF and non-SPF macaques. We also demonstrated that chronic RhCMV infection modulated immune development over the lifetime of the host, evident in a more inflammatory response of juvenile RhCMV-infected macaques compared to SPF-juveniles. Second, we investigated the functional capacity of the infant immune system to control viral replication. We found that infant rhesus macaques were rapidly infected with RhCMV via the oral route and shed virus more frequently in bodily fluids and in higher titers when compared to adult macaques. Lastly, we used cross sectional human blood samples to test the hypothesis that the propensity towards Th2 response in infants is due to altered cytokine-receptor mediated signaling in $CD4^+$ T cells. Our data showed that $CD4^+$ T cells from cord blood (CB) and older infants differed in Th1 and Th2-associated cytokine receptor expression from adults. We also found that cytokine-signaling factors (JAKs and STATs) were differentially phosphorylated after cytokine treatment. Lastly, *in vitro* stimulation through the TCR and exogenous addition of IFNγ revealed differences in JAK/STAT related gene expression and cytokine productions between CB and adult $CD4^+$ T cells. Together, our findings imply that cytokine signaling is developmentally controlled because responsiveness to cytokines increased with age in infant $CD4^+$ T cells.

The assessment of important functional parameters in $CD4^+$ T cell development, such as cytokine and TCR signaling, from birth to adulthood can reveal mechanistic insights into

how and why cellular immunity changes with age. How lifelong infections and other perturbation affect these immunological processes will likely impact both disease outcome and the generation of immunity. Unraveling the molecular mechanisms involved in these processes may aid in pediatric vaccine designs and therapeutic interventions.

To my loving family:

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PREFACE

The unpublished material covered in Chapter 2 is a product of collaboration where M.G. dela Peña and K. Oxford are first co-authors and Kara Jensen, Koen Van Rompay, Joseph Rigdon, Katie Mollan, Michael Hudgens, Jennifer Rice and Peter Barry are contributing authors. Kristina Abel is the lead principal investigator.

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LIST OF ABBREVIATIONS

Ab	Antibody
AP1	Activator protein 1 family transcription factor
Ag(s)	Antigen(s)
APC	Antigen presenting cells
BATF	B-cell activating transcription factor-like
BCG	Mycobacterium bovis Bacillus Calmette-Guerin
BM	Breast-milk
CCR	C-C chemokine receptor
CB	Cord blood
CD	Cluster of Differentiation
CCL	Chemokine (CC-motif) ligand
CCL20	Chemokine (CC-motif) ligand-20
CCR	Chemokine (CC-motif) receptor
CNPRC	California National Primate Research Center
CRP	C-reactive protein
CXCR	CXC chemokine receptor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FSC	Forward scatter

FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
GC(s)	Germinal center(s)
H	Hours
HCMV	Human cytomegalovirus
HIV/AIDS	Human Immunodeficiency Virus / Acquired Immunodeficiency Syndrome
HBV	Hepatitis B virus
IFN	Interferon
IFNγ	Interferon-gamma
IFNγR	Interferon-gamma-receptor (1 or 2)
Ig	Immunoglobulin
IL	Interleukin
IL2Rα	Interleukin 2 –receptor alpha
IL2Rβ	Interleukin 2 –receptor beta
IL4Rα	Interleukin 4 –receptor alpha
IL13Rα1	Interleukin 13-receptor alpha1
iTreg	Inducible regulatory T cell
Iono	Ionomycin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK	Janus kinase (1 or 2)
LSM	Lymphocyte separation medium
MΦ	Macrophage

MFI	Mean fluorescence intensity
mg	Milligram
min	Minutes
ml	Milliliter
Mo.	Months
MS	Multiple sclerosis
MVA	Modified Vaccinia Ankara
µg	Microgram
µl	Microliter
mDCs	Myeloid dendritic cells
nTregs	Natural regulatory T cells
NF-κB	Nuclear factor kappa B
NFAT	Nuclear factor of activated T cells
NFATc2	Nuclear factor of activated T cells c2
ng	Nanogram
non-SPF	Non specific pathogen free
NS	Not significant
NHPs	Non-human primates
NT	Not tested
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

pg	Picogram
PMA	Phorbol 12-myristate 12-acetate
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
RM	Rhesus macaques
RhCMV	Rhesus cytomegalovirus
RORγt	Retinoid acid-related orphan receptor (ROR) γt
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SEM	Standard error of the mean
SIV	Simian Immune Deficiency Virus
SLE	Systemic Lupus Erythematosus
SPF	Specific pathogen free
SOCS	Suppressor of cytokine signaling
SSC	Side scatter
STAT	Signal transducer and activator of transcription
T-bet/Tbx21	T box transcription factor/protein 21
T(CM)	T central memory T cell
T(E/EM)	T effector/effector memory T cell
Tfh	T follicular helper cell
Th	T helper cell
T(N)	Naïve T cell
TNFα	Tumor necrosis factor alpha

TNFβ	Tumor necrosis factor beta
TLRs	Toll-like receptors
Treg	T regulatory cell
US	United States

CHAPTER 1: GENERATION OF INFANT $CD4^+$ T CELL IMMUNITY

Introduction

The immune system is designed to protect the host from infectious diseases. In an infant environment, the immune system is faced with unique challenges because once born, the infant encounters a world full of novel microorganisms, pathogens and environmental substances. When confronted with these novel antigens of self or foreign origin, the infant must establish tolerance to self-antigens (Ags), but also be capable of mobilizing the immune system quickly to mount a protective response against infectious agents. Compared to adults, cellular and molecular differences have been observed in infant immune responses. These altered, generally lower, responses are reflected in the infant's increased susceptibility to infectious pathogens, and in higher morbidity and mortality rates.

The focus of this work is to discuss the features of $CD4^+$ T cell responses in infants and how they influence the generation of protective immunity. Specifically, the goals of my research were to (i) characterize the immune development of $CD4^+$ T cells in infants, (ii) determine infant $CD4^+$ T cell response to exogenous cytokine stimulations, and (iii) assess the effectiveness of infant immunity in the control of a viral infection.

Chapter 1 will examine the background behind $CD4^+$ T cell development, differentiation and their role in antiviral immunity. The subsequent chapters will discuss in more detail the phenotypic and functional changes in infant $CD4^+$ T cells using cross-sectional human blood samples and longitudinal blood samples collected from rhesus

macaque infants during the first year of life. My data from these studies confirm that infant $CD4^+$ T cells show altered cytokine responses compared to adults after both polyclonal and TCR-mediated T cell activation (Chapter 2). Furthermore, I could evaluate how the infant immune responses changed from birth throughout the first year of life in the context of a chronic viral infection (Chapter 3). To define the underlying molecular mechanisms for these age-specific differences, I examined infant $CD4^+$ T cell signaling in response to various cytokines. Indeed, I was able to identify differences in interleukin (IL) signaling, specifically, interferon gamma (IFNγ), IL4 and IL2 signaling of infant compared to adult $CD4^+$ T cells (Chapter 4). The understanding of the kinetics of immune maturation in infants on the molecular level should provide valuable insights for pediatric vaccine design and implementation.

$CD4^+$ T cell development and function in infants

$CD4^+$ T cells originate from pluripotent hematopoietic stem cell precursors found in the fetal liver. These precursor cells populate the fetal thymus and will differentiate into distinct cell population during thymopoiesis, with the majority of cells developing into T cells bearing the αβ T cell receptor (TCR)(21), (25). These T cells can be further classified by the presence of specific co-receptors into CD4 or CD8 T cells. Studies show that commitment to $CD4^+$ or $CD8^+$ T cell require the kinases Lck or Zap70 for their development and peptide recognition through MHC class II or I complexes, respectively (6). Both T cell populations undergo proliferative expansion in the periphery to maintain clonal size and repertoire (129).

$CD4^+$ T helper cells (Th) play a central role in effectively orchestrating different immune cells to generate an appropriate immune response to protect the host from infections

and simultaneously, control the response to avoid detrimental outcomes such as autoimmunity. The classic functions of $CD4^+$ Th cells are (1) to promote antibody production by B cells, (2) to activate and maintain cytotoxic $CD8^+$ T cells and (3) direct effector function via the production of specific cytokines (166), (137). Other non-helper functions of $CD4^+$ T cells include activation of macrophage (MΦ) and the regulation and recruitment of immune cells (e.g. neutrophils) (57), (43). Numerous studies have shown that the functional capacity of infant $CD4^+$ T cells differs from that of adults (4), (115), (164). The reasons for impaired infant $CD4^+$ T cell function, however, have not been fully elucidated. Altered $CD4^+$ T cell responses in infants compared to adults could be due to inefficient priming by antigen-presenting cells (APCs), $CD4^+$ T cell intrinsic factors that prevent optimal activation of their responses, or could be the result of active suppression by regulatory cells. In fact, regulatory $CD4^+$ T cells are more abundant in infants than in adults (56), (160), (52). Similarly, it has been demonstrated that pathogen-recognition receptors (PRRs) such as Toll-like receptors (TLRs), are expressed at lower levels on infant compared to adult APCs (72, 79), (24). The differentiation of naïve $CD4^+$ T cells towards a specific Th lineage and effector function further depends on the presence of specific cytokines. Infant $CD4^+$ T cell responses are generally thought of as Th2 biased, meaning that infant $CD4^+$ T cells are more prone to produce IL4, IL5, or IL13 than IFNγ, the characteristic cytokine of Th1 $CD4^+$ T cells. Consistent with this observation, it has been shown that the IFNγ promoter in infant $CD4^+$ T cells is hyper-methylated compared to adult $CD4^+$ T cells (154, 155). Lower production of effector cytokines might increase the vulnerability of infants to infections and delay pathogen clearance.

The analysis of the phenotype and function of $CD4^+$ T cells in the periphery can provide information on an infant's immunological status. Longitudinal studies on lymphocyte populations during the first year of life revealed age-related changes in the $CD4^+$ T cell compartment. In healthy children, absolute $CD4^+$ T cell numbers increased during the first months of life and decreased around 9 to 15 months of age (126). The circulating infant $CD4^+$ T cells, when compared to adults, were found to be recent thymic emigrants, phenotypically resembled adult naïve T cells, and were highly sensitive to growth-promoting cytokines (129) (126), (142), (74). Interestingly, while effector memory $CD4^+$ T cells were absent in umbilical cord blood (CB), $CD4^+$ T cells with a central memory phenotype were easily detected at birth and this population remained relatively stable post-birth (126). A more detailed analysis of CB revealed that the predominantly naïve infant $CD4^+$ T cells expressed less CD45RA, and that the central memory $CD4^+$ T cells might represent a transitional phenotype(119). When assessed for function, these infant naïve $CD4^+$ T cells ($CD45RA^+$) required longer duration of T cell receptor (TCR) engagement compared to mature or Ag-primed (memory; $CD45RO^+$) $CD4^+$ T cells. In the presence of polarizing cytokines, infant $CD4^+$ T cells up-regulated CD45RA similar to that of adult levels (37). Additional research showed that during secondary stimulations, naïve CB $CD45RA^+$ cells differentiated more quickly than their adult counterparts into functionally equivalent $CD45RO^+$ T cells supposedly counteracting their slower response after primary stimulation (119). Naïve $CD4^+$ $CD45RA^+$ and memory $CD4^+$ $CD45RO^+$ T cells differ in their early signal transduction events, suggesting a role of the different isoforms of CD45 and other associated proteins (53). Indeed, TCR specific stimulations of purified naïve CB $CD4^+$ $CD45RA^+$ resulted in proliferation, but also in more cell death compared to adults, further

underlining the distinct responses of infant T cells (26). Determining the functional fates of these subset of cells will aid in understanding key differences in the development of infant and adult effector and memory $CD4^+$ T cell populations.

Differentiation of distinct effector $CD4^+$ T cell lineages

The immune system encounters a myriad of microorganisms and appropriate generation of effector $CD4^+$ T cells is crucial to achieve protective immunity. The process of effector differentiation in $CD4^+$ T cells has been shown to be influenced by the "priming conditions"; that is the nature of the pathogen and the cytokine milieu provided by the cells of the innate immune system in response to a specific pathogen (166, 167). The different $CD4^+$ T cell lineages arise from common precursors, naïve $CD4^+$ T cells that have emerged from the thymus and are circulating in the periphery but have not yet encountered their cognate antigen(s) (Ags). APCs, e.g. dendritic cells will recognize pathogen-associated molecular patterns (PAMPs) derived from microbial proteins via PRRs, express co-stimulatory ligands and present the Ag-peptides complexed to MHC class II to naïve $CD4^+$ T cells. Once naïve $CD4^+$ T cells encounter their cognate Ags through their specific T cell receptors (TCRs), they get activated, proliferate, undergo multiple rounds of cell division and differentiate into distinct, yet plastic, lineages (Table 1). Decades of research have determined that naïve $CD4^+$ T cell precursors must undergo many steps of cell division and that differentiation is determined by many factors including antigen dosage, type and duration of exposure and the cytokine milieu, before these cells can acquire the effector function necessary for their specific function (136). In addition, the strength of the TCR signaling influences T cell differentiation (144). Weak TCR stimulation induces a calcium signal that triggers IL4- synthesis while stronger TCR stimulation induces mitogen-activated

protein kinase (MAPK) that control IFNγ production (11). Moreover, during Ag encounter, naïve $CD4^+$ T cells must be exposed to IL2 to sustain cell division and differentiation towards highly differentiated effector cells. Defects in IL2 production will result in poor generation of effector and memory cells (136).

Originally, only two distinct $CD4^+$ T cell lineages, Th1 and Th2 were identified (166). Now, at least 3 more Th lineages have been discovered (Table 1), with their unique functions being primarily determined by the cytokine patterns they produce (99), (167), (51). The cellular identity of these differentiated $CD4^+$ T cell lineages is achieved through epigenetic changes, where specific and heritable pattern of gene expression are imposed on cells and its progeny without changing the DNA sequence (8). Specifically, the identity of these $CD4^+$ T cells is controlled by complex dynamic interplays of transcription networks and epigenetic modifications such as cis-regulatory DNA elements that affect binding of transcription factors, chromatin remodeling and transcription of genes (8). Intriguingly, there is great flexibility in the $CD4^+$ T cell commitment to a certain effector T cell subset (20), (99), (86). Table 1 below summarizes the main criteria that distinguish the different $CD4^+$ T helper cell lineages.

Table 1: $CD4^+$ T cell Populations

Population	Induction	Surface markers	Transcription Factors	Major Cytokine	Immunity
Th1	IL12, IFNγ	CXCR3, IL12Rβ2, CCR5	T-bet, Eomes, STAT4 and 1	IFNγ, IL2, TNFα, Lymphotoxin α	Intracellular viruses and bacteria
Th2	IL4	CRTH2, CCR6	Gata-3, c-maf, STAT6	IL4, IL13, IL15 & IL5	Parasite and allergies
Th17	*IL6 & TGFβ ; **IL1β, IL6, &IL23	CCR6, CCR4	RORγt, STAT3	*IL17A&F & IL21; **IL17A&F, IL22, IL26	extracellular bacteria and fungi
Th9	TGFβ and IL4	CCR3, CCR6, CXCR3	PU.1, GATA3, STAT6, IRF4	IL9	Parasite expulsion, allergies
iTreg	*TGFβ, IL2, retinoic acid (RA); **TGFβ & IL2	$CD25^{high}$, $CD127^{low}$, CTLA-4	FOXP3	TGFβ, IL10	Autoimmunity

*mice;
**humans

Pioneering studies demonstrated that unresponsiveness in the infant T cells was similar to T cell tolerance to self-Ags, where T cells are educated not to react to self-Ags and was termed neonatal tolerance (164). However, there is increasing evidence that infants are immunocompetent, and other effector $CD4^+$ T cell populations, including Th1 $CD4^+$ T cells can develop. Under appropriate conditions, infant $CD4^+$ T cells showed competence in producing cytokines related to Th1 and Th2 when stimulated through the TCR (30). Other studies reported that even when CB naïve $CD4^+$ T cells were stimulated with myeloid-dendritic cells (mDCs) derived from adult monocytes, they showed a lower activation profile and impaired Th1 differentiation (impaired IL12 expression, and signaling molecule, STAT4) compared to naïve $CD4^+$ T cells from adult peripheral blood (28). The mechanisms for neonatal tolerance and immune deviation are still elusive but research in neonatal mice

showed that wild type, Stat4-deficient (IL12-Th1 development), and Stat6-deficient mice (Th2 development) efficiently established tolerance regardless of immune deviation (27). The classification of Th responses into Th1 and Th2 has great historical relevance in infant immunity, as the infant immune response is generally considered to have a Th2 type. The caveat remains though that even when appropriately stimulated infant $CD4^+$ T cells will respond but the response might not be suitable to effectively combat infections.

Th1 cells were one of two T helper lineages first discovered in 1986 by Timothy Mossman and Robert Coffman (20), (96). These cells protect the hosts against intracellular pathogens such as viruses and mycobacteria. IL12 produced by DCs and MΦ is the main cytokine that drives the development of Th1 cells. Surface markers such as IL12Rβ2, CXCR3 and CCR5 identify these $CD4^+$ Th1 cells as well as the transcription factors T-bet, Eomes and STAT1. Th1 cells mainly produce IFNγ, but also secrete IL2, TNFα and lymphotoxin α (166), (167), (139). IFNγ, a pleiotropic cytokine, activates MΦ and increases their phagocytic and microbicidal activity (166), (130). Not only is IFNγ the major product of Th1 cells, it is also sufficient to further support Th1 development as it maintains responsiveness to IL12 along with strong TCR signals in co-stimulation with CD28 and IFNγ secreted from innate cells (151). A strong Th1 response translates into effective control of intracellular pathogens while aberrant responses result in organ destruction seen in autoimmune disorders. Infants have been described to have lower Th1 responses compared to adults making them vulnerable to many infectious diseases early in life. Studies on infant immune responses to vaccines and pathogens like human cytomegalovirus (HCMV) revealed that some functions of their immune system are comparable to adults such as the $CD8^+$ T cell response but their ability to mount a protective Th1 response is defective, due to lower IFNγ

production by their $CD4^+$ T cells (87). HCMV infected children, whether they acquired the virus in utero or during infancy, have low proliferative and IFNγ responses against HCMV antigens, and show prolonged excretion of the virus in bodily fluids. Nevertheless, the immune system of neonatal mice and humans is not deficient, but altered, and potent Th1 response, e.g. in response to vaccination of *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) vaccine, can develop (106). The mechanisms responsible for variable Th1 responses against different pathogens are not understood.

Th2 responses are thought to protect the host against extracellular pathogens (e.g. bacteria and helminthes). Their signature cytokine products include IL4, IL5 and IL13, and they express the transcription factors Gata-3, c-maf and STAT6. Although the main function of Th2 cells is supposed to be in the control of humoral immunity, there is increasing evidence that IL4 and IL13 can activate MΦ (166), (167), (146). These so-called alternatively activated MΦ are important in homeostasis and tissue repair. Uncontrolled Th2 responses can result in allergy and asthma, and are associated with elevated IgE, eosinophilia, increased mucus production and smooth muscle hyper-contractility (146). Early studies in neonatal mice suggested that that neonatal T cell response was skewed towards the Th2-lineage (4), (3). It was also demonstrated that the Th2 cytokine IL4 could utilize an alternative receptor, IL13Rα1, in mice and drive apoptosis of Th1 cells (78). Although neonatal mice developed both Th1 and Th2 responses during the primary response in this system, the recall response was Th2-driven and the Th1 cells underwent apoptosis.

$CD4^+$ Th17 cells were first described in 2003 and are characterized by the expression of the transcription factor RORγt, production of the chemokine CCL20 and the cytokines IL17A and F, and IL22 (167), (159). In contrast to mice where the development of Th17 is

contingent on TGFβ and IL6, IL23 and IL1β are needed for their induction in humans. Although Th17 responses are associated with the development of autoimmune disorders such as multiple sclerosis (MS), psoriasis and rheumatoid arthritis (RA), they also confer protection against both bacteria and fungi, especially at epithelial surfaces by regulating innate immunity (159), (33). Recent studies have shown that the infant immune environment is capable of inducing Th17 differentiation and this capacity is achieved within the first three months of life (39). The delay in Th17 differentiation might be due to intrinsic mechanisms that result in reduced production of RORC2 mRNA, affecting the regulation of RORγt expression (35). Other studies reported that development of Th17-lineage capacity was inversely correlated with developmental age. Infant but not adult naïve $CD4^+$ T cells had the propensity to develop into Th17 cells because infant $CD4^+$ T cells express higher levels of Th17 related genes such as IL23R, RORγt and STAT3, than adults (19). These data suggest a developmental regulation of this $CD4^+$ T cell subset, favoring Th17 development early in life.

Regulatory T cells or Tregs are another subset of $CD4^+$ T cells that are specialized in ~~s~~uppressive function to induce and attenuate immunological tolerance to self or non-self antigens (123, 124). This $CD4^+$ Treg cell lineage can be divided into two populations, natural Tregs (nTregs), which arise during thymic development, and inducible Tregs (iTregs), which are derived from naïve $CD4^+$ T cell circulating in the periphery and develop during the adaptive immune response. The nTregs constitutively express the CD25 molecule, also known as IL2Rα chain, and the transcription factor, forkhead box P3 (FOXP3) (123, 125). These cells act to control self-reactive T cells in the periphery that have escaped thymic negative selection and thus, are responsible for self-tolerance, and hypo-responsiveness to tumor antigens. In contrast, iTregs suppress aberrant IL4 and IL13 responses, especially at

mucosal barriers (e.g. lungs), by restraining type-2 immune activation in the steady state (146). Inhibitory cytokines such as TGFβ and IL10 produced by Tregs have a role in modulating both protective and aberrant responses (34). Interestingly, neonatal T cells were found to have higher frequencies of natural regulatory T cells compared to adults. The numbers of $FOXP3^{+}$ $CD25^{high}$ –expressing $CD4^{+}$ T cells in infants increase rapidly in circulation during the first days of life (56), (160), (52), (87). Similarly, $CD4^{+}$ $CD25^{high}$ Tregs were also found in higher frequency in fetal but not adult lymph nodes (LN) (93) . Tregs are already present at birth, have a memory phenotype, and express homing receptors than adults (52). Depletion of $CD4^{+}$ $CD25^{high}$ Tregs increases T cell proliferation and IFNγ production of $CD25^{neg}$ $CD4^{+}$ T cells (93). The presence of Tregs early in life likely has a role in controlling non-specific activation and establishing peripheral tolerance, but at the same time may also impede the generation of effective immunity.

Recently, another specialized subset of $CD4^{+}$ T cells, Th9 cells, that produces the cytokine IL9, was defined. Though IL9 was described in the 1980s, recent research showed that Th9 cells mediate allergic inflammation, have protective roles in helminthes infection and autoimmunity. Such functions seemed to be distinct from Th1 and Th17-mediated immunity (51). PU.1, an ETS family transcription factor, has been identified as a key transcription factor in Th9 development. Th9 differentiation also required the cytokines TGFβ and IL4, as cells lacking the signaling components of these cytokines failed to express IL9 (62). Microarray analysis revealed that Th9 development required a transcriptional network that involved the expression of activator protein 1 (AP1) family transcription factor BATF (B-cell activating transcription factor-like) (61). Recent studies also revealed a role of the IL2-STAT5 axis in the differentiation of this new Th subset (82). Th9-IL9-producing

cells have pleiotropic actions and affects a myriad of cells ranging from lymphocytes, mast cells, epithelial cells, smooth muscles and hematopoietic stems (51), (67). The role of Th9 in infant immunity is still elusive but it is possible that infants may preferentially develop this response as Th9 cells were discovered in the context of a Th2 response (86).

In addition to these Th subsets that can be found in blood and tissues, T follicular helper (Tfh) cells are found in lymphoid tissues. They have an important role in inducing antibody production by B cells and in the generation of long-lived high affinity plasma and memory B cells (84). Tfh develop when naïve $CD4^+$ T cells receive signals through their TCR, co-stimulatory signals, and when the co-polarizing cytokines IL6 and IL21 induce c-maf and up-regulate the transcriptional repressor Bcl-6 (84). Tfh cells express CXCR5, which allow their migration towards the B cell follicle, and ICOS on their cell surface (32), (84). Moreover, they have a distinctive transcriptional profile that reflects their role as professional B cell helpers where they ensure that only germinal center (GC) B cells with the highest affinity for the specific Ag will survive, differentiate and become long-lived plasma cells (84). It has been implied that in persistent infections, $CD4^+$ T cell differentiation could be redirected towards the generation of $CD4^+$ Tfh to ensure continued humoral immune responses (44). Though limited data are available about $CD4^+$ Tfh in human infants, recent studies in newborn mice demonstrated that both the neonatal environment and T cell-intrinsic factors influenced the development of Tfh responses and the use of adjuvants in immunization could result in adult like GC responses (92). Tfh cells seemed to develop in vaccinated newborn mice but were impaired in function and had defective localization in GCs (36). The frequency of these cells and the mRNA of their associated transcription factor, Bcl-6 and cytokine IL21 were decreased and was associated with defective antibody

responses. Interestingly, IL4, a Th2 associated cytokine seemed to modulate the function of Tfh cells by promoting their development, emergence, localization to and formation of GCs (36). These findings suggest an important role of Tfh in antibody development and may have implications in the reduced antibody responses observed in infants.

The development of the distinct $CD4^+$ T cell lineages discussed depends on the complex interplay of cytokine networks. The next section will describe in more detail the role of cytokine signaling in the generation of effector $CD4^+$ T cells, especially regarding the Th1 and Th2 fates.

Cytokine signaling contributes to effector $CD4^+$ T cell generation

$CD4^+$ T cell differentiation is a dynamic process. The acquisition of the unique and plastic fates of effector $CD4^+$ T cells requires the activation of a complex cytokine and transcription factor network. During antigen encounter, the cytokine milieu is essential in determining the fate of a naïve $CD4^+$ T cell and the generation of an adaptive immune response. Infants have been demonstrated to be inefficient in producing certain effector cytokines, particularly, IFNγ, the major product of Th1 cells. The reduced production of this cytokine is associated with the infant's susceptibility to intracellular pathogens. Thus, it is necessary to understand whether cytokine-mediated signaling in infant $CD4^+$ T cells differ from adults and whether such mechanism can explain the unique immune status of infants. Understanding the role of cytokine signaling in the generation of protective immunity will likely provide additional parameters to put in consideration when implementing better interventions that are effective in the pediatric populations.

Cytokine levels in the periphery can be used to indirectly assess cellular immunity as they serve as effector proteins of immune cells, particularly $CD4^+$ T cells. Analysis of IFNγ and IL4 concentration in normal sera showed that IL4 was produced earlier than IFNγ in CB, at day 1 and day 5 after birth (118). Stimulation with non-specific stimulants or mitogens (Staphylococcal enterotoxin B (SEB), lipopolysaccharide (LPS) or phorbol 12-myristate 12-acetate and ionomycin (P/I) revealed that the cytokine-producing capacity in infants increased from birth to 1 year of age but remained weaker compared to their mothers or other adults (76). Early comparative studies in neonates, older children and adults demonstrated that the Th1-cytokine-producing capability increased with age and was correlated with expression of CD45RO, suggesting antigen exposure dependency (58). Th2 cytokine production was minimal across all age groups (31). Additionally, when purified $CD4^+$ T cell populations were used ($CD4^+$ $CD45RA^+$ T cells or $CD4^+$ $CD45RO^+$) and stimulated by polyclonal activation, adult, but not CB purified cells secreted the cytokines IFNγ, IL2, and IL4 and seem to be impacted by the environment (30), (41), (54). Some of the $CD4^+$ T cell data appeared contradictory and difficult to interpret because the various studies employed different conditions to measure Th1 or Th2 associated cytokines in the infant. Therefore, they might not necessarily depict an accurate picture of the infants' potential to secrete and, equally important, respond to these effector cytokines. Determining which signaling components are differentially activated in infant compared to adult $CD4^+$ T cell will be critical in identifying factors leading to distinct IFNγ and IL4 production in infants and their skewed differentiation to $CD4^+$ Th2 cells.

Cytokines regulate cellular responses by long-term transcription-dependent or short-term transcription-independent mechanisms (18). The former, which represents the

traditional signaling route, is primarily the JAK/STAT signal transduction pathway. Cytokines activate specific Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) molecules to affect gene transcription or repression (Figure 1). Given that the JAK/STAT signaling pathway can affect a wide range of immune responses ranging from immune defense, differentiation, proliferation and even oncogenesis, this pathway is strictly regulated. Regulation is achieved through numerous levels including receptor expression, kinase activation, cytoplasmic transcription phosphorylation and negative regulator expression. Its relevance is highlighted by the ability of viruses to escape the immune system by inactivating the different components of the JAK/STAT pathway (70).

For cells to be receptive to cytokines, they must possess the necessary receptors on the cell surface. IL4 binds to type I receptors which are characterized by the presence of 4 conserved cysteine residues Trp-Ser-X-Trp-Ser, the WSXWS sequence motif, and the fibronectin type II modules in the extracellular domain, whereas IFNγ utilizes Type II receptors (R1 and R2) without the WSXWS sequence motif, and have tandem fibronectin type III domains (77), (73). The IL4 receptor complex is composed of the IL4Rα/IL13Rα1 and γ_c (common gamma chain), while the IFNγ receptor complex consists of 2 chains, the p90 binding chain, IFNγR1 and p35, the accessory signal transducing chain or IFNγR2 (78), (77), (122). Receptor density and ligand affinity can modulate the effects of cytokines. For example, IFNγR2 expression in human T cells is higher in the cytoplasm compared to the cell surface, possibly suggesting intracellular trafficking of receptors to control the effects of its ligand (122). IL4 receptor expression on human T cells can be affected by various signaling pathways, and by IL4 itself at both the transcriptional and posttranscriptional level (40). In tissues, IL4 is secreted by Th2 cells, Tfh cells and basophils, but there are potential

other sources such as mast cells, eosinophils, NK T cells and innate lymphoid cells 2 (ILC2) (146). In response to infection, innate immune cells such as natural killer (NK) and natural killer T (NKT) cells are the first source of IFNγ. During the adaptive phase when antigen-specific T cells are generated, both $CD4^+$ and $CD8^+$ T cells gain the ability to produce IFNγ. However, naïve $CD8^+$ T cells by default, are programmed to be IFNγ-producing cytotoxic T cells while $CD4^+$ T cells differentiate into different lineages where only Th1 cells produce substantial amounts of IFNγ (128).

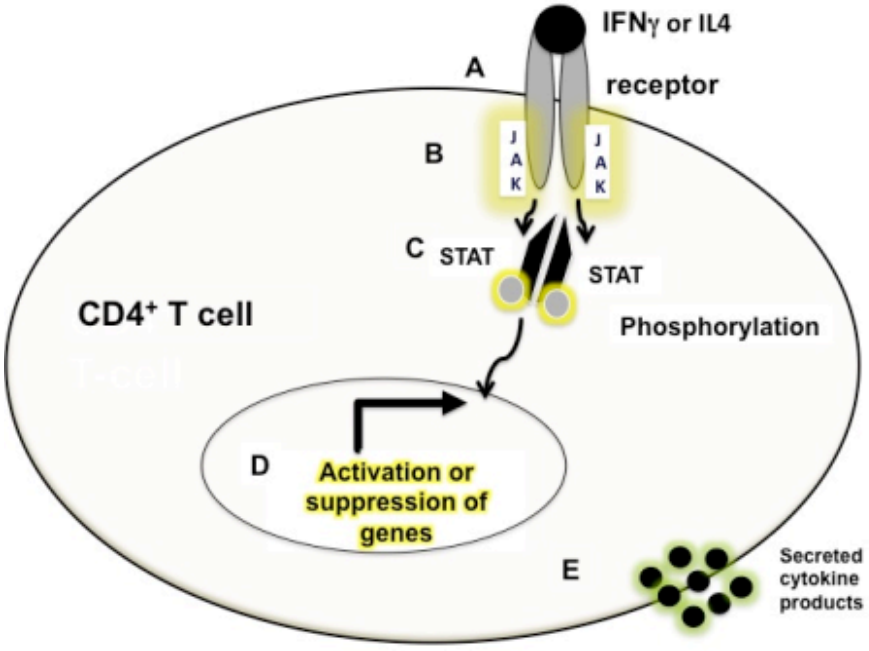

Figure 1: Schematic of cytokine signaling in a $CD4^+$ T cell. A. Cytokines such as IFNγ or IL4 will bind to their respective receptors. Receptor- associated JAKs are phosphorylated (B) and activate STATs (C) which then dimerize and translocate to the nucleus (D), where they activate or suppress genes and thereby determine the production of effector molecules, e.g. cytokines (E).

In a non-activated state, cytokine receptors exist as individual subunits in the plasma membrane and lack kinase activity (18). When cytokine-binding to its receptor is initiated,

downstream molecules are activated to relay the signal to the cell's nucleus to affect gene expression. This signal is communicated inside the cell through receptor dimerization and phosphorylation of tyrosine residues on the cytoplasmic tail of receptors by JAKs, which serve as docking sites for the SH-2 domain of STATs, which themselves are phosphorylated by JAKs, allowing STAT molecules to homo- or heterodimerize and translocate to the nucleus to activate or suppress gene transcription (133), (103, 111), (105), (108).

The Janus kinase family is composed of 4 members: JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase 2) (133). The receptors undergo conformational changes, which permit their associated JAKs to trans-phosphorylate each other, and by phosphorylating conserved tyrosine residues in the cytoplasmic tail of their coupled receptors. JAK 1 is involved in the signaling of the IL4-receptor family (IL4R, IL13R) whereas both JAK1 and JAK2 are associated with the IFNγ receptor complex. These kinases have been shown to be essential for host immunity as knockout of these components results in embryonic and perinatal lethality, or at a minimum hypersensitivity to infectious pathogens (133). Moreover, enhanced expression of JAKs and related receptors in effector $CD4^+$ T cells associated with chronic inflammatory diseases like multiple sclerosis underlines their significance (33).

There are 7 mammalian STATs (STAT1, 2, 3 4, 5a, 5b, & 6) described to date and their actions involve binding to response elements associated with the promoter of their target genes and modulating transcription through recruitment of transcriptional co-activators or repressors (108), (111), (127). STAT1 was discovered to mediate IFNγ signaling to target genes that promote inflammation and antagonize proliferation, whereas STAT6 transduces signals from IL4 to polarize naïve $CD4^+$ T cells into Th2 effectors and promote B cell function by binding to IgE promoter on B cells or IL4 promoter on T cells (127), (95), (58).

Mutations in STAT1 are associated with susceptibility to viral and mycobacterial infections while STAT4 variant alleles are involved in the pathophysiology of systemic lupus erythematosus (SLE), an autoimmune disease (104).

The onset of JAK/STAT signaling is rapid and so is their termination. The termination or decay in signal involves down regulation of receptors, JAKs and STATs. STATs are dephosphorylated by phosphatases and are inactivated by suppressor of cytokine signaling (SOCS) proteins (127). There are 8 members within the SOCS family, and they are SOCS1 through SOCS7 and CIS (75). SOCS 1, 2 3 and CIS are present in cells at low or undetectable levels but are rapidly induced by cytokines such as IL4, IFNγ, IL2, IL3, IL6 and others (75). Moreover, SOCS 1 and 3 can inhibit signaling by IL4 and IFNγ. Indeed, research in the field has demonstrated that SOCS proteins regulate the differentiation of $CD4^+$ T cells into Th1 or Th2 lineage. (42). Thus, tight regulation of cytokine signaling via JAK/STAT is vital, as excessive stimulation will result in tissue damage.

Considering that IFNγ and IL4 cytokines have diverse effector roles in the immune system, different transcriptional programs must be activated by these cytokines. Complex populations such as peripheral blood mononuclear cells (PBMCs) will most likely activate or express a different transcriptional program than purified populations. The transcriptional response of PBMC to IFNγ was shown to be restricted to a subset of type I interferon-inducible genes and the amplitude of the response increased with IFNγ dose (150). Comprehensive profiling of genes specifically expressed by human activated Th1 and Th2 cells have offered insights into the molecular basis of Th1/Th2 development and especially in human diseases in which these 2 subsets are involved (98). $CD4^+$ T cells from CB showed reduced expression of NFATc2 (nuclear factor of activated T cells c2), a critical

transcription factor necessary for the up-regulation of multiple cytokines known that can amplify T cell allogeneic responses, and also regulate IFNγ, and IL4 cytokine responses (65). In contrast, in graft-versus-host disease, cytokine gene array and RNAse protection assay revealed that Th2 responses in CB were lower compared to adults due to the reduced mRNA expression of c-maf, an important transcription factor regulating Th2 cytokine expression (65). Independent of the study setting, gene transcription in infants and adults significantly differed. Equally important, the components of the JAK/STAT signaling pathway are undeniably pivotal in determining how cytokines influence cellular processes such as T helper differentiation. Epigenetic studies had also shown that naïve T cells that have been stimulated by antigens seemed to show low, nonselective transcription of *Ifng* and the Th2 cytokine cluster (*Il4* locus) (8). Moreover, differentiations of certain lineage rely on additional signals such as cytokines, that induce differentiation, as the genes are poised in the chromatin context to be either epigenetically activated or silenced (8) There seemed to be regulatory mechanisms in place to control both the production and the response to cytokines. In the unique infant environment, how tolerance and T cell responses against pathogens are balanced are not well understood. Clearly, an intricate crosstalk between multiple immunological processes including signaling and epigenetic changes will influence the generation of $CD4^+$ T cell immunity.

Role of $CD4^+$ T cells in Antiviral Immunity

The importance of $CD4^+$ T cell immunity is highlighted in their ability to influence the function of other lymphocytes. In the context of viral infections, infants compared to adults have increased susceptibility to viral infections such as HCMV, HIV, and Hepatitis B virus (HBV) resulting in high morbidity and mortality worldwide (115), (116). The viral

burden imposed by these viruses illustrates that infants have an altered $CD4^+$ T cell response that are inefficient in controlling these pathogens. Similarly, infant responses to vaccination are generally weaker compared to adults supporting the need for better ways to optimize infant vaccination strategies. This section will discuss the current understanding of $CD4^+$ T cell immunity in the context of HCMV infection as substantial aspect of my graduate work was focused on modeling CMV infection in infant rhesus macaques.

HCMV infection *in utero* is the most prevalent congenital infection, afflicting newborns in the developed world. Recent data estimated that congenital CMV infection occurs in up to 6.2% of live births following primary infection, re-infection or maternal HCMV reactivation (116). CMV-infected newborns suffer severe disease and may develop neurodevelopmental sequelae, particularly sensori-neural deafness (116). Interestingly, perinatal acquisition of CMV, most likely via breast milk (BM) by healthy infants, is asymptomatic and their viral load is not as high as in congenitally infected infants. However, these HCMV infected infants and children who acquire HCMV from contaminated saliva have prolonged viral shedding compared to adults with primary HCMV infection (5).

In contrast to robust and comparable CMV-specific $CD8^+$ T cell responses to adults, CMV-specific $CD4^+$ T cell responses in infants are persistently deficient. Specifically, IFNγ production by these CMV-specific $CD4^+$ T cells was highly reduced in children even after a year since acquiring CMV (143). Concomitant with diminished IFNγ production, IL2 production and expression of the co-stimulatory molecule, CD40-ligand, were also reduced. More recent CMV studies also reported that CMV-specific $CD4^+$ T cell responses were very low in children under 2 years of age but become detectable with increasing age (83). Taken together, these data suggest that failure to control CMV in the young is associated with the

age-dependent development of $CD4^+$ T cell immunity. The molecular mechanisms underlying this differential antiviral response of infant and adult $CD4^+$ T cells are still not clear.

The thesis work describe here investigates immune ontogeny, maturation and cytokine signaling. In Chapter 2, we took advantage of the non-human primates (NHPs), rhesus macaques (*Macaca mulatta*), to identify age-related differences in immune system development in infants, juveniles and adults in the context of a viral infection. We compared 2 groups of animals that were raised to be specific pathogens free (SPF) and naturally infected with RhCMV. We explored whether age and viral burden affect frequency and function of immune cells. Chapter 3 describes SPF infants and adults that were experimentally infected with RhCMV. We determined the shedding of RhCMV in bodily fluids to evaluate the development of adaptive immunity in the infant and adult animals. Chapter 4 describes our work done using human samples. We described the differences in cytokine signaling in $CD4^+$ T cells as an effort to reveal a molecular mechanism that might play a role in the unique function of infant $CD4^+$ T cells. A deeper understanding of immune development should help in elucidating what molecular mechanisms are influencing $CD4^+$ T cell immunity in infants.

CHAPTER 2: THE INTERPLAY BETWEEN IMMUNE MATURATION, AGE, CHRONIC VIRAL INFECTION AND ENVIRONMENT

OVERVIEW

Maturation and age are dynamic processes. Associated with people living longer, numerous health ailments are increasing from cardiovascular diseases to cancer suggesting dysregulation in immune function with age or immunosenescence. One possible reason that leads to immunosenescence is exposure to a lifetime inflammatory environment caused by both clinical and subclinical infections described as "inflammaging". Determining T cell development, maturation, and function from birth to adulthood are important parameters to assess changes in cellular immunity with age and the impact of a lifelong viral infection. In this study, we use rhesus macaques of specific (SPF) and non-specific pathogen free (non-SPF) status to characterize basic immunological changes in peripheral blood cell populations with age and how they are affected by chronic cytomegalovirus infection (RhCMV). Our findings indicated that age-related changes in major blood cell populations from birth to adulthood are common to both SPF and non-SPF macaques. We also demonstrated that chronic RhCMV infection modulates immune development over the lifetime of the host, evident in juvenile RhCMV-infected macaques. These altered immune responses in non-SPF animals may predispose them for inflammaging in late adulthood. These data support the idea that rhesus macaques could serve as a model system to study inflammaging and the possible factors leading to immunosenescence.

INTRODUCTION

Immune development and aging are dynamic processes that are governed by many distinct, yet interacting factors. Lifespans are mostly increasing worldwide, yet there have been concomitant increases in age-related morbidities, particularly cardiovascular diseases, increased susceptibility to seasonal infection, cancers, and neurodegenerative disorders. A unifying link between the increased disease rates from these disparate diseases observed in aging populations is the progressive decline in immune functions brought on by both intrinsic and extrinsic factors. This phenomenon has been termed "immunosenescence", and the etiologies of immunosenescence are incompletely defined. A central driving force in decline in immune function is the inherent process of progressive thymic involution, which results in an extremely restricted generation of naïve T cells by the fifth or sixth decades of life. However, studies over the past several years have emphasized the critical importance of extrinsic factors, which when layered over thymic involution, can produce a "conspiracy of immune dysfunction" that can increase rates of morbidity and mortality in aging individuals.

Several hypotheses have been presented to explain the mechanistic basis for increased disease susceptibility associated with aging. Many of these focuses on the age-related development of pro-inflammatory state that is observed in many aging individuals, a process that have been termed "inflammaging". Multiple studies have demonstrated increased production of inflammatory cytokines with age, and the causes for inflammaging are likely multifactorial and remain to be determined (94), (16). Several polymorphisms in genes for IL6, IL10, and TLR4 have been associated with increased inflammation during aging (9), (10), (22), (46), (47), (60), (45). Interestingly, this association was more pronounced in men than in women suggesting that hormones may influence the aging process. In fact, a recent

review discussed principle age-related alterations of the endocrine system that could influence immunity (60).

An alternative theory points to chronic infections as major driver in immunosenescence, and, in fact, the processes of immunosenescence and inflammaging may be inextricably intertwined. Among the various known chronic viral pathogens, human cytomegalovirus (HCMV) in particular, has been suggested to be associated with reduced immune function in older individuals (68), (141), (23), (48). HCMV is ubiquitous throughout the world with adult seroprevalence rates of 50-100%. HCMV, a member of *Herpesvirales* order of viruses, is considered to be a virus with low pathogenic potential in immune competent hosts, but like all *Herpesvirales* members, it establishes a lifelong persistence within the infected individual. Persistence is characterized by the presence of cells harboring latent HCMV genomes that periodically and asymptomatically reactivate to produce progeny virions that can be secreted in bodily fluids. Although HCMV has low pathogenic potential in an immune competent host, evidence indicates that the virus-host relationship is unlike that for any other virus, and this relationship has important clinical implications in relation to immunosenescence. The implications stem from persistent antigenic stimulation of the immune host, resulting in an extraordinarily large devotion of the infected host's immune repertoire to HCMV.

A seminal study by Sylwester et al quantified the CD4 and CD8 T cell responses to the HCMV proteome in 32 healthy long-term HCMV-infected individuals (138). On average, almost 10% of memory CD4 and CD8 T cells are specific to HCMV antigens, far exceeding in magnitude the frequency of antigen-specific T cells to any other pathogen. Other studies have indicated that there are progressive oligoclonal expansions of some of the HCMV-

specific T cell populations. The majority of HCMV-specific effector memory cells are terminally differentiated, considered to be dysfunctional and thereby, immunosenescent. Furthermore, the hypothesis has been presented that the vast expansion of HCMV-specific T cells consumes the available finite "niche" for immune cells and interferes with the development of effector and memory cells targeting other pathogens (48). At the same time, the persistence of antigens continuously induce stress signals promoting inflammation (48). Although seminal studies like the OCTA, NONA and NHANES cohort studies have shown a statistical association between aging, reduced immune function, increased C-reactive protein (CRP) levels and HCMV seroprevalence, cause and effect cannot be conclusively distinguished (156), (157), (7), (140).

The underlying mechanisms of and associations between chronic infections and immune function could be best determined and tested in an animal model that closely recapitulates characteristics of human development, physiology, immunology, virology, and longevity. We propose that rhesus macaques represent such a model. Wild and captive populations of rhesus macaques are ubiquitously infected with rhesus cytomegalovirus (RhCMV), as well as other persistent pathogens. Furthermore, RhCMV-infected monkeys show similar age-associated immune changes as those observed in HCMV-infected humans have been noted in these, including decreased $CD4^+$ to $CD8^+$ T cell ratios and the accumulation of terminally differentiated $CD8^+CD28^-$ T cells (63), (112), (100). In captivity, these animals live for an average of 25 years and a maximum of approximately 40 years. Although their lifespan is shorter compared to humans, macaques undergo similar developmental, hormonal and immunological changes, but in an accelerated fashion compared to humans.

The current study characterizes basic immunological parameters in peripheral blood cell populations in relation to 1) age, 2) housing environment and 3) the presence or absence of a persistent virus (RhCMV). Taking advantage of the unique resource of rhesus macaques that were bred and raised be Specific Pathogen Free (SPF), for specific viruses (described below), we compared the immune development of SPF infant macaques from birth throughout their first year to non-SPF infant macaques that were raised in a similar nursery environment. Once the animals were transferred to outdoor corrals at one year of age, we asked whether the SPF status of an animal would affect immune modulation induced by pathogenic or other stimuli. Specifically, we investigated how immune responses to RhCMV evolve over the lifetime of a chronically infected animal in correlation with virological parameters, and whether immune cell populations of RhCMV-infected animals would display distinct functional characteristics compared to SPF animals without RhCMV infection of the same age.

Our results show that age-related changes in major blood cell populations from birth to adulthood are common to both SPF and non-SPF macaques. In addition though, our study clearly demonstrates that chronic RhCMV infection modulates immune development over the lifetime of the host. These altered responses were already evident in juvenile RhCMV-infected macaques, thereby potentially predisposing these non-SPF animals for inflammaging in late adulthood. These data support the idea that rhesus macaques could serve as a model system to study inflammaging, and provide a foundation for future studies to delineate specific mechanisms of RhCMV-induced immunosenescence with the long-term goal of developing therapeutic interventions, targeted to young adults, to prevent the onset of inflammaging.

MATERIALS AND METHODS

Animals

Genetically outbred infant rhesus macaques (*Macaca mulatta*) of Indian (n=13; 8 male and 5 female infants) or Chinese (n=10; 8 female and 2 male infants) origin were obtained from the specific pathogen-free (SPF) colony at the California National Primate Center (CNPRC). SPF status was defined as being seronegative for simian type D retrovirus (SRV), simian immunodeficiency virus (SIV), simian T cell lymphotropic/leukemia virus (STLV), Cercopithecine herpesvirus 1 (CHV-1; herpes B virus), RhCMV, simian foamy virus (SFV), and rhesus rhadinovirus (RRV). In addition, 11 non-SPF infant macaques from the conventional colony were included for comparison. SPF and non-SPF infant macaques were removed from their dams at birth and nursery-reared indoors. After 9 months to 1 year, they were relocated to outdoor SPF or non-SPF animal corrals, respectively. SPF juvenile macaques (n=28; 3-5 years), and non-SPF juvenile (n=50; 3-5 years) or adult macaques (SPF n=8, 12-12yrs) and non-SPF adults (n=98, 12-20 years) macaques were of Indian origin and housed outdoors (Table 1). Representative of the CNPRC colony's effort to increase breeding age female macaques, the gender distribution was shifted toward female macaques that represented 65% of SPF juveniles, 42% of non-SPF juveniles, and 70.5% of adult non-SPF macaques. The Institutional Animal Care and Use Committee of the University of California, Davis (UC Davis), which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, approved all animal protocols in advance of any procedures.

Sample collection and processing

Animals were immobilized with (10mg/kg) ketamine-HCl intramuscularly (IM) prior to all sample collections. Complete blood counts (CBC) were performed at each blood collection. Infant EDTA blood samples were collected longitudinally starting at birth and subsequently every month for one year. Plasma was removed from whole blood by centrifugation and stored at -80°C for antibody measurement. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via density centrifugation using lymphocyte separation medium (LSM) (MP Biomedicals, Solon, OH) or Accu-Paque Lymphocyte (Accurate Chemical & Scientific Corp., Westbury, NY) density gradient as described (91), (1), (2). Due to the low blood volume, and therefore relatively low cell counts, infant PBMCs were immediately used for functional analyses. PBMCs from juvenile and adult macaques were cryopreserved and batch-analyzed.

Complete blood count (CBC)

EDTA blood samples were analyzed on a HORIBA Pentra 60+ electronic cell counter (HORIBA Diagnostics, Irvine, CA). A standard blood smear was prepared from the EDTA sample and stained with a Wright-Giemsa stain (Harleco, EM Scientific, Gibbstown, NJ). Manual 100-cell differentials were performed for the differentiation of white blood cells. The relative percentage of each cell type was obtained and then multiplied by the total white blood cell count to get the absolute numbers of each cell population.

T cell stimulation

Infant PBMCs were resuspended with RPMI 1640 media (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated FBS (Cellgro, Manassas, VA) and L-glutamine-

penicillin-streptomycin antibiotic cocktail (Sigma, St Louis MO) at 1.0 x10^6 cells/ml. Negative controls were cultured in media only. To evaluate polyclonal T cell responses, PBMC were stimulated with phorbol 12-myristate 12-acetate (PMA) at 10ng/ml and Ionomycin (I) at 125ng/ml (Sigma). The cells were incubated for a total of 12 hours at 37°C and 5% CO_2 with Brefeldin A (3.0μg/ml) being added after the first hour (eBioscience, San Diego, CA). At the end of the culture period, cells were stained for surface antibodies, fixed, and permeabilized for intracellular cytokine staining (see below) as described previously (91), (1), (2).

In addition, infant PBMCs were stimulated through the TCR with anti-CD3 and anti-CD28 antibodies. Microtiter tissue culture plates were coated overnight with 10μg/ml of pure CD3 (SP34-2) and 10μg/ml pure CD28 (L293) in 100 μl of PBS/ well. The next day, plates were washed 3x with PBS. Cells at 1x10^6 /ml were then aliquoted at 100 μl/well and cultured for 24 hours at 37°C and 5% CO_2. Supernatants were collected and stored at -80^0C until analysis using the Milliplex MAP Nonhuman Primate Cytokine Panel (EMD Millipore, Billerica, MA).

Similarly, 1.25 x 10^6 PBMCs/ml from juvenile and adult macaques were resuspended in RPMI with 10% FBS and stimulated with 25μg/ml concanavalin A (Con A) or 25μg/ml RhCMV lysate prepared from an infected cell extract exhibiting 100% cytopathic effect. Negative control cultures consisted of cells in media only. Cell culture supernatants were collected after 48 hours and were immediately analyzed in duplicates for multiple soluble markers using multiplex bead arrays (Bioplex Suspension Array System with Bioplex Manager 4.0 software by Bio-Rad Laboratories, Hercules, CA, or Milliplex MAP Nonhuman Primate Cytokine Panel by EMD Millipore).

Flow cytometric analysis:

T cell or B cell subpopulations were analyzed for activation or memory cell differentiation using standard surface staining protocols (91), (1), (2). The ability of the $CD4^+$ and $CD8^+$ T cells to produce cytokines in response to PMA/ Ionomycin stimulation was evaluated using the following logical gating strategy: PBMC gate (FSC-A vs. SSC-A), singlets (FSC-H vs. FSC-A), T cells ($CD3^+CD4^+$ or $CD3^+CD4^-$ = $CD8^+$ T), followed by gating for IFNγ, IL2 and/or TNFα within the $CD4^+$ or $CD8^+$ T cell populations. Samples were acquired on a FACS Aria flow cytometer (BD Biosciences). A minimum of 300,000 events were acquired for T cell stimulation responses and 30,000 events for phenotyping studies. Data were analyzed using FlowJo Software (Tree Star, Ashland, Oregon) and Boolean gating strategies were applied when appropriate. Cytokine $CD4^+$ or $CD8^+$ T cell responses were considered positive if their frequencies were at least 2 times higher than in unstimulated (media) PBMCs and if their actual value was ≥0.01%. Data are reported as percentage of $CD4^+$ or $CD8^+$ T cells.

Juvenile and adult whole blood samples were stained with direct labeling of whole blood with antibodies for CD3, CD4, CD8, CD20, CD25, CD28, CD45RA, CD95, HLA-DR, CD195 (CCR5) (BD Biosciences, San Jose, CA), and CD38 (Nonhuman Primate Reagent Resource or StemCell Technologies). Whole blood samples were divided, stained with antibody cocktails to analyze T cell maturation and activation, washed, red cells were lysed, and then PBMC were fixed using a Coulter TQ Prep System (Coulter Corporation, Hialeah, FL). Samples were acquired on a FACS Calibur and analyzed with CellQuest software (BD Biosciences, San Jose, CA)

RhCMV antibody measurement

RhCMV-specific binding antibodies were determined by ELISA as previously described (85), (163). Briefly, 96-well Immulon 4HBX plates (Fisher-Thermo) were coated overnight at 4°C with whole RhCMV lysate (0.5mg/ml) Plasma samples were diluted at 1:100. Anti-Rhesus IgG peroxidase-conjugated antibody was used to detect anti-RhCMV IgG (KPL, Gaithersburg, Md.). The specificity and reproducibility of the ELISA was confirmed using animal sera that were previously confirmed to be RhCMV positive and a plasma pool of seronegative animals. The limit of detection (LOD) was based on the average OD values plus 0.1 OD obtained for the plasma pool of RhCMV negative animals at the lowest dilution of 1:50. Antibody titers are reported as the lowest plasma dilution at which OD values were above the OD value for the RhCMV-negative plasma pool. RhCMV neutralizing antibodies (nAb) were determined as described previously (Abel, 2008, 2011). Data are reported as the reciprocal of the dilution that resulted in 50% neutralization (NT_{50}).

RhCMV quantitation

Oral saliva swabs were collected from both RhCMV-seropositive non-SPF juveniles and adults. RhCMV was quantitated as previously described (59), (131). Real-time PCR for RhCMV gB was performed using the following primer and probe pair: forward primer: 5'-TGC GTA CTA TGG AAG AGA CAA TGC-3', reverse primer: 5'-ACA TCT GGC CGT TCA AAA AAA C-3' (Invitrogen, Carlsbad, CA), and probe (5'-3') TET-CCA GAA GTT GCG CAT CCG CTT GT-TAMRA (Applied Biosystems, Carlsbad, CA). RhCMV was quantitated based on a RhCMV gB plasmid standard curve spanning $10^{\wedge 0}$ to $10^{\wedge 6}$ copies. The viral load was calculated as RhCMV gB copies per ml for saliva. The limit of detection was

10 copies of RhCMV gB.

Statistical analysis

The data are presented as median values if not indicated otherwise. Results between two or more groups were compared by Student's t-test or one-way ANOVA, respectively, using GraphPad Prism, version 5 (GraphPad, Inc., La Jolla, CA). Nonparametric Mann-Whitney tests or Kruskal-Wallis tests with Dunn's Multiple Comparisons were applied when we observed large variation within a group, which is not untypical due to the outbred nature of rhesus macaques. An exact Wilcoxon signed-rank test was used to test the null hypothesis that *in vitro* cytokine responses to PMA/ Ionomycin stimulation in 40 to 48 week old infants are not different from responses in infants between 0 to 4 weeks of age. To perform this statistical analysis, absolute numbers of cytokine producing $CD4^+$ or $CD8^+$ T cells per 1 million $CD4^+$ or $CD8^+$ T cells were used instead of percentages. An estimate of location shift and corresponding 95% confidence interval were also computed. Adjustments for multiple testing were not applied due to the exploratory nature of the study and the relatively small sample sizes.

RESULTS

Infant immune development in a nursery setting

SPF infant macaques were reared in an indoor nursery in small groups of 2-3 animals per cage. Infants born to non-SPF dams were housed under identical conditions but in a separate room of the nursery. Therefore, both infant groups were housed in a similar environment with minimal pathogen exposure, received identical food, and had no contact with older animals that could potentially harbor transmittable pathogens. These controlled

conditions allowed us to define general immune changes in infant peripheral blood cell populations after birth. Furthermore, by including equivalent numbers of SPF Indian and SPF Chinese infant macaques, we could determine whether the genetic origin of the animals influenced early immune development. Non-SPF macaques were of Indian origin only.

The longitudinal analysis of major blood cell populations in SPF infants revealed population-specific patterns of developmental changes during the first year of life (Figure 1; Table 1). Neutrophils constituted the most abundant cells at birth, but rapidly declined during the first 12 weeks. Monocytes showed a similar decline. In contrast, lymphocyte numbers increased steadily. At birth, the vast majority of lymphocytes represented T cells; B cell numbers were relatively low (Figure 2). Both T and B lymphocyte numbers increased after birth, but the increase in absolute B cell numbers was much more pronounced (Figure 2A-D). On average, B cell numbers increased more than 5 times, while T cell numbers less than doubled. Between the two main T cell subsets, $CD4^+$ T cells outnumbered $CD8^+$ T cells at birth with CD4:CD8 T cell ratios ranging from 2.5 – 8.8 (Figure 2E). After birth, the $CD8^+$ T cell population showed a marked expansion (1.0 to 8.8-fold increase), whereas $CD4^+$ T cell numbers only slightly increased (0.8-3.0 fold increase) from birth to 3 months of age. As a result, the CD4:CD8 T cell ratio declined (Figure 2E), eventually stabilizing at a 2:1 ratio of CD4:CD8 T cells, which is typical for juvenile and adult macaques (Table 2). The expansion of T cells occurred concurrently with rising $Ki67^+$ $CD4^+$ and $Ki67^+CD8^+$ T cells during the first weeks after birth (Figure 2F). Consistent with the larger increase in $CD8^+$ T cell numbers, the percentage of $Ki67^+$ T cells was higher in the $CD8^+$ than the $CD4^+$ T cell population. Thus, during the first few months of life, the various blood cell populations

changed significantly in their absolute frequencies and their relative proportions to each other (Tables 1 and 2), reaching steady state levels between 12-24 weeks of age (Figures 1 and 2).

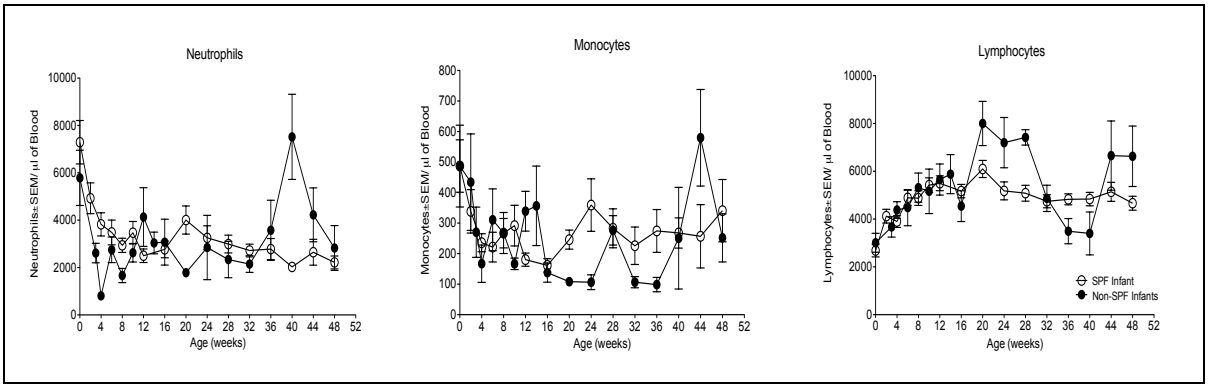

Figure 1: Longitudinal changes in major blood population during the first year of life. The graphs show longitudinal changes in the frequencies of absolute neutrophils (left panel), monocytes (middle panel), or lymphocyte numbers (right panel) in peripheral blood of SPF (open circles) and non-SPF (closed circles) infant macaques from birth to 1 year. Data are represented as mean absolute numbers ± SEM per microliter of blood. A total of 23 SPF infants were included and 11 non-SPF infants were followed until week 16, but only 4 non-SPF macaques were followed until week 52.

Table 1: Peripheral Blood Mononuclear Cell Populations

Cell Population	**Median Cell Numbers/ μl Blood** (*Minimum-Maximum No.*)				
	Infants (40weeks)	**Juveniles** (3-5yrs)	*Infants vs. Juveniles*	**Adults (>12yrs)**	*Juveniles vs. Adults*
SPF	**n=23**	**n=20**		**n=8**	
Lymphocytes	4608 (*2596-7314*)	1949 (*1107- 3088*)	<0.0001	710 (*342-2388*)	p=0.0014
Monocytes	182 (*45-637*)	328 (*229-855*)	<0.0001	938 (*476-2030*)	<0.0001
Neutrophils	1952 (*990-4900*)	10502 (*6897-15355*)	<0.0001	11274 (*10032-19694*)	NS
Non-SPF	**n=11**	**n=50**		**n=98**	
Lymphocytes	2874 (*2016-5838*)	3037 (*756-8073*)	NS	1248 (*262-3888*)	<0.0001
Monocytes	534 (*261-990*)	266 (*0-1620*)	NS	291 (*0-1494*)	NS
Neutrophils	6764 (*4026-12525*)	9576 (*1936-21156*)	NS	8280 (*2520-20169*)	0.0119
Comparison of SPF versus Non-SPF Populations					
Lymphocytes	NS	p<0.0001 (SPF<non-SPF)		NS	
Monocytes	NS	NS		p=0.0002 (SPF>non-SPF)	
Neutrophils	p=0.0024 (SPF<non-SPF)	NS		p=0.0068 (SPF>non-SPF)	

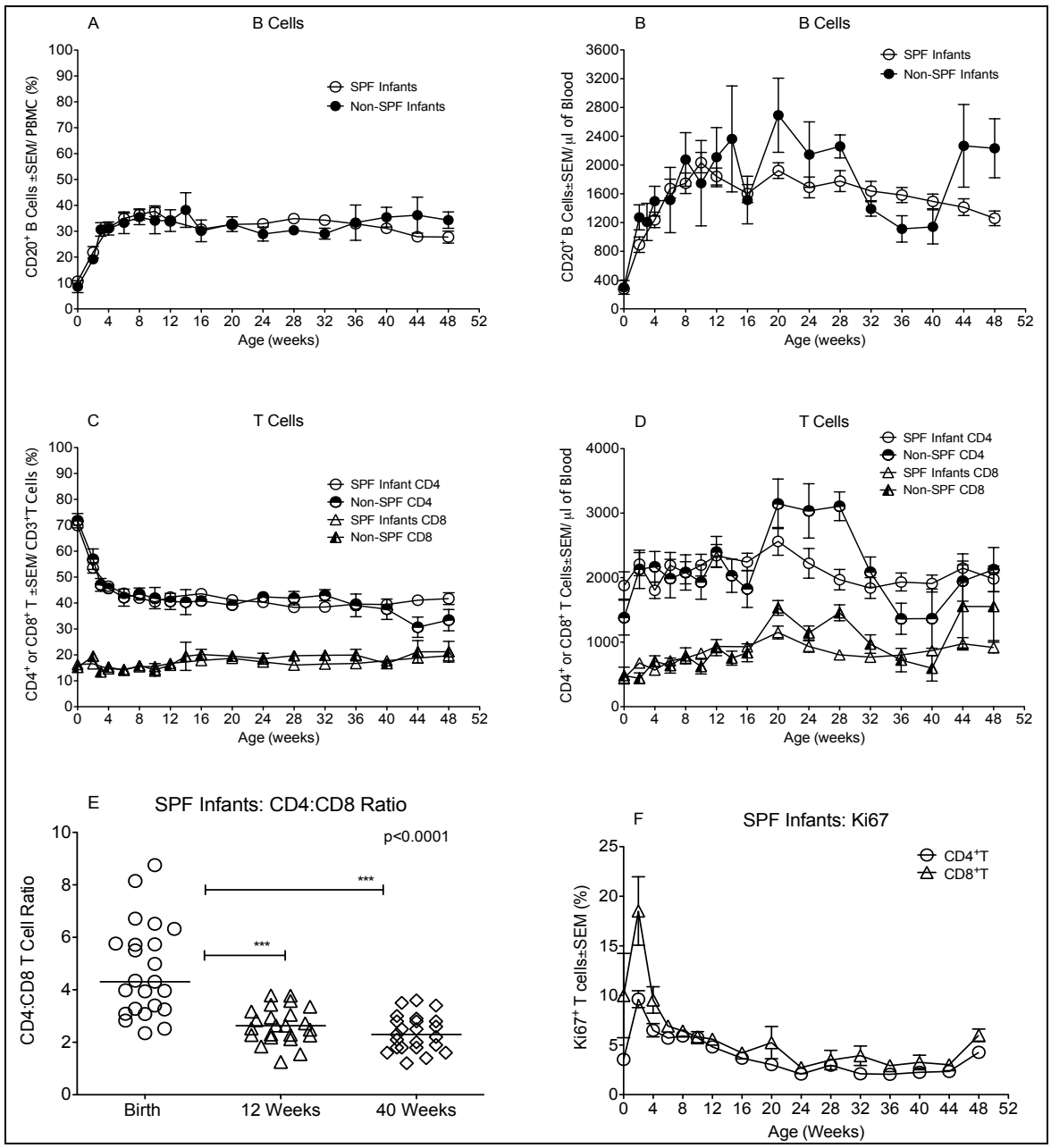

Figure 2: Developmental changes in T and B cell populations in infant macaques. Panel A: The average percentages ± SEM of B cells in PBMCs of SPF (open symbols) or non-SPF (closed symbols) infant macaques from birth to 48 weeks of age. The corresponding changes in absolute numbers of B cells are shown in Panel B. Panels C and D illustrate the changes in $CD4^+$ and $CD8^+$T cells within the CD3 T cell population during this time. Panel E shows the decline in the CD4:CD8 T cell ratio of SPF infants from birth to 40 weeks. Each symbol represents an individual SPF infant macaque; horizontal bars indicate the median value in

each age group. In Panel F, the average percentages ± SEM of $Ki67^+CD4^+$ (open circles) or $Ki67^+CD8^+$ (open triangles) T cells are graphically represented.. Statistically significant differences between groups are shown by capped lines and star symbols (*p<0.05, **p <0.01,***p<0.001).

Table 2: Lymphocyte Populations

Cell Population	**Median Cell Numbers/ µl Blood** (*Minimum-Maximum No.*)				
	Infants	**Juveniles**	*Infants vs. Juveniles*	**Adults**	*Juveniles vs. Adults*
SPF	**n=23**	**n=20**		**n=8**	
All T cells ($CD3^+$)	2633 (*1373-4811*)	1257 (*336-1899*)	<0.0001	486 (*194-1760*)	p=0.0031
$CD4^+$ T ($CD3^+CD4^+$)	1891 (*1217-3598*)	828 (*459-1319*)	<0.0001	124 (*15-734*)	<0.0001
$CD8^+$ T ($CD3^+CD8^+$)	832 (*506-1843*)	397 (*209-1109*)	<0.0001	363 (*179-1026*)	NS
CD4:CD8 Ratio	2.3 (*1.2-3.6*)	2.0 (*0.7-3.9*)	NS	0.6 (*0.3-0.8*)	<0.0001
B cells ($CD20^+$)	1464 (*649-2716*)	474 (*136-808*)	<0.0001	224 (*97-736*)	p=0.0252
Non-SPF	**n=11**	**n=50**		**n=98**	
All T cells ($CD3^+$)	1657 (*854-3676*)	1155 (*311-3730*)	NS	745 (*103-2798*)	<0.0001
$CD4^+$ T ($CD3^+CD4^+$)	1169 (*592-2531*)	753 (*195-2202*)	NS	433 (*44-1606*)	<0.0001
$CD8^+$ T ($CD3^+CD8^+$)	488 (*262-1145*)	310 (*99-1401*)	NS	250 (*43-1276*)	0.0139
CD4:CD8 Ratio	2.2 (*1.9-4.0*)	2.4 (*1.4-4.0*)	NS	1.8 (*0.2-4.2*)	<0.0001
B cells ($CD20^+$)	824 (*246-3472*)	1445 (*269-4317*)	NS	366 (*69-1341*)	<0.0001
Comparison of SPF versus Non-SPF Populations					
All T cells ($CD3^+$)	NS	NS		NS	
$CD4^+$ T ($CD3^+CD4^+$)	NS	NS		p=0.0025 (SPF < non-SPF)	
$CD8^+$ T ($CD3^+CD8^+$)	NS	p=0.0247 (SPF > non-SPF)		NS	
CD4:CD8 Ratio	NS	p=0.0132 (SPF < non-SPF)		p=0.0132 (SPF < non-SPF)	
B cells ($CD20^+$)	NS	p<0.0001 (SPF < non-SPF)		NS	

The post-birth immune development was also marked by phenotypic changes in T cell maturation. As expected, in the newborn, the majority of $CD4^+$ and $CD8^+$ T cells expressed a naïve phenotype, defined as $CD3^+CD45RA^+CCR7^+$ or $CD3^+CD28^+CD95^-$ (Figure 3). Even in SPF infants, the low, but continuous postnatal exposure to environmental (air- water-, and food-borne antigens) and pathogen-derived stimuli since birth led to a steady increase in both central memory (T_{CM}) and effector/effector memory ($T_{E/EM)}$ T cells. Consistent with their primary function as helper $CD4^+$ T cells or effector $CD8^+$ T cells, respectively, T cell maturation and differentiation was more pronounced in $CD8^+$ T cell population. At 1 year of age, close to 90% of the circulating $CD4^+$ T cells still expressed a naïve T cell (T(N)) phenotype and less than 5% of all $CD4^+$ T cells represented effector $CD4^+$ T cells (Figure 3). In contrast, 15% of $CD8^+$ T cells had developed into effector or effector memory T cells. B cells matured similarly over the first year. There was a continuous decline in naïve ($CD19^+CD20^+CD27^-IgD^+$) B cells accompanied by a concurrent increase in unswitched ($CD27^+IgD^+IgM^+$), switched ($CD27^+IgD^-$) and in double negative (IgD^-CD27^-) B cells (Figure 4).

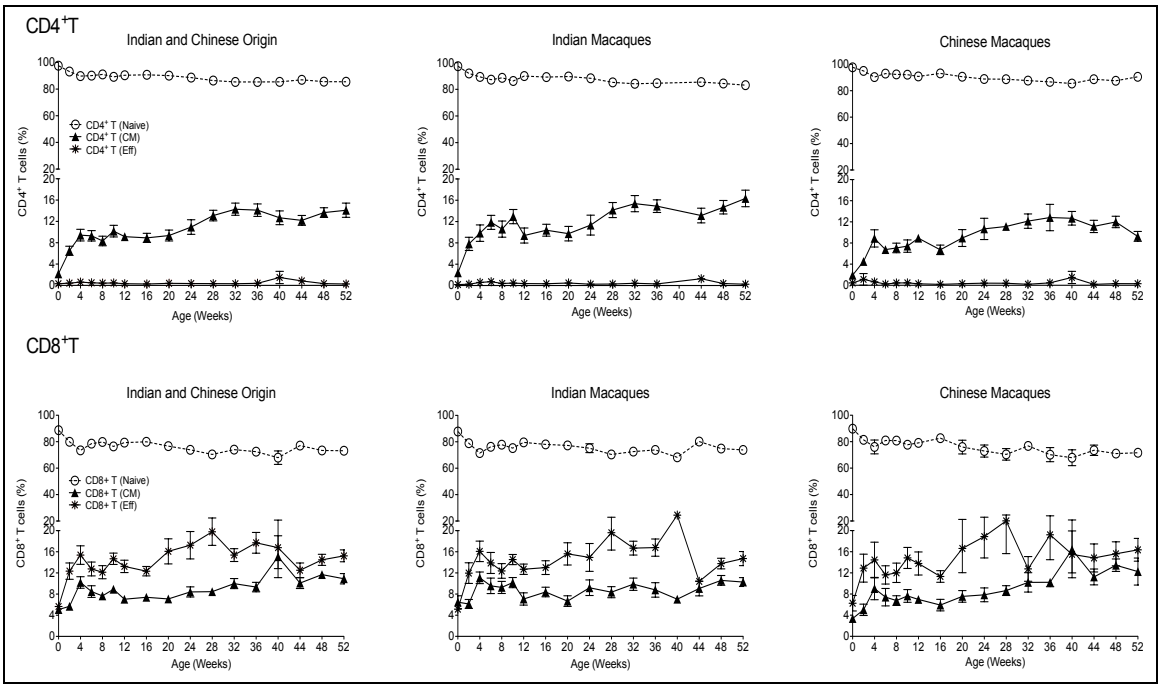

Figure 3: Memory T cell development in SPF infant macaques. The graphs show the longitudinal changes of naïve (open circles), central memory (stars), and effector/effector memory (closed triangles) in $CD4^+$ (top row) and $CD8^+$ T cells (bottom row) of infant SPF macaques of Indian (left panels, n=13) or Chinese (middle panels; n=10) origin. The combined average ± SEM data each for in $CD4^+$ or $CD8^+$ T cell subpopulation in Indian and Chinese infants are presented in the right-hand panels.

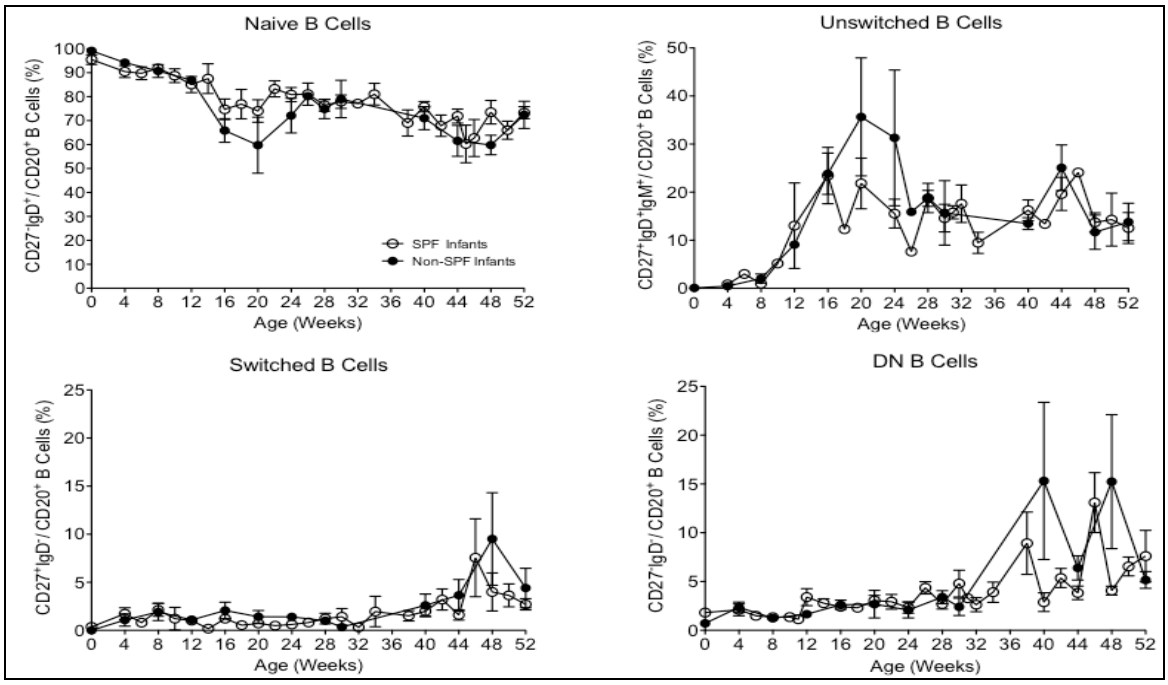

Figure 4: B cell differentiation in infant macaques. The normal developmental changes in peripheral blood B cell populations from birth to 1 year of age in SPF (open symbols) and non-SPF (closed circles) infant macaques. Shown are mean values ± SEM for naïve, unswitched, switched and double negative B cell populations based on phenotypic characterization using CD27 and IgD.

Non-SPF infants mirrored the developmental changes in the blood of SPF infants (Tables 1 and 2). Thus, in the absence of easily transmittable pathogens such as RhCMV, both SPF and non-SPF infant macaques peripheral blood cell populations matured in an analogous manner (Figures 1-4). The larger data variation in non-SPF infants was likely the result of the smaller group size because while we followed 11 non-SPF infant macaques until week 16, only 4 non-SPF macaques were analyzed from birth to one year. It should be noted that the non-SPF infants were not serologically characterized for infection status. However, the non-SPF infants were raised under identical conditions (i.e. separation from the dam at

birth and nursery reared away from infected cohorts) that the CNPRC has used to generate the SPF cohort. Therefore, it is presumed that the non-SPF infants were, in fact, SPF. We did not observe any differences in immune maturation between Indian or Chinese macaques (Figure 3 as a representative example and data not shown), and thus concluded that macaque origin did not affect early immune development of peripheral blood cells.

RhCMV immunity in juvenile and adult macaques

At one year of age, SPF and non-SPF infant macaques were transferred to separate SPF or non-SPF outdoor field cages, respectively. Thus, during the development from infants into adolescent and adult macaques, SPF and non-SPF macaques continued to be exposed to similar environmental pathogens. Because SPF macaques were bred to be free of RhCMV, juvenile or adult SPF animals could not transmit RhCMV to younger animals upon cohabitation. Continued regular monitoring confirmed that RhCMV remained undetectable in all animals of the SPF colony. In contrast, the transition of the young non-SPF macaques to a cohabitation with older non-SPF macaques prompted their exposure to pathogens endemic within the non-SPF colony, such as RhCMV.

We have previously documented that about >90% of conventional non-SPF macaques housed in outdoor corrals become infected with RhCMV between 5-12 months of age (13), (149), (161). Moreover, virological surveys revealed that the frequency and magnitude of viral shedding on naturally RhCMV infected animals were higher in younger compared to adult monkeys (Oxford and Barry, unpublished observations). This could explain why many adult monkeys in our study had undetectable virus in the saliva at a single time point. When we tested juvenile non-SPF macaques, we could detect RhCMV in saliva (Figure 5A). In fact,

juvenile non-SPF macaques shed RhCMV more frequently (78% vs. 28%), and at significantly higher titers (5.47×10^4 copies/ml; 95% CI: 6665 to 102833) than adult non-SPF macaques (2.31×10^3 copies/ml; 95% CI: 348 to 4265, respectively) (Figure 5A). Furthermore, all juvenile non-SPF macaques included in the current study had seroconverted and developed neutralizing antibodies to RhCMV glycoprotein B. The RhCMV gB–specific neutralizing antibody titers of the juvenile macaques were comparable to those of the adult non-SPF macaques (Figure 5B), suggesting that once maximum neutralizing antibody titers were reached, they persisted. This result, similar to HCMV in humans, is consistent with the lifelong persistence of RhCMV in the animals without causing disease, despite occasional virus shedding (13). Albeit neutralizing antibody titers were weakly associated with RhCMV shedding in juvenile macaques (Figure 5C), such a correlation was not observed in the infrequently shedding adult macaques (Figure 5D). Thus, we concluded that the magnitude of the neutralizing antibody response was not correlated to antigen load in the animals.

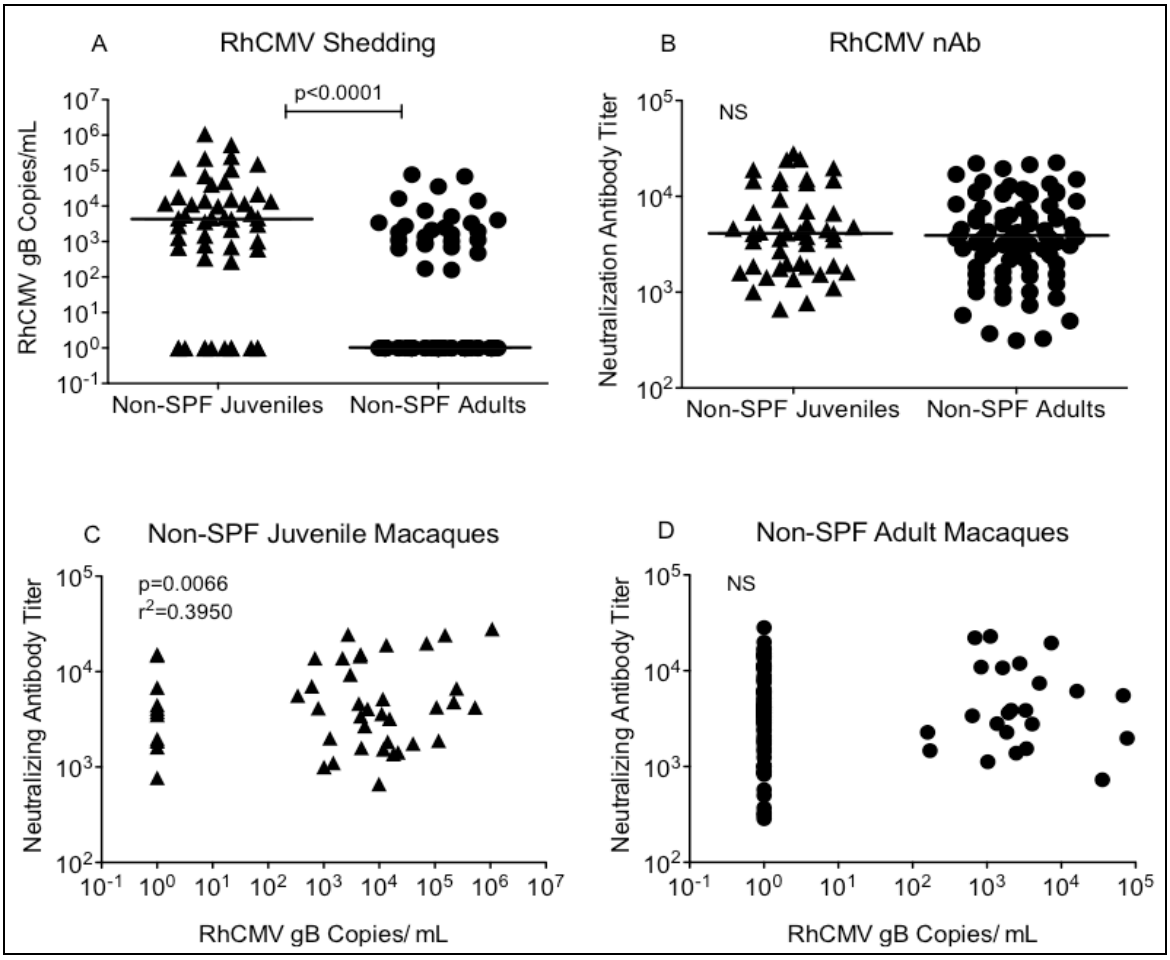

Figure 5: Viral and immune parameters of RhCMV infection. Panel A shows the quantity of RhCMV shed orally by juvenile (n=50, filled triangles) or adult (n= 98, filled hexagon) non-SPF macaques. Each symbol represents an individual animal; horizontal lines indicate the median of the age-group. In Panel B, neutralizing plasma antibody titers for RhCMV-infected juvenile and adult mcaques are shown. The legend is similar to Panel A. Neutralizing antibodies were correlated with oral RhCMV shedding in juvenile (Panel C) or adult (Panel D) macaques. Statistically significant differences are indicated by P values.

In contrast to neutralizing antibodies, RhCMV-specific T cell responses further increased with age. In response to *in vitro* stimulation of PBMCs with RhCMV lysate, juvenile non-SPF macaques produced only low levels of cytokines (Figure 6). Although not

every animal produced all cytokines, each juvenile animal produced at least one cytokine in response to RhCMV lysate stimulation. Consistent with the RhCMV-induced expansion of memory T cells with age, adult macaques produced significantly higher levels of certain cytokines, in particular the pro-inflammatory cytokines IFNγ, IL6 and IL1β, than juvenile non-SPF rhesus macaques (Figure 6). Median values of TNFα and RANTES were also higher in adults than in juveniles, but this difference did not reach statistical significance (data not shown). These results were consistent with the previously documented expansion of RhCMV-specific T cells in macaques and HCMV-specific T cells in humans (117), (152), (153).

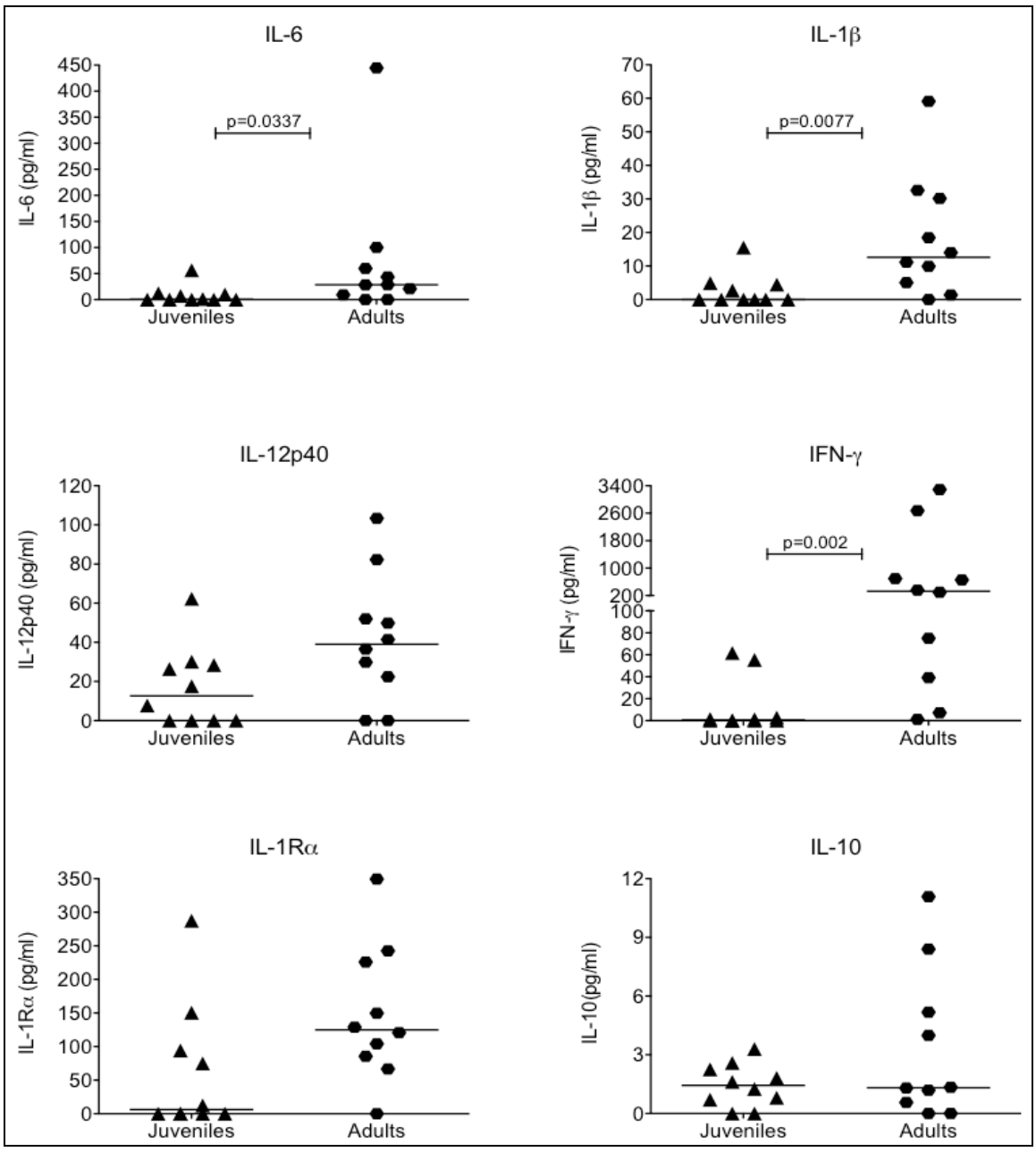

Figure 6: Cytokine responses to *in vitro* RhCMV stimulation. Culture supernatants collected after rhesus PBMCs from juvenile (n=10) or adult (n=10) non-SPF macaques were stimulated for 48 hours with RhCMV lysate and analyzed by multiplex analysis. Each graph represents the amount of a specific cytokine produced in response to RhCMV lysate stimulation expressed in pg/mL. Each symbol is representative of a single animal and horizontal bars indicate median cytokine levels for each age group. Statistically significant differences are indicated by P values.

SPF status modulates immune maturation in juvenile and adult macaques

Having confirmed that RhCMV infection was one distinguishing factor between SPF and non-SPF juvenile and adult macaques, we compared lymphocyte maturation and function between these two groups of macaques to determine whether chronic RhCMV infection was associated with modulated immune development or responsiveness.

Lymphocyte numbers in juvenile SPF macaques were significantly lower ($p<0.0001$) than in 1-year old infant macaques (Table 1). Both T and B cells were significantly decreased in SPF juvenile compared to SPF infant macaques. This trend was not observed in non-SPF macaques, with non-SPF juvenile macaques having significantly higher lymphocyte numbers (median: 3037/ml blood) than juvenile SPF macaques (median: 1949/ml blood; Table 1). This difference in absolute lymphocyte numbers appeared to be primarily the result of higher B cell numbers in non-SPF juvenile macaques, because T cell frequencies were comparable between SPF and non-SPF juvenile macaques (Table 2). Interestingly, juvenile SPF macaques had fewer differentiated B cells than non-SPF juvenile macaques (Figure 7). It is tempting to speculate that the higher frequencies of switched and double negative B cells in non-SPF juveniles could be related to the development of RhCMV-specific antibodies in these animals, but further studies would be required to confirm this hypothesis. Over time, these differences in peripheral blood B cell maturation between SPF and non-SPF macaques waned and adult SPF and non-SPF macaques showed similar percentages of naïve and differentiated B cells.

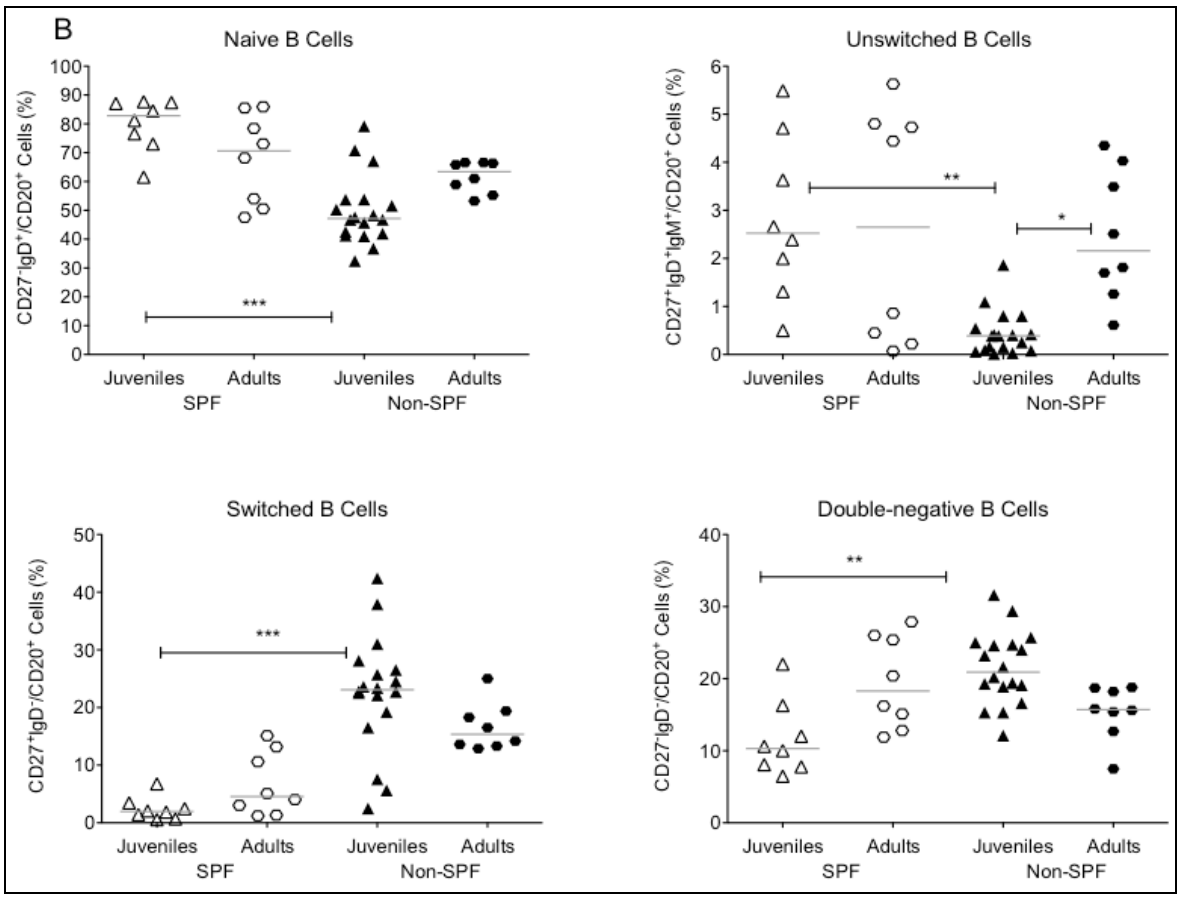

Figure 7: Memory B cell development in juvenile and adult macaques. B cell sub populations as described in Figure 4 were analyzed in SPF juvenile (n=8) and adult (n=8) macaques and compared to the same populations in blood of non-SPF juvenile (n=18) and adult (n=8) macaques. Open and closed symbols represent SPF and non-SPF macaques, respectively. Horizontal lines indicate median values per age groups. Statistically significant differences between groups are shown by capped lines and star symbols (*p<0.05, **p <0.01,***p<0.001).

Despite similar total T cell frequencies, differences between SPF and non-SPF macaques started to emerge when we examined T cell subpopulations in more detail. From infant to adult age, there was a significant decrease in $CD4^+$ T cell numbers of SPF and non-SPF macaques (Table 2). $CD8^+$ T cell frequencies seemed to reach steady state level in juvenile macaques. More importantly though, as the individual animals became exposed to a

greater number of various environmental and pathogenic stimuli with age, the maturation status of their T cells changed. Compared to infant macaques, juvenile and adult macaques had fewer numbers of naïve T cells and increasing numbers of more differentiated T cells (Figure 8). As expected, we observed a similar age-related decline in naïve $CD4^+$ ($CD45RA^+CD95^-$) and $CD8^+$ ($CD45RA^+CD28^+$) T cell populations of SPF and non-SPF macaques (Figure 8). However, naïve $CD4^+CD45RA^-CD95^-$ T cells were higher in SPF compared to non-SPF adult macaques, whereas the more differentiated effector-like ($CD45RA^{+/-}CD95^+$) $CD4^+$ T cells were higher in non-SPF adults (Figure 8). Differentiated $CD8^+$ T cell subsets did not differ between SPF and non-SPF adult macaques, but juvenile non-SPF macaques had more $CD45RA^-CD28^+$ $CD8^+$ T cells (Figure 8). Thus, in addition to age-related changes in B and T cell populations between SPF and non-SPF macaques we also observed differences in T and B cell phenotypes, suggesting that both age and chronic viral infection, i.e. RhCMV, influenced B cell maturation and the differentiation of $CD4^+$ and $CD8^+$ T cell subsets in peripheral blood.

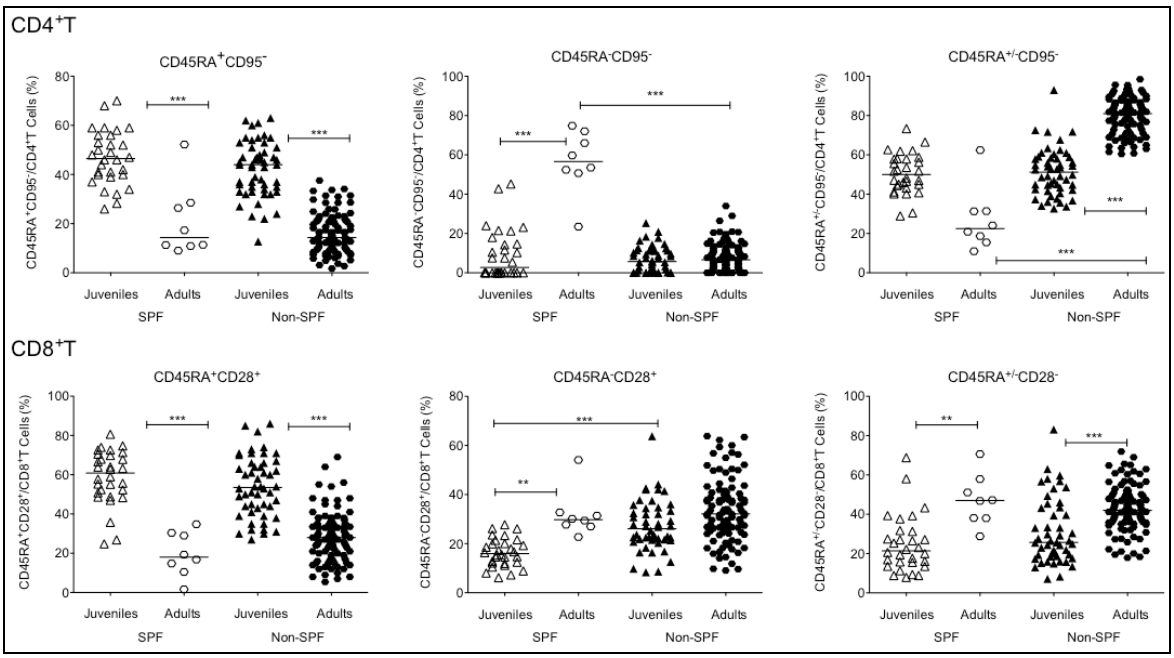

Figure 8: Comparative analysis of memory T cell populations in SPF and non-SPF juvenile and adult macaques. Percentages of naïve (left panels), and various differentiated (middle and right panels) $CD4^+$ (top row) and $CD8^+$ (bottom row) T cell populations in juvenile and adult SPF and non-SPF macaques. Each symbol represents an individual animal with horizontal bars indicating the median value for each group. Statistically significant differences between groups are shown by capped lines and star symbols (*p<0.05, **p <0.01,***p<0.001).

Assessment of T lymphocyte function in relation to age and RhCMV serostatus

One important question that evolved from these data was how age-associated T cell maturation was reflected in T cell function, and specifically, whether and how chronic RhCMV infection would affect the magnitude or quality of T cell responses. Table 3 summarizes the development of single cytokine responses in $CD4^+$ and $CD8^+$ T cells from birth to one year of age in SPF infant macaques. Infant $CD4^+$ T cells generally exhibit a Th2-biased response. Indeed, in the first 4 weeks of life, few $CD4^+$ T cells from SPF infant macaques produced IFNγ, the principle Th1 characterizing cytokine, in response to

polyclonal T cell stimulation. IFNγ-producing $CD4^+$ T cells gradually increased from <0.015 % in the first month to >0.1% of total $CD4^+$ T cells by 6 months of age (gray-gradient) (Table 3; p=0.002). This was consistent with the gradual increase in effector T cells, which were the primary cytokine-producing T cell population (data not shown). However, despite a similar increase of effector $CD8^+$ T cells from birth to one year, the majority of animals had equivalent $CD8^+$ T cell IFNγ responses ranging from 0.1 to 1% throughout the first year (Table 3, p=0.111). These data, combined with the fact that newborns were generally able to mount good IL2 and TNFα $CD4^+$ T cell responses (Table 3), were indicative of $CD4^+$ T cell-intrinsic factors that could interfere with IFNγ production in neonates. In fact, TNFα responses did not change significantly over time, and IL2-producing $CD4^+$ and $CD8^+$ T cells actually declined in the first year (p<0.001; Table 3).

Table 3: Cytokine Responses in infant T cells

Age (weeks)	Animal No.	Cytokine-producing $CD3^+CD4^+$T Cells (%)					Cytokine-producing $CD3^+CD8^+$T Cells (%)				
		<0.015	≥0.015<0.1	≥0.1<1.0	≥1.0<10.0	≥10.0	<0.015	≥0.015<0.1	≥0.1<1.0	≥1.0<10.0	≥10.0
IFN-γ											
0	13	**9**	**4**	0	0	0	2	3	8	0	0
2	21	**10**	**6**	4	1	0	4	5	9	3	0
4	20	5	**8**	**7**	0	0	5	0	8	7	0
6	16	2	**9**	**5**	0	0	0	1	9	6	0
8	22	3	**10**	**9**	0	0	0	1	16	5	0
10	23	7	**13**	**3**	0	0	0	5	17	1	0
12	22	4	**13**	**5**	0	0	2	4	13	3	0
16	21	4	**13**	**3**	1	0	2	1	16	2	0
20	22	4	**13**	**5**	0	0	0	6	14	2	0
24	22	1	**11**	**10**	0	0	1	2	19	0	0
28	22	3	**8**	**9**	0	0	0	2	14	6	0
32	21	3	5	**10**	**3**	0	1	2	14	4	0
36	20	1	5	**11**	**3**	0	0	2	13	5	0
40	17	0	2	**14**	**1**	0	0	1	12	4	0
44	19	1	3	**14**	**1**	0	1	0	14	4	0
48	18	3	5	**10**	**0**	0	0	2	15	1	0
IL-2											
0	13	0	0	2	4	7	1	0	1	7	4
2	21	1	3	1	11	5	2	2	2	11	4
4	20	2	3	0	6	9	4	1	2	9	4
6	16	0	0	1	6	9	0	0	0	12	4
8	22	0	0	1	8	13	0	0	2	15	5
10	23	0	0	5	13	5	0	0	3	16	4
12	22	1	2	4	9	6	1	0	4	16	1
16	21	1	2	4	10	4	1	1	0	15	4
20	22	1	1	5	9	5	1	1	4	16	0
24	22	2	2	7	11	0	2	1	10	9	0
28	22	2	4	1	13	2	3	4	4	9	0
32	21	1	3	4	10	3	4	4	5	3	1
36	20	3	2	6	9	0	3	7	6	3	0
40	17	8	1	4	4	0	4	4	5	3	1
44	19	3	6	5	5	0	3	7	6	3	0
48	18	12	2	1	3	0	10	4	1	3	0
TNF-α											
0	13	1	0	3	4	5	0	1	3	6	3
2	21	4	2	8	7	0	2	1	7	11	0
4	20	4	3	7	6	0	4	1	5	10	0
6	16	0	0	7	9	0	0	0	7	9	0
8	22	0	1	5	15	1	0	0	9	12	1
10	23	0	0	3	13	7	0	0	2	19	2
12	22	0	1	2	14	5	0	0	3	16	3
16	21	0	0	3	13	5	0	0	2	16	3
20	22	1	1	3	11	6	1	1	3	15	3
24	22	2	0	2	16	2	2	0	2	17	1
28	22	1	1	7	9	4	1	1	8	11	1
32	21	1	2	10	7	1	1	0	10	10	0
36	20	5	0	10	4	1	4	1	8	7	0
40	17	2	1	1	10	3	3	0	2	12	0
44	19	0	0	0	12	7	0	0	3	16	0
48	18	0	0	1	10	6	0	0	5	12	1

To measure physiologically more relevant responses, we stimulated SPF infant PBMC with anti-CD3 and anti-CD28 antibodies to activate T cells directly through the T cell receptor (TCR). Supernatants were collected at 24 hours and analyzed for cytokines important in T cell activation and $CD4^+$ T helper cell lineage differentiation. This experiment included 8 SPF infants that ranged from 3 to 9 weeks in age and 8 additional 10 to 16 week-

old SPF infants. As we could not detect any statistically significant differences between these two groups (data not shown), we combined the data from both infant groups (Total n=16) and compared them to cytokine levels induced by TCR stimulation in SPF juvenile and SPF adult macaques. There was an age-dependent increase in cytokine responses to TCR stimulation. Infants had significantly lower IFNγ, IL17A, IL10, and IL6 responses than SPF juvenile macaques (Figure 9). Although there was also a trend towards higher IL2 and TNFα levels in juvenile macaques, these differences did not reach statistical significance. Median cytokine responses further increased when SPF juvenile macaques were compared to SPF adult macaques. In addition to these quantitative changes, the quality of the response differed between age groups. Consistent with a Th2-biased response of infants, the ratio of IFNγ:IL4 was lowest in infant and highest in adult SPF macaques (Figure 10). An impairment of Th responses was further supported by the fact that the IFNγ:IL10 was significantly lower in infant compared to adult SPF macaques.

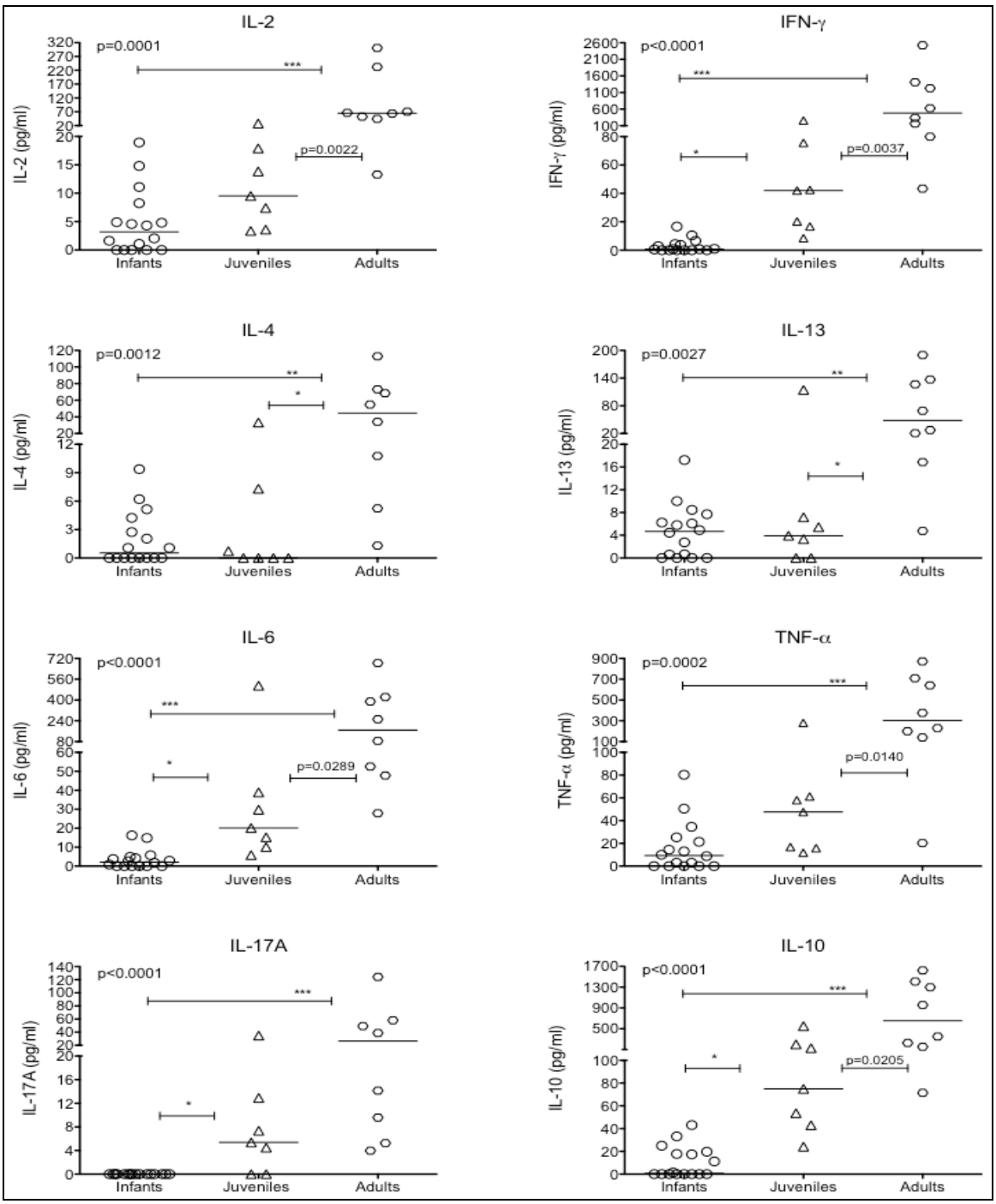

Figure 9: TCR-induced cytokine responses in PBMCs of SPF macaques of different age groups. The various graphs show the production of specific cytokines by PBMCs of infant, juvenile and adult SPF macaques after 24 hours of stimulation with anti-CD3 and anti-CD28 antibodies. Data are expressed in pg per milliliter (pg/ml) of culture supernatant. Cytokine levels between the three distinct age groups were compared using the Kruskal-Wallis test and

P values are indicated in the upper left hand corner of each graph. Statistical significant differences between two groups by subsequent Dunn's comparison are shown with capped lines and star symbols (*p<0.05, ** p<0.01,*** p<0.001).

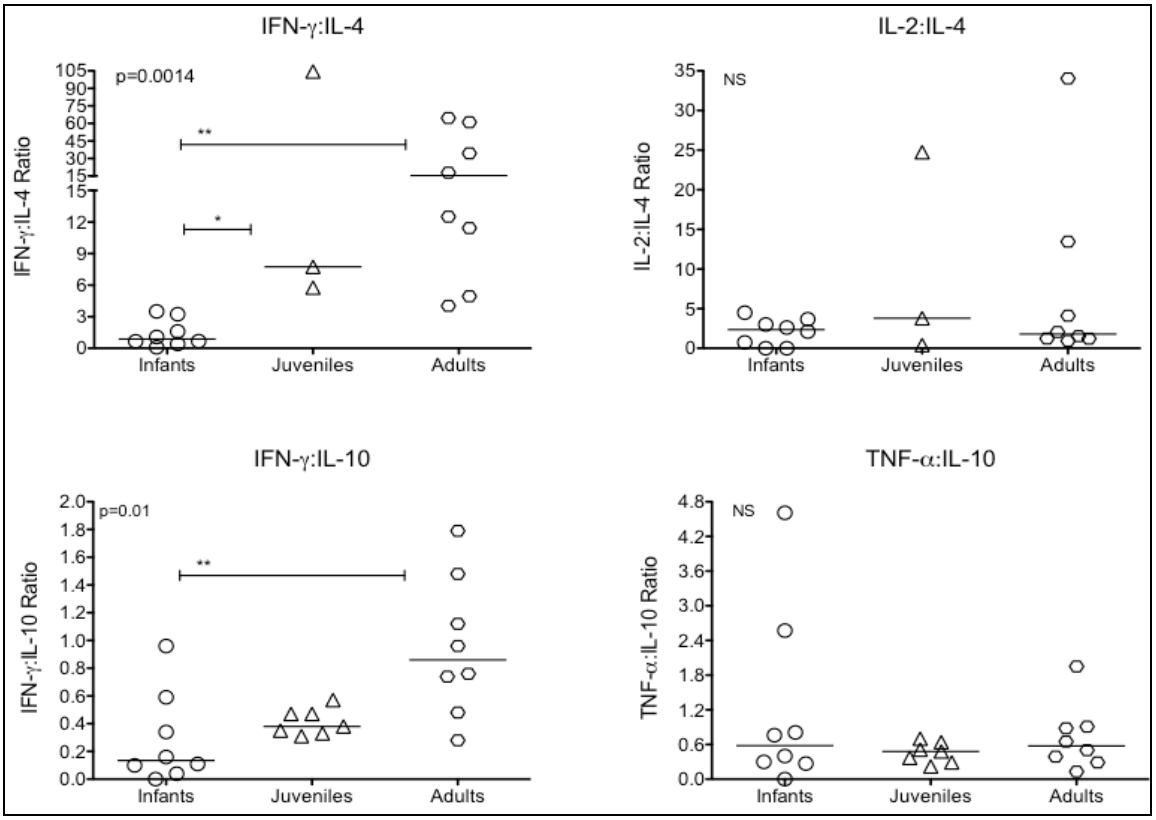

Figure 10: Changing cytokine ratios in response to TCR stimulation with age. Consistent with lower IFNγ responses in infants, the raio of IFNγ:IL4 or IFNγ:IL10 increase with age (left top and bottom graphs, respectively), whereas no age-related differences were observed in the IL2:IL4 or the TNFα:IL10 ratios (right top and bottom graphs, respectively). Statistical significant differences between two groups by subsequent Dunn's comparison are shown with capped lines and star symbols (*p<0.05, ** p<0.01,*** p<0.001).

To assess the potential impact of RhCMV infection on T cell function, we compared cytokine responses between SPF and non-SPF juvenile macaques after Con A stimulation. Most pro-inflammatory cytokines, including IL6, TNFα (Figure 11A), IL1β, and TNFβ (data

not shown) were expressed at higher levels in non-SPF than in SPF juvenile macaques. Although SPF juveniles produced significantly more IL12 and had a higher IL12:IL10 ratio than non-SPF juveniles (Figure 11B), the SPF juveniles showed lower IFNγ responses (Figure 11A). The significantly lower ratio of IFNγ:IL4 in SPF compared to non-SPF juvenile macaques was indicative of a more Th2-dominant response in the SPF animals, whereas T cell responses in non-SPF juvenile macaque seemed to be more biased towards inflammatory responses (Figure 11). Adult non-SPF macaques showed similar responses to juvenile non-SPF macaques. Although the adult non-SPF macaques produced significantly higher levels of some anti-inflammatory cytokines (e.g. IL10, IL1Rα) in response to Con A stimulation (Table 4), they also produced more TNFα and the Th1-associated chemokine RANTES (Table 4). These data were reminiscent of the increased induction of some inflammatory cytokine in RhCMV-stimulated PBMCs of adult compared to juvenile non-SPF macaques (Figure 6). The persistence of activated T cells into adulthood was further supported by the fact that adult $CD4^+$ T cells contained similar frequencies of CCR5 and CD25 positive $CD4^+$ T cells compared to juvenile non-SPF macaques (Figure 12). Frequencies of CXCR3-positive $CD4^+$ T cells were even higher in adult macaques, consistent with their further expansion of memory $CD4^+$ T cells (Figure 12).

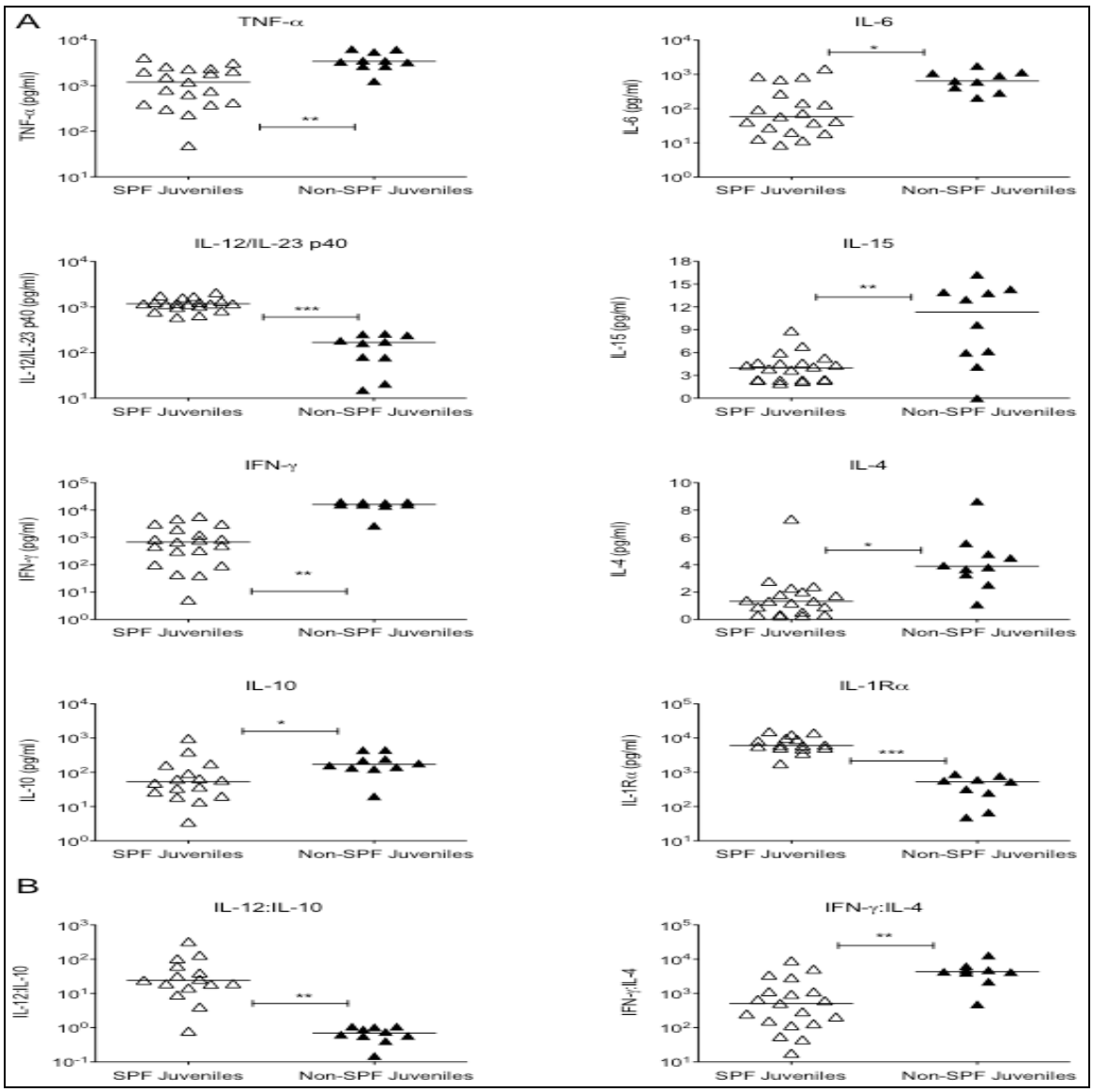

Figure 11: Comparative cytokine responses of juvenile SPF and non-SPF macaques to Con A stimulation. Panel A. The individual graphs show the production of specific cytokines in PBMC of SPF or non-SPF juvenile macaques after *in vitro* stimulation with Con A. Cytokines were measured in culture supernatants at 48 hours. Panel B shows how the cytokine ratios of IL12:IL10 and IFNγ:IL4 differ between juvenile SPF and non-SPF macaques. Each symbol represents an indivudal animal; horizontal lines indicate the median of the age-group. Capped lines with P values indicate statistically significant differences between the two groups determined by Mann-Whitney test (*p<0.05, **p <0.01,***p<0.001).

Table 4. Cytokine Responses To *In Vitro* Con A Stimulation

Cytokine	**Median Cytokine Levels in pg/ ml** (Minimum-Maximum)				
	SPF Juveniles	**Non-SPF Juveniles**	*SPF vs. Non-SPF Juveniles*	**Non-SPF Adults**	*Non-SPF Juveniles vs. Adults*
Inflammatory					
IL-6	58.0 (8.6-1451)	647.7 (207.6-1778)	p<0.05	1267.0 (268.2-2253)	NS
TNF-α	1198.0 (48.2-4103)	3436.0 (1260.0-6368.0)	p<0.001	6181.0 (2246-9517)	p<0.05
IL-1β	23.4 (0.3-71.4)	113.7 (25.5-259.0)	p<0.001	226.3 (80.9-795.9)	NS
TNF-β	334.7 (31.5-1089.0)	1185.0 (104.6-2307.0)	p<0.001	753.2 (232.3-1465.0)	NS
IL-12p40	1174.0 (592.9-2068.0)	167.3 (15.1-260.8)	p<0.0001	215.8 (96.2-606.6)	NS
IL-15	4.0 (1.9-8.9)	11.3 (0-16.2)	p<0.001	11.1 (7.6-15.6)	NS
CXCL-10	33.0 (16.2-45.0)	13.1 (3.0-23.4)	p<0.0001	10.6 (0.1-22.0)	NS
Anti-Inflammatory					
IL-10	53.5 (3.5-974.9)	171.2 (20.0-453.0)	p=0.0251	489.7 (72.3-978.1)	p=0.0115
IL-1Rα	6073.0 (1775-15352)	529.7 (48.2-887.4)	p<0.0001	937.8 (266.6-3574.0)	p=0.0279
Th differentiation					
IL-2	2451.0 (19.4-21229.0)	4892.0 (40.6-7656.0)	NS	3511.0 (814.1-23975)	NS
IFN-γ	681.0 (5.2-5832.0)	16058.0 (2704-20164)	p<0.001	19656 (14315-20941)	NS
IL-4	1.3 (0.3-7.3)	3.9 (1.1-8.6)	p<0.05	9.3 (4.2-21.7)	p=0.0015
IL-9	259.2 (126.2-512.0)	20.5 (9.8-47.9)	p<0.0001	38.2 (24.2-103.3)	p=0.0089
Chemokines					
RANTES	11017.0 (800.3-37070)	970.5 (151.8-2041.0)	p<0.0001	1818.0 (930.2-3434.0)	p=0.0089
Eotaxin	13.3 (4.1-24.1)	21.3 (8.0-31.2)	p=0.0301	21.7 (1.5-25.5)	NS
Cytokine Ratios					
IL-12:IL-10	24.1 (0.8-326)	0.7 (0.2-1.1)	p<0.001	0.3 (0.2-0.8)	NS
IFN-γ:IL-4	508.2 (17.8-8997.0)	4329.0 (484.6-12917)	p<0.001	2032.0 (886.0-4871.0)	p=0.0350
IL-1β:IL-1Rα	0.004 (0.0-0.0096)	0.36 (0.04-3.69)	p<0.0001	0.27 (0.06-0.88)	NS
RANTES:Eotaxin	611.3 (64.1-8074.0)	41.0 (16.7-158.1)	p<0.0001	79.8 (52.9-1353.0)	p=0.0435

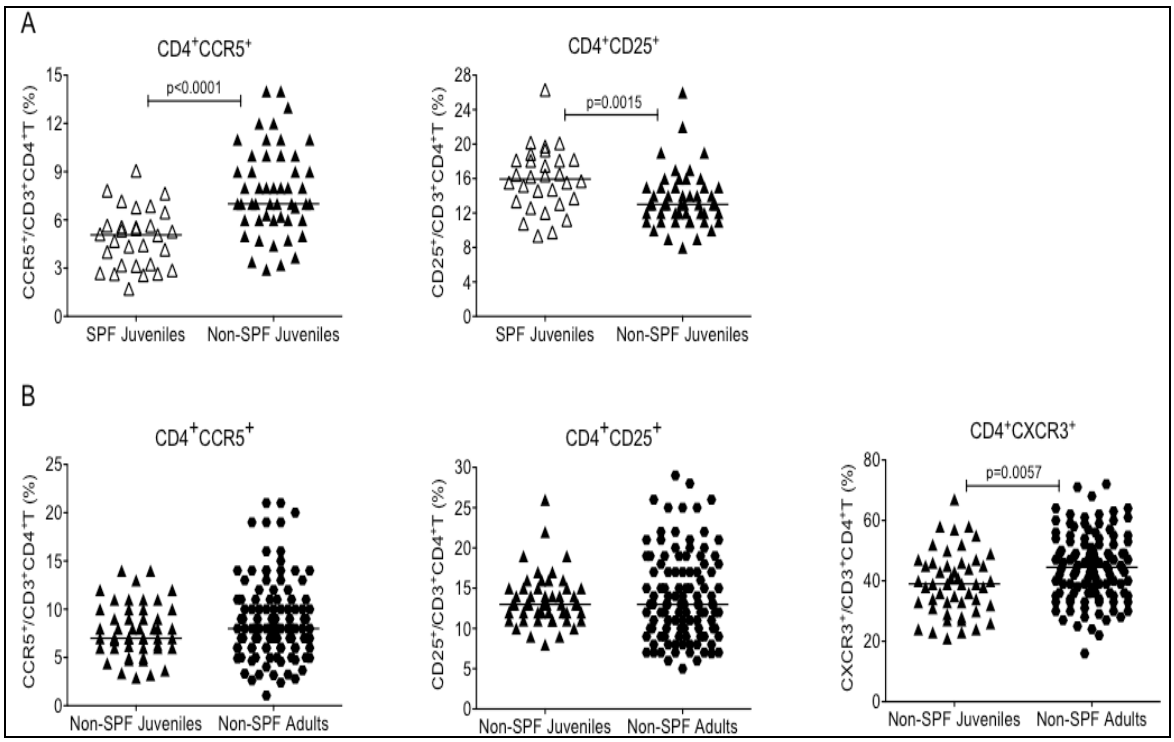

Figure 12: CD4$^+$ T cell activation in relation to SPF status and age. Data in Panel A demonstrate that CD4$^+$ T cell activation as measured by the percentage of CCR5-positive CD4$^+$ T cells is higher in non-SPF compared to SPF juvenile macaques. In contrast, frequencies of CD25-positive CD4$^+$ T cells are higher in SPF juveniles, likely because CD25 is not only a T cell activation marker, but also expressed on regulatory T cells. In Panel B , frequencies of CCR5- and CD25-positive CD4$^+$ T cells do not differ between non-SPF juvenile and adult mcaques. However, adult non-SPF mcaques have higher frequencies of CXCR3-positive CD4$^+$ T cells in their blood. Each symbol represents an individual animal, horizontal bars represent the median value per group. Statistical significant differences are indicated by P values.

DISCUSSION

First and foremost, the goal of the study was to provide evidence that the available SPF and non-SPF colonies of rhesus macaques can serve as a tool to study the impact of chronic infections such as HCMV on the immune system. Particularly, we aimed to show that chronic RhCMV infection could modulate immune responses throughout the lifespan of

the host. Our approach was threefold. We generated normative data for neonatal and infant immune development of peripheral blood cell populations of SPF and non-SPF macaques from birth throughout the first year of life. Under the controlled housing conditions we applied, namely limited pathogen exposure and identical diet, SPF status and origin of the infant did not influence neonatal and infant immune development. Indirectly, these results imply that SPF breeding of rhesus macaques is not associated with major inherent changes in their genetic immune repertoire. This is important as rhesus macaques are widely used as animal model for human diseases. We would anticipate though that infants raised outdoors and infants that are breastfed by their mothers would differ in their immune development from the nursery SPF and non-SPF infants included in the current study due to a different microbial flora at mucosal sites and an earlier and broader pathogen exposure (110, 121), (135). In fact, our comparison of juvenile SPF and non-SPF macaques demonstrates that the infection with RhCMV could modulate host immune responses. Finally, our data show that changes introduced in certain immune parameters by RhCMV of juvenile macaques will persist in the chronically infected animal.

The analysis of blood cell populations from birth to 1 year shows that the infant immune system undergoes tremendous changes after birth. Cell populations changed in frequencies and in their relative abundance to each other. Our results in infant macaques are remarkably similar to normal developmental changes observed in human infants (142), (38). Generally, homeostatic levels were reached by about 3 months of age. Functional maturity of the various cell populations, however, required more time. Here, we specifically focused on T cell immunity. Although neutralizing antibodies are likely essential for the prevention of HCMV or RhCMV infection, it is well established that $CD4^+$ and $CD8^+$ T cell responses are

critical in controlling HCMV infection in humans and RhCMV infection in rhesus macaques (132), (117), (152), (145, 147) Consistent with previous reports, we observed minimal IFNγ production by infant $CD4^+$ T cells in the first few months of life (143). There was a gradual significant increase in the ability to produce IFNγ, a cytokine important for $CD4^+$ Th1 and $CD8^+$ T cell antiviral responses, in the first year of life. Interestingly though, inflammatory cytokines like TNFα could be produced evenly well by infant $CD4^+$ and $CD8^+$ T cells at birth and at 1 year. Overall, infant T cells responded with lower cytokine production to unspecific as well as TCR-specific stimulation compared to juvenile and adult macaques, independent of their SPF status. Higher cytokine responses with age were consistent with increasing frequencies of differentiated effector and memory T cells. As expected, these normal developmental changes were observed in both SPF and non-SPF, RhCMV infected animals. Interestingly though, the acquisition of RhCMV in juvenile non-SPF macaques appeared to be associated with some deviation in immune parameters between SPF and non-SPF macaques. Juvenile non-SPF macaques had higher frequencies of differentiated B cell subsets compared to SPF juveniles. Although, our studies were not designed to determine a causative relationship, one could speculate that the induction of RhCMV-specific antibodies was one factor driving the development of more mature B cells in non-SPF macaques. As has been reported previously, chronic RhCMV infection is associated with memory T cell expansion. Indeed, our data confirm that the total effector $CD4^+$ T cell population is significantly higher in adult SPF than in adult non-SPF macaques. While we did not determine the actual frequencies of RhCMV-specific memory T cells, we assessed the functional response of RhCMV-specific T cells. Consistent with memory T cell inflation, we observed higher cytokine responses in adult compared to juvenile non-SPF macaques.

Unspecific T cell stimulation also resulted in higher production of pro-inflammatory cytokines in adult compared to juvenile macaques, but the ratio of pro-to anti-inflammatory cytokines decreased slightly with age.

Altered T cell responsiveness with aging was not restricted to RhCMV-specific T cell responses. In comparison to SPF macaques, the response of non-SPF (RhCMV-infected) macaques to polyclonal T cell stimulation showed a bias towards higher inflammatory cytokine induction. This bias was most pronounced when juvenile SPF and non-SPF animals were compared. We hypothesize that the active replication of RhCMV in juvenile macaques, as evident in their more frequent shedding patterns of RhCMV in oral fluids, contributed to the more inflammatory cytokine profile of non-SPF compared to SPF juvenile macaques. Furthermore, these qualitatively distinct responses in juvenile macaques were indicative of early predisposition of juvenile RhCMV-infected animals to "inflammaging" in adult macaques, and supported our hypothesis that chronic RhCMV infection could broadly modulate host immune responses. To define the underlying mechanisms leading to inflammaging, future longitudinal and comparative studies between animals of SPF and non-SPF colonies are needed. Such studies could address questions examining (i) whether RhCMV infection leads to epigenetic changes in T or B cell populations, (ii) whether immune modulation would be reversible if recognized earlier in life, (iii) which processes regulate and control inflammaging, (iv) how normal aging or immunosenescence differ from inflammaging, and (v) whether genetic factors contribute to inflammaging in chronically infected humans. Further, these two cohorts of macaques can be used to assess vaccine and adjuvant responsiveness in relation to both aging and persistent pathogen loads. We now have large cohorts of SPF and non-SPF macaques available to perform a thorough analysis of

immune modulation in relation to normal aging in the context of viral infection in blood and relevant tissues.

In summary, our results confirm that RhCMV infection in macaques is highly similar to HCMV infection in humans and that the development of the major immune cell populations in blood occurs analogous to humans. The results of the current study support the further exploration of rhesus macaques as a model system to study the impact of chronic viral infections, specifically RCMV infection, on the modulation of host immune responses on otherwise, healthy monkeys and during aging using the now available SPF and non-SPF rhesus macaque colonies. RhCMV, similar to humans, is naturally acquired during early childhood and persists in rhesus macaques throughout their lifespan (13), (85), (112) (114), (161). Previously, we demonstrated that RhCMV-specific binding and neutralizing antibodies develop during primary infection (163). Our current data show juvenile and adult non-SPF macaques had comparable titers of RhCMV-specific neutralizing plasma antibody titers. These data suggest that once elicited, neutralizing antibodies to RhCMV persist at high levels. Furthermore, we concluded that the magnitude of the neutralizing antibody response was not dependent on antigenic load because we observed significantly higher and more frequent viral shedding in juvenile compared to adult RhCMV-infected macaques. In contrast, there was an age-dependent increase in RhCMV-specific T cell responses, a result consistent with the previously reported memory inflation in HCMV and RhCMV infection (71, 112, 113).

CHAPTER 3[1]. USE OF SPECIFIC-PATHOGEN-FREE (SPF) RHESUS MACAQUES TO BETTER MODEL ORAL PEDIATRIC CYTOMEGALOVIRUS INFECTION

OVERVIEW

The immune system of infants undergoes many developmental changes after birth and these changes may affect their ability to respond to viral pathogens like human cytomegalovirus (HCMV). A member of the β-herpes family of viruses, HCMV establishes a lifelong infection in an immunocompetent host without clinical symptoms. Interestingly, HCMV-infected children shed virus longer and in higher titers than adults. The limited functional capacity of the infant immune system contributes to infants' reduced ability to control viral replication compared to adults. As predicted, infant rhesus macaques were rapidly infected via the oral route when compared to adults. Moreover, infant T cell responses were of lower magnitude compared to adult macaques. Lastly, viral shedding was observed more frequently in the infants' bodily fluids compared to their adult counterparts. The utility of this nonhuman primate model of infant RhCMV will improve our understanding of the temporal development and differences of the infant immune system's response to viral pathogens and potentially provide insights into the design of effective vaccines tailored to infants early in life.

[1] This chapter previously appeared as a Short Paper article in the Journal of Medical Primatology. The original citation is as follows: dela Pena MG, Strelow L, Barry PA, and Abel K. Use of Specific-Pathogen-Free (SPF) Rhesus Macaques to Better Model Oral Pediatric Cytomegalovirus Infection. J Med Primatol, 2012 June; 41(3):225-9.

INTRODUCTION

Worldwide, human cytomegalovirus (HCMV) infection is the most common congenital infection, affecting ≈0.7% of all fetuses. Congenitally infected infants can suffer lifelong neurological sequelae and clinically healthy babies at birth can develop neurological complications in the first years of life (50), (66), (147). In the United States (US), 0.5-2% of all infants acquire HCMV in utero. Seronegative young infants can acquire HCMV through BM or in day care settings, e.g. through HCMV-contaminated saliva on toys (134), (90), (101). Similar to HCMV-infected adults, infants generally do not develop clinical symptoms upon HCMV acquisition, but, in contrast to adults, shed virus for prolonged periods of time (101). As their infection goes largely unnoticed, HCMV-infected children can transmit the virus to seronegative pregnant women who either have or do not have preconceptional immunity to HCMV. It is now well established that HCMV can re-infect HCMV-immune women. In the absence of an HCMV vaccine, interventions aimed at stopping or reducing HCMV shedding in infants could provide an effective means of preventing congenital HCMV infection.

The host factors responsible for prolonged viral shedding in infants are only poorly understood. To overcome sample limitations from young children, we sought to develop an infant rhesus CMV infection model because RhCMV infection in adult macaques is highly similar to human HCMV infection (15), (85). Previously, a direct comparison of RhCMV-specific immune responses between infant and adult macaques in correlation to virological outcome was not possible, because RhCMV is ubiquitous in macaque colonies and >90% of animals have seroconverted by 6 months of age. The generation of Specific Pathogen Free

(SPF) macaque colonies in which animals are bred to be free of multiple viruses, including RhCMV, now enables controlled RhCMV pathogenesis studies in different age groups (14).

MATERIALS AND METHODS

Animals and Rhesus Cytomegalovirus (RhCMV) Infection

Infant and adult rhesus macaques (*Macaca mulatta*) were obtained from the specific pathogen-free (SPF) colony at the California National Primate Center (CNPRC). These animals were seronegative for the Rhesus Cytomegalovirus (RhCMV) prior to study entry, and also seronegative for simian herpes B virus, simian immunodeficiency virus (SIV), simian retrovirus (SRV), simian T-cell lymphotropic virus type 1 (STLV1), simian foamy virus (SFV) and rhesus rhadino virus (RRV). The infant macaques were nursery reared and adult animals were housed indoors for the duration of the study. All animal procedures were approved by the University of California at Davis Institutional Animal Use and Care Committee (IACUC) and in accordance with the guidelines provided by the American Association for Accreditation of Laboratory Animal Care Standards.

RhCMV infection was performed via oral administration of a natural RhCMV isolate with a blunted syringe at 1x 10^6 PFU per ml, a dose based on successful infection of adult monkeys (unpublished data, Dr. Peter Barry). The oral inoculation route was chosen to mimic mother-to-child transmission of HCMV in humans by breast milk (BM). Infant macaques (n=5) were inoculated at 1 month after birth. Adult macaques (n=3) were between 4 and 5 years of age at the time of RhCMV exposure. The animals were followed for 1 year and then released back into the non-SPF colony where RhCMV infection is ubiquitous.

Sample collection and processing

Animals were anesthetized with 10mg/ml ketamine-HCl prior to all sample collections. EDTA blood samples were collected longitudinally every 2 weeks for 12 weeks and then once a month for 1 year. Plasma was harvested by centrifugation and stored at -80°C for antibody measurement and virus quantitation. Saliva was harvested by placing 2 sterile Weck-Cel spear sponges soaked with PBS (Beaver Visitec International, Waltham, MA) between the gums and cheeks on each side. Saliva supernatant was separated by centrifugation and stored immediately at -80°C. Urine was collected via cystocentesis, the total volume was recorded and immediately stored -80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via density centrifugation using Lymphocyte Separation Medium (LSM) (MP Biomedicals, Solon, OH) as described (91).

RhCMV-specific T cell response

To measure RhCMV-specific T cell responses, PBMCs were resuspended with RPMI 1640 media (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated FBS (Cellgro, Manassas, VA) and L-glutamine-penicillin-streptomycin antibiotic cocktail (Sigma, St Louis MO) at 1.0 $x10^6$ cells/ml. RhCMV antigens pp65 (lower matrix phosphoprotein), IE1 (immediate early antigen), and whole virus lysate were used at concentrations of 1.0µg/ml, 5.0µg/ml and 5.0µg/ml, respectively. Negative control received no stimuli (media only). PBMC stimulated with phorbol 12-myristate 12-acetate (PMA) (Sigma) at 10ng/ml and Ionomycin (I) (Sigma) at 125ng/ml served as a positive control. The cell cultures were incubated for 6 hours at 37°C, 5% CO_2 with Brefeldin A at 3.0µg/ml being added after the first hour (eBioscience, San Diego, CA). At the end of the culture period, cells were stained

for surface antibodies, fixed, and permeabilized for intracellular cytokine staining as described previously (91).

Flow cytometric analysis

The following antibody panels were used to determine T cell activation: CD3 (SP34-2)-Pacific Blue CD4 (L200)-PerCPCy5.5, CD8 (RPA-T8)-PeCy7 and Ki67 (B56)-FITC (B56), T cell differentiation: CD3, CD4, CD8, CD45RA (5H9)-APC, CCR7 (3D12)-PeCy7, or T cell cytokine production: CD3, CD4, IL2 (MQ1-17H12)-APC, IFNγ (B27)-Alexa700 (B27), TNFα (MAb11)-PeCy7 (eBiosciences), and CD107a/b (H4A3/H4B4)-FITC. Antibodies were purchased from Beckton-Dickinson (BD) unless indicated otherwise. Flow cytometric samples were acquired on the LSRII flow cytometer (BD Biosciences, San Jose, CA). A minimum of 300,000 events were acquired for antigen-specific responses and 30,000 events for T cell activation. FlowJo Software was used to analyze the data (Tree Star, Ashland, Oregon). The ability of the $CD4^+$ and $CD8^+$ T cells to produce cytokines were evaluated using the following logical gating strategy: PBMC gate (FSC-A vs. SSC-A), Singlets (FSC-H vs. FSC-A), T cells ($CD3^+CD4^+$, $CD3^+CD4^-$ = $CD8^+$ T), followed by gating for IFNγ, IL2 and/or TNFα (within $CD4^+$ or $CD8^+$ T cells. Boolean gating was applied to assess the frequency of multifunctional T cells. Antigen-specific $CD4^+$ or $CD8^+$ T cell responses were considered positive if their frequencies were at least 2 times higher than in unstimulated PBMC and if their actual value was ≥0.015%.

RhCMV antibody measurement

RhCMV-specific binding antibodies were determined by ELISA as previously described (85), (163). Briefly, 96-well Immulon 4HBX plates (Fisher-Thermo) were coated

overnight at 4°C with whole RhCMV lysate (25μg/ml). Plasma samples were diluted at 1:100. Anti-Rhesus IgG peroxidase-conjugated antibody was used to detect anti-RhCMV IgG (KPL, Gaithersburg, Md.). The specificity and reproducibility of the ELISA was confirmed using animal sera that were previously confirmed to be RhCMV positive and a plasma pool of seronegative animals. The limit of detection (LOD) was based on the average OD values plus 0.1 OD obtained for the plasma pool of RhCMV negative animals at the lowest dilution of 1:50. In addition, plasma samples were serially diluted after the initial rise in antibodies during the acute infection (week 10) and once during chronic infection when RhCMV-specific antibodies had stabilized. Antibody titers are reported as the lowest plasma dilution at which OD values were above the OD value for the RhCMV-negative plasma pool.

RhCMV neutralizing antibodies (nAb) were determined as described previously (85), (2), (1). The following time points were used to cover the induction, rise and persistence of nAb: weeks 0, 6, 10, 16 and 40. Plasma from seronegative animals served as negative control. Data are reported as the reciprocal of the dilution that resulted in 50% neutralization (NT_{50}).

RhCMV quantitation

RhCMV was quantitated in blood and bodily fluids as previously described (59, 131). DNA was extracted from plasma and saliva using the DNEasy Blood and Tissue Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). DNA from urine was extracted using the Norgen DNA Urine Isolation Kit (Norgen Biotech, Canada). Subsequently, real-time PCR for RhCMV gB was performed using the following primer and probe pair: forward primer: 5'-TGC GTA CTA TGG AAG AGA CAA TGC-3', reverse primer: 5'-ACA TCT GGC CGT TCA AAA AAA C-3' (Invitrogen, Carlsbad, CA), and

probe (5'-3') TET-CCA GAA GTT GCG CAT CCG CTT GT-TAMRA (Applied Biosystems, Carlsbad, CA). Each PCR reaction was ran in duplicate. The same amount of input DNA was used for RhCMV amplification from PBMC samples (100ng). However, due to the low DNA yield in some bodily fluids, the input DNA amount varied between 40 to 100 ng. RhCMV was quantitated based on a RhCMV gB plasmid standard curve spanning 10^0 to 10^6 copies. The viral load was calculated as RhCMV gB copies per ml for bodily fluids. The limit of detection was 10 copies of RhCMV gB.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Data between infant and adult animals were compared using the unpaired Student t-test. Data with $P< 0.05$ were considered to be significantly different between groups.

RESULTS AND DISCUSSION

To mimic oral HCMV infection in children, 4-week old infant (n=5) and 5-year old young adult SPF macaques were orally (via the buccal pouch) infected with a natural RhCMV isolate (1x 10^6 PFU / ml) using a blunted syringe, and followed for 1 year. All infants, but only 1 of 3 adult animals, seroconverted within 2-4 weeks (85), (163), (162) (Figure 1A). The 2 seronegative adults received a second RhCMV dose at week 16. One animal seroconverted within 4 weeks, the other animal remained seronegative for 8 weeks, but had RhCMV antibodies by week 12 after the 2nd infection (Figure 1A). The data suggest that infants compared to adults have enhanced susceptibility to *oral* RhCMV infection. In addition to binding antibodies, all animals developed RhCMV-specific neutralizing

antibodies (2), (1) (Figure 1B). Although two of the adults showed the highest 50% neutralization titers (NT_{50}), no statistically significant differences in NT_{50} values were detected between infant and adult animals. As all infant and 2 adult animals seroconverted within 4 weeks of RhCMV infection, animal #A3 likely became naturally infected, because the adult animals were co-housed. Possible infection via the natural route as opposed to the experimental oral exposure may explain the high NT_{50} for animal #A3. Animals #A1 and #A2 had qPCR-detectable RhCMV DNA in saliva at a single time point (59, 131) (Table 1), but it could not be conclusively determined whether these animals were actively shedding and transmitted RhCMV to #A3, because the RhCMV copy number was below the cut-off value (10 copies/ml). Consistent with observations in human infant HCMV infection, infant macaques showed pronounced high titer RhCMV shedding in saliva and urine (5), (107), (143), (165) (Table 1).

Table 1: RhCMV detection.

Weeks Post-Infection	-4	0	2	4	6	8	10	12	16	20	24	28	32	36	40	44	48	52	Compartment
INFANTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Plasma
I.1	-	NS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	Saliva
	NS	NS	NS	NS	NS	NS	NS	NS	NS	++	+++	NS	+	+	NS	NS	NS	NS	Urine
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Plasma
I.2	-	NS	-	-	+/-	-	-	-	-	-	-	+/-	-	+/-	+/-	-	+/-	-	Saliva
	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	-	NS	+++	NS	NS	NS	NS	NS	Urine
	-	-	-	+/-		+/-	+/-	-	-	-	-	-	-	-	-	-	-	-	Plasma
I.3	-	-	-	-	-	-	-	-	+++	+/-	++	+++	++	++	+/-	++	+	+/-	Saliva
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	+++	NS	NS	NS	NS	Urine
	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Plasma
I.4	-	-	-	-	-	-	-	+/-	-	+	NS	++	+	+/-	+	+	+/-	NS	Saliva
	NS	NS	NS	NS	NS	+/-	NS	+	+	++	++	+	+++	NS	+	NS	+++	NS	Urine
	-	-	-	-	-	-	-	+/-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Plasma
I.5	-	-	+	+/-	++	+	++	+/-	+/-	+/-	+	-	+/-	+/-	-	+/-	NS	NS	Saliva
	NS	NS	NS	NS	NS	NS	NS	+/-	-	NS	+	+	NS	NS	NS	NS	-	NS	Urine
ADULTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Plasma
A.1	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	Saliva
	-	-	NS	NS	NS	NS	NS	NS	NS	-	-	NS	NS	NS	-	-	NS	NS	Urine
	-	-	-	-	-	-	-	-	-	-	-	-	-	-					Plasma
A.2	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-					Saliva
	-	NS	-	-	-	-	NS	-	NS	-	-	NS	NS	NS					Urine
	-	-	-	-		-		-	-	-	-	-							Plasma
A.3	-	-	-	-		-		-	-	-	-	-							Saliva
	-	-	-	-		-		-	NS	-	NS	NS							Urine

NS = no sample available NT = not tested +/- >1, but less than 10 copies (LOD)

- negative + >10, but less than 10^4 ++ >10^4<10^5 +++ >10^5

Better control of virus shedding in adults could not be explained by antibody responses because binding and neutralizing titers did not differ between infant and adult animals and persisted in both groups throughout the study period. Around the time of seroconversion, all animals showed an increase in Ki67 positive cells within the total $CD4^+$ and $CD8^+$ T cell populations, with the frequencies of Ki67 positive $CD8^+$ T cells being significantly higher in adults than in infants (data not shown). Analysis of antigen-specific T cells in longitudinally collected blood samples by intracellular cytokine staining (IL2, IFNγ, and TNFα) showed that all animals developed $CD4^+$ and $CD8^+$ T cell responses to RhCMV lysate, RhCMV pp65 and RhCMV IE1 (Figure 1C) (2), (1), (91). There were no differences, however, in the magnitude, persistence, or quality of infant and adult T cell responses.

Generally, RhCMV-specific T cells produced only a single cytokine, IFNγ or TNFα. Dual-positive cytokine responses were detected in 4 of 5 infant and 2 of 3 adult animals within the $CD4^+$, and in 4 of 5 infants and 1 of 3 adult animals within the $CD8^+$ T cell population (data not shown). These results are in contrast to the age-dependent increase in HCMV-specific T cells and to a similar age-dependency of RhCMV-specific T cell responses in *non*-SPF macaques (own unpublished data) (29), (143). As 5 year-old macaques are comparable to 15 to 19 year old human teenagers, the age difference between the two animal groups in the current study might have been too small. Alternatively, the SPF status could have affected immune responsiveness.

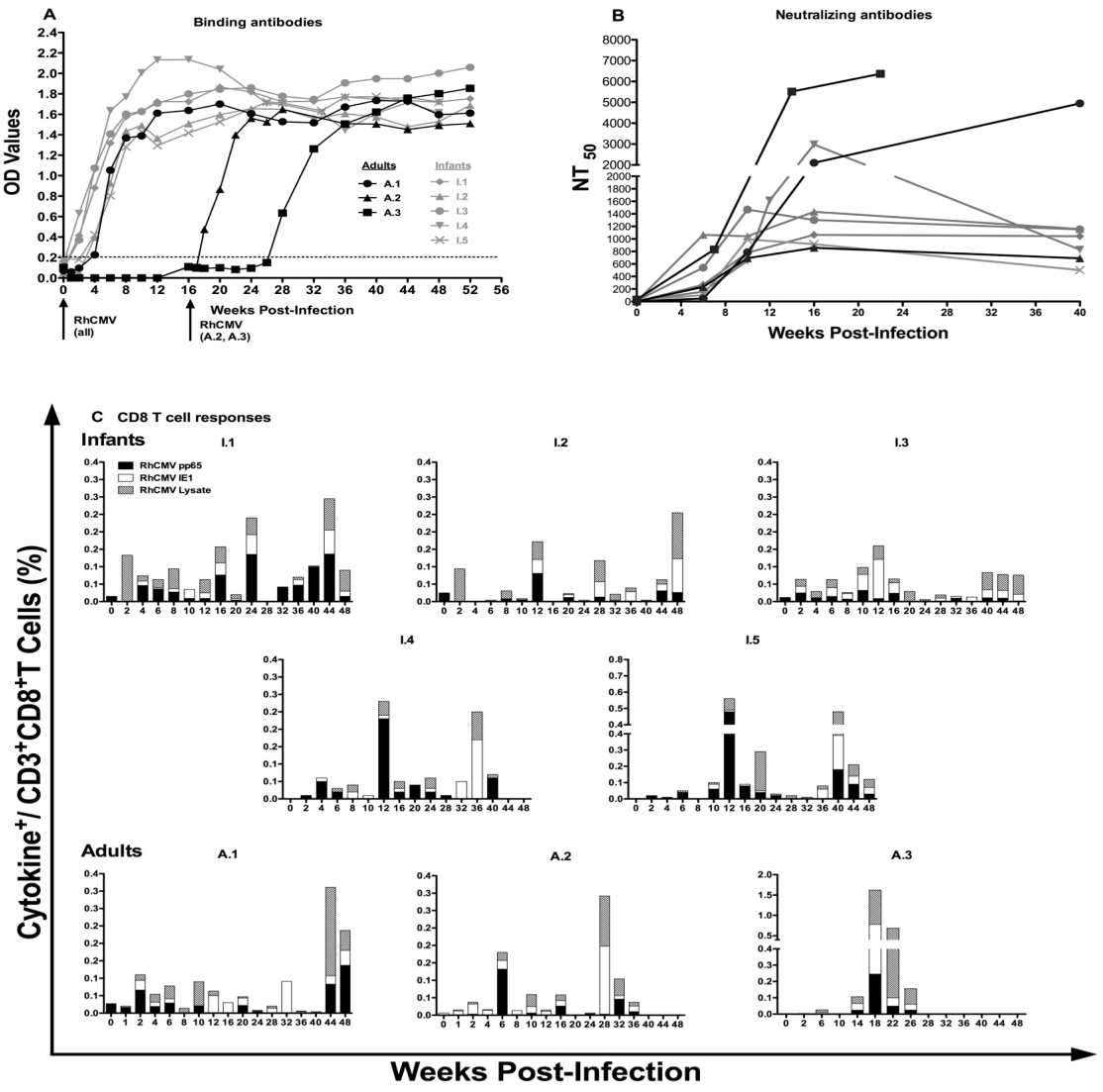

Figure 1: Immune responses to oral RhCMV infection. A. RhCMV-specific antibody development. OD values for RhCMV-specific binding antibodies in longitudinally collected plasma samples (1:100 dilution). Infant animals are depicted in grey symbols and lines and adult animals in black symbols and lines. Arrows below the x-axis indicate the times of oral RhCMV exposure. The weeks post infection for all animals are based on the first oral RhCMV exposure at week 0. The dashed line shows the threshold for a sample to be considered RhCMV antibody positive based on the analysis of a pool of plasma collected from RhCMV-negative animals. B. Neutralizing antibody titers. Neutralizing antibodies for RhCMV were determined at weeks 0, 6, 10, 16 and 40 post RhCMV infection. Note that for animal A.3 only samples from weeks 0, 7, 14 and 22 were available. Shown are the NT_{50}

titers for individual infant and adult macaques. C. RhCMV-specific T cell responses in peripheral blood. Shown are the total frequencies of single cytokine positive cells determined by Boolean gating analysis within the $CD8^+$ T cell populations in individual animals. The sum of cytokine positive cells specific for RhCMV pp65 (black bars), RhCMV IE1 (white bars) and RhCMV lysate (striped bars) are shown at individual time points post RhCMV infection. Animal numbers are indicated on top of each graph.

In this proof-of-concept study, we established an oral RhCMV infection model in infant SPF rhesus macaques. The findings can be summarized as follows: (i) The susceptibility to oral RhCMV infection appears to decline with age, as 2 of 3 older animals required multiple oral RhCMV exposures to become infected, although this route proved to be 100% effective in infants. Future studies should determine host factors enhancing susceptibility to RhCMV infection within the oral microenvironment. (ii) Similar to HCMV infection in humans, infant macaques shed RhCMV more persistently and at higher titers compared to adult macaques. Viral shedding in saliva and urine might represent the most reliable marker to assess control of CMV infection, and efficacy of drug treatments and vaccines. (iii) Larger animal studies are needed to define immune parameters associated with better control of RhCMV in adult compared to young animals. In the limited study here, differences in the magnitude or quality of RhCMV-specific T and B cell responses could not be detected. However, this was the first time SPF animals were available, and as the SPF colony ages, more detailed virological and immunological studies, including tissue analysis, could be performed with larger animals groups.

CHAPTER 4: ALTERED CYTOKINE SIGNALING IN INFANT $CD4^+$ T CELLS

OVERVIEW

$CD4^+$ Th1 and Th2 cells elicit their function by producing distinct pattern of cytokines effective for intracellular pathogens and parasitic expulsion, respectively. $CD4^+$ T cell differentiation is dependent on the infecting pathogen and the cytokine milieu generated by responding immune cells. A preferential $CD4^+$ Th2 response is generated in the infant and Th1 in the adult against infectious diseases caused by intracellular pathogens. Such bias make infants, regardless of gestation, more susceptible to infectious diseases and experience greater morbidity and mortality compared to adults. Since infant $CD4^+$ T cells are thought to be Th2-biased, we asked whether infant $CD4^+$ T cells can respond to exogenous IFNγ. Our study tests the hypothesis that the propensity towards Th2 responses in infants is due to diminished cytokine-receptor mediated signaling in $CD4^+$ Th1 cell differentiation. We also tested whether IL4 and IL2 cytokine signaling pathways are affected by gestational age. Our data showed that $CD4^+$ T cells from cord blood of preterm (PT) and full term (FT) infants differ in Th1 and Th2-associated cytokine receptor expression from adult $CD4^+$ T cells. Moreover, i*n vitro* stimulation of whole blood with cytokines resulted in lower phosphorylation of JAK2 and STAT1 in FT infants compared to adult $CD4^+$ T cells after IFNγ treatment. Moreover, purified $CD4^+$ T cells from FT infants showed differential up-regulation of IFNγ-induced genes after TCR and IFNγ stimulations. This treatment also resulted in generally higher cytokine production from adult compared to FT infant $CD4^+$ T cells. A better understanding of the role of cytokine signaling in immune development is

important to delineate whether inefficient cytokine-receptor binding and downstream signaling components contribute to the immature status and impaired function of infant $CD4^+$ T cells. Unraveling the molecular mechanisms involved may aid in pediatric vaccine designs.

INTRODUCTION

Cytokines present in the local environment are central in differentiation and commitment of $CD4^+$ T cells into T helper 1 (Th1) or T helper 2 (Th2) lineages. Both IFNγ and IL12 are important for Th1 differentiation while IL4 is needed for Th2. Th1 cells secrete the cytokines IFNγ and IL2 to mediate protection against intracellular bacteria and viruses, whereas Th2 cells mainly produce IL4 and facilitate allergic inflammation and parasite expulsions (166), (167). Cytokines elicit their cellular actions by binding to specific receptors expressed on the cell's membrane. Cytokine-receptor interaction results in the phosphorylation of receptor-associated Janus kinase (JAKs) family members (JAK1, 2, 3 and TYK2) (133), (58), (49, 103). The signal is then amplified and transduced by phosphorylating cytokine-specific transcription factors, the signal transducer and activators of transcription (STATs) (STAT1, 2, 3, 4, 5ab and 6). The transcription factors translocate to the nucleus where they regulate cytokine-specific gene expression. Cytokines and their respective transcription factor networks are critical in the establishment of the differentiated $CD4^+$ T helper cell's functional identity.

Numerous research studies have been conducted to understand the unique status of the infant immune system. The results demonstrated that infant and adult $CD4^+$ T cells have distinctive capacities to produce cytokines such as IFNγ, IL2 or IL4 (4), (3), (164), (31), (55). One particular feature that stands out is that infants and young children have a reduced ability

to produce the Th1-related cytokine, IFNγ. Moreover, studies showed the infant's immune response to recall antigens selectively induces the death of Th1 cells by apoptosis, while concurrently, Th2-asssociated cytokine receptors were expressed at higher levels (88) (78). Interestingly, little is known about the responsiveness of infant $CD4^+$ T cells to IFNγ or IL4. Specifically, we sought to determine whether the signaling machinery via the JAK/STAT pathway is operational in infant $CD4^+$ T cells when exogenous IFNγ, IL4 or the T-cell growth factor, IL2, are provided. Not only are these three cytokines involved in $CD4^+$ T cell differentiation, they are important for growth, maintenance and function of the distinct effector T helper subsets. Thus, we hypothesized that the preferential development of Th2 response in infants is due to decreased activation of the components of the JAK/STAT signaling machinery needed for commitment towards Th1 development.

Our results show that that the activation of different components of the cytokine-receptor signaling pathway associated with Th1 development was reduced in infant compared to adult $CD4^+$ T cells. Specifically, we found differences in IFNγ, IL4 and IL2 signaling between infants and adults. Moreover, gestational age at birth (preterm (PT) vs. full term (FT) infants) was associated with unique signaling properties, and we observed an age-dependent increase in activation of cytokine signaling factors in infants from birth to 1 year of age. Our results showed that the percentage of $CD4^+$ T cells expressing IFNγR1 was significantly lower at birth compared to adult $CD4^+$ T cells. However, on a per cell basis, infant $CD4^+$ T cells at birth expressed more IFNγR1 than adult $CD4^+$ T cells. In contrast, frequencies of IFNγR2 positive $CD4^+$ T cells and the expression of IFNγR2 on $CD4^+$ T cells did not differ between infant and adult $CD4^+$ T cells. Furthermore, both JAK2 and STAT1 activation in response to IFNγ were reduced in $CD4^+$ T cells at birth compared to adults but

responsiveness increased throughout the first year of life. In contrast to IFNγ, IL4 signaling in infant $CD4^+$ T cells at birth was comparable to that observed in adult $CD4^+$ T cells. In fact, both the percentage of $CD4^+$ T cells expressing IL4Rα and the number of IL4Rα receptors on a per cell basis was higher in infants than in adults. Although STAT6 phosphorylation in response to IL4 was reduced in CB $CD4^+$ T cells of PT and FT infant, by 2-5 months of age, STAT6 phosphorylation was as effective as in adult $CD4^+$ T cells. Surprisingly, frequencies of IL2Rα positive $CD4^+$ T cells were significantly lower in infants at birth, and were still significantly lower compared to adults at 1 year of age. However, the absolute number of IL2Rα receptors per $CD4^+$ T cell was higher in infant compared to adult $CD4^+$ T cells. Similar to the activation of the IFNγ signaling pathway in infant $CD4^+$ T cells, STAT5 phosphorylation in response to IL2 was reduced in infant $CD4^+$ T cells at birth compared to adult $CD4^+$ T cells, but increased throughout the first year of life. Differences in the activation of various components in cytokine signaling pathways were not only observed between the total $CD4^+$ T cell populations of infants and adults, but these age-dependent differences were also observed within the naïve-T(N), central memory-T(CM) and effector/effector memory-T(E/EM) $CD4^+$ T cell subsets. Lastly, *in vitro* stimulation through the TCR and exogenous addition of IFNγ revealed that FT infant and adult $CD4^+$ T cells showed differences in the induction of genes encoding IFNγ, IL2, CXCL9 and other genes related to JAK/STAT signaling pathway. At the protein level, infant $CD4^+$ T cells, in general, produced lower amounts of cytokines and chemokines compared to adult $CD4^+$ T cells after stimulation through TCR only and in combination with exogenous IFNγ. Together, our data revealed that cytokine signaling is developmentally controlled as responsiveness to cytokines by infant $CD4^+$ T cells increased with age. Such progression is likely associated with

immune maturation and antigen exposure. Our findings may shed light on possible molecular mechanisms that are the basis for the unique status of the infant immune system and offer insights in the design of pediatric vaccines and interventions.

MATERIALS AND METHODS

Human subjects

All study populations were recruited from the University of North Carolina, Chapel Hill. Cord blood samples from full term (FT) infants collected with citrate-phosphate-dextrose (CPD) anticoagulant were acquired either directly at delivery at the University of North Carolina Chapel Hill (UNC-CH) hospital with prior approval from the patient and/or requested from the Carolinas Cord Blood Bank (Duke Medicine, NC). Cord blood samples from preterm (PT) infants were collected in Ethylenediaminetetraacetic acid (EDTA)-containing blood tubes. EDTA blood samples from older infants (2-12+ months [Mo.]) were obtained during routine doctor visits after obtaining signed consent (IRB protocol 11-1906, UNC Chapel Hill). Adult EDTA-blood samples were collected after written informed consent obtained from healthy blood donors of the UNC Center for AIDS Research Immunology Core Laboratory study (IRB protocol# 96-0859, UNC Chapel Hill).

Sample processing

Whole blood was used for JAK and STAT experiments. For all other assays, peripheral blood mononuclear cells (PBMC) were used. PBMCs were isolated by density gradient centrifugation using Lymphocyte Separation Medium (LSM, MP Biomedicals, OH). PBMCs were resuspended in serum free media (AIM V, GIBCO-Life Technologies, NY) supplemented with L-glutamine-penicillin-streptomycin antibiotic cocktail (Sigma, St Louis MO) at a concentration of $2x10^6$ cells/ml. Cells were used for surface and cytokine receptor

quantification and for $CD4^+$ T cell purification for gene and protein expression experiments.

Flow cytometric analysis

To determine T cell maturation, PBMCs were stained with the following antibody combinations: CD3 (SP34-2)-Pacific Blue, CD4 (L200)-PerCPCy5.5, CD8 (RPA-T8)-Alexa Fluor700, CD20 (2H7)-APC-H7, CD45RA (5H9)-FITC, and CCR7 (3D12)-PeCy7. The cytokine receptor expression on the surface of T cells was measured by staining with these T cell specific antibodies at 4°C in combination with the relevant cytokine receptor antibody (all in PE): IFNγR1 (CD119-GIR-208), IFNγR2 (2HUB-159, Biolegend, San Diego, CA), IL4Rα (hIL4R-M57), IL2Rα (CD25-clone-4E3, Miltenyi Biotec, Auburn, CA), or IL2Rβ (CD122-clone-Mikβ1). All antibodies, unless indicated otherwise, were purchased from BD Biosciences (San Diego, CA). The $CD4^+$ T cell subsets are abbreviated and defined as follows: T(N)= Naïve (CD45RA+/CCR7+); T(CM)= Central memory ($CD45RA^-$ /$CCR7^+$); and T(E/EM)= Effector/effector memory ($CD45RA^{+/-}$/$CCR7^-$). Cytokine receptor expression is reported as percentage of $CD4^+$ T cell subpopulations or quantitated on a per cell basis using a linear equation based on a standard curve generated with Quantibrite PE beads (BD Biosciences).

Phosflow Analysis

Activation of JAK2 and STAT molecules was measured by Phosflow analysis. Briefly, JAK2 activation was measured after whole blood was stimulated for 10 min with 10μg/ml of human recombinant IFNγ1b (Miltenyi Biotec, Auburn, CA). STAT phosphorylation was measured after activation of whole blood for 15 min with either 10μg/ml of human recombinant IFNγ1b, 0.1μg/ml of recombinant rhesus IL4 (R&D, Minneapolis, MN) or 0.1μg/ml of recombinant human IL2 (Miltenyi Biotec, Auburn CA).

Note: Recombinant human and rhesus IL4 were tested separately and yielded comparable results (data not shown). The following antibodies were used to identity $CD4^+$ T cells and maturity status: CD3 (SP34-2)-PeCy7, CD4 (L200)-PerCPCy5.5, CD27 (L128)-PE and STAT1 (pY701)-Alexa Fluor488 (Cell Signaling Technology, Danvers MA) or STAT6 (pY641)- Alexa Fluor488, or STAT5 (pY694)- Alexa Fluor647 to determine STAT phosphorylation after IFNγ, IL4 or IL2 treatment, respectively or JAK2 (D4A8-Tyr1008)-Alexa647 (Cell Signaling Technology, Danvers MA) phosphorylation after IFNγ treatment. The $CD4^+$ T cell subsets were also abbreviated and defined as follows: T(N)= naïve ($CD27^+$); T(CM)= Central memory ($CD27^{low}$); and T(E/EM)= Effector/effector memory ($CD27^-$) Unstimulated whole blood served as negative controls. The flow cytometric staining procedures were adapted according to BD Phosflow protocols with the following buffers: Lyse/fix, Perm Buffer III, and Phosflow buffer (PBS +2% heat-inactivated FBS +0.09% Sodium Azide). Cells positive for JAK2 or STAT phosphorylation are reported as percentage of $CD4^+$ T cells after subtraction of values from negative controls (unstimulated sample).

All flow cytometric samples were acquired on a LSRII flow cytometer (BD Biosciences, San Jose, CA). A minimum of 300,000 events was acquired for both receptor expression and phosphorylation. Data were analyzed using FlowJo Software (Tree Star, Ashland, Oregon). Antibodies were titrated prior to use and gates for certain cell populations were based on FMO controls.

$CD4^+$ T cell enrichment/purification

$CD4^+$ T cells were purified from both cord and adult blood using the Stem Cell $CD4^+$ Enrichment kit, according to the manufacturer's protocols (STEMCELL Technology,

Vancouver, Canada). Purity of $CD4^+$ T cells was confirmed by flow cytometry and purity exceeded 97%. Cells were cultured at a concentration of $2x10^6$ /ml under the following conditions: 1) unstimulated (Aim V media), 2) T cell receptor (TCR) stimulation using 1μg of anti-CD3 (UCHT1) and anti-CD28 (L293) antibodies, and 3) a combination of TCR (anti-CD3 and anti-CD28) stimulation plus 10μg/ml of human recombinant IFNγ1b added at the start of culture. TCR stimulation was prepared by first coating 96-well round bottom plates overnight with 1μg of pure anti-CD3 and anti-CD28 antibodies per well. Unbound antibodies were removed by washing 3x with sterile PBS. A total of 10 wells/condition received 200,000 cells/well and cells were incubated at 37°C, 5% CO_2 for 24 h. The 10 wells from each condition were harvested and the supernatants were stored at -80^0C for cytokine analysis by Luminex Technology and cells were used for PCR array.

Profiling of multiple cytokines and chemokines using a Luminex® –based assay

Supernatants from the 3 conditions described above were tested for cytokine production using the Milliplex Mag Human Cytokine/Chemokine Panel containing 20 analytes (IFNγ, IL10, IL5, IL17A, IL1α, IL1β, IL9, IL2, IL6, IL7, IL8, TNFα, TGFα, GM-CSF, IL12p70, PDGF-AA, IP10, MCP1, MIP1α, and MIP1β). All procedures were performed as described in the manufacturer's protocol and were acquired on a Luminex xMAP® MagPix platform (EMD Millipore, MA).

Real time RT-PCR analysis of JAK-STAT signaling

RNA was isolated from cells under the 3 conditions described above using the Qiagen RNEasy Mini Kit (Qiagen, Germany). DNase treatment with RNAse-Free DNAse Set (Qiagen) was performed to eliminate genomic DNA contamination. RNA was reverse transcribed using the RT^2 First Strand kit (Qiagen) and cDNA was subsequently amplified

using the RT^2 JAKSTAT Signaling Pathways PCR Array. PCR was performed according to the manufacturer's protocol. Data analysis was performed using the web-based software for PCR arrays on the SA Bioscience website (http://www.sabiosciences.com/dataanalysis.php)

Statistical Analysis

Receptor expression and JAK/STAT phosphorylation results are expressed as median values. All graphs and analyses were performed using GraphPad Prism for Mac Version 5.0. Significant differences between multiple groups were determined by Kruskal-Wallis test followed by Dunn's Multiple Comparison test. P values of $p<0.05$ were considered significant.

RESULTS

Expression of Th1-associated receptors, kinase and cytoplasmic transcription factor is age-dependent

In order for cytokines to exert their functions, they must bind to their respective receptors, activate their associated JAKs and respective STAT molecules. The magnitude and kinetics of the cell's response might depend on the number of receptors present on the cell surface and the availability of the signaling components downstream. We first assessed both the frequency and absolute number of receptors related to Th1 or Th2 responses on resting $CD4^+$ T cells. Our data show that infants have altered expression of both Th1 and Th2 cytokine- related receptors. Specifically, the frequency of IFNγR1-expressing $CD4^+$ T cells was higher in adults compared to CB (PT or FT infants) $CD4^+$ T cells regardless of the gestational age of the infant (Figure 1). Even infants between 2-12+ months of age had lower frequencies of IFNγR1-expressing $CD4^+$ T cells than adults. Considering the distinct cell differentiation of infant and adult $CD4^+$ T cells, with infant blood containing predominantly

T(N) $CD4^+$ T cells, we deemed it important to confirm these findings in differentiated $CD4^+$ T cells. Surprisingly, we observed lower frequencies of IFNγ$R1^+$ $CD4^+$ T cells in adults compared to PT and FT infants at birth when we specifically analyzed T(N) $CD4^+$ T cells while the adults contained higher frequencies of IFNγ$R1^+$ T(E/EM) $CD4^+$ T cells. We could not detect statistical differences within the T(CM) $CD4^+$ T cells (Figure 1A-D, left). Intriguingly, IFNγR1 expression was mainly confined to the T(E/EM) compartment. However, when we compared absolute numbers of IFNγR1 on a per cell basis, we observed that $CD4^+$ T cells at birth had significantly higher numbers of IFNγR1 per cell compared to adults across all subsets (Figure 1E-H, right panel). The age-related differences in receptor numbers were irrespective of gestational age as both PT and FT infants at birth had higher numbers of IFNγR1 on a per cell basis, and this was true for all $CD4^+$ T cell subsets. As the IFNγ receptor exists as a heterodimer, we also assessed the expression of the signaling chain, IFNγR2. Although we observed higher frequencies of IFNγR2 –expressing T(N) and T(E/EM) $CD4^+$ T cells in adults, (Figure 2A-D), absolute numbers of IFNγR2 on a per cell basis did not differ between the various age groups. (Figure 2E-H).

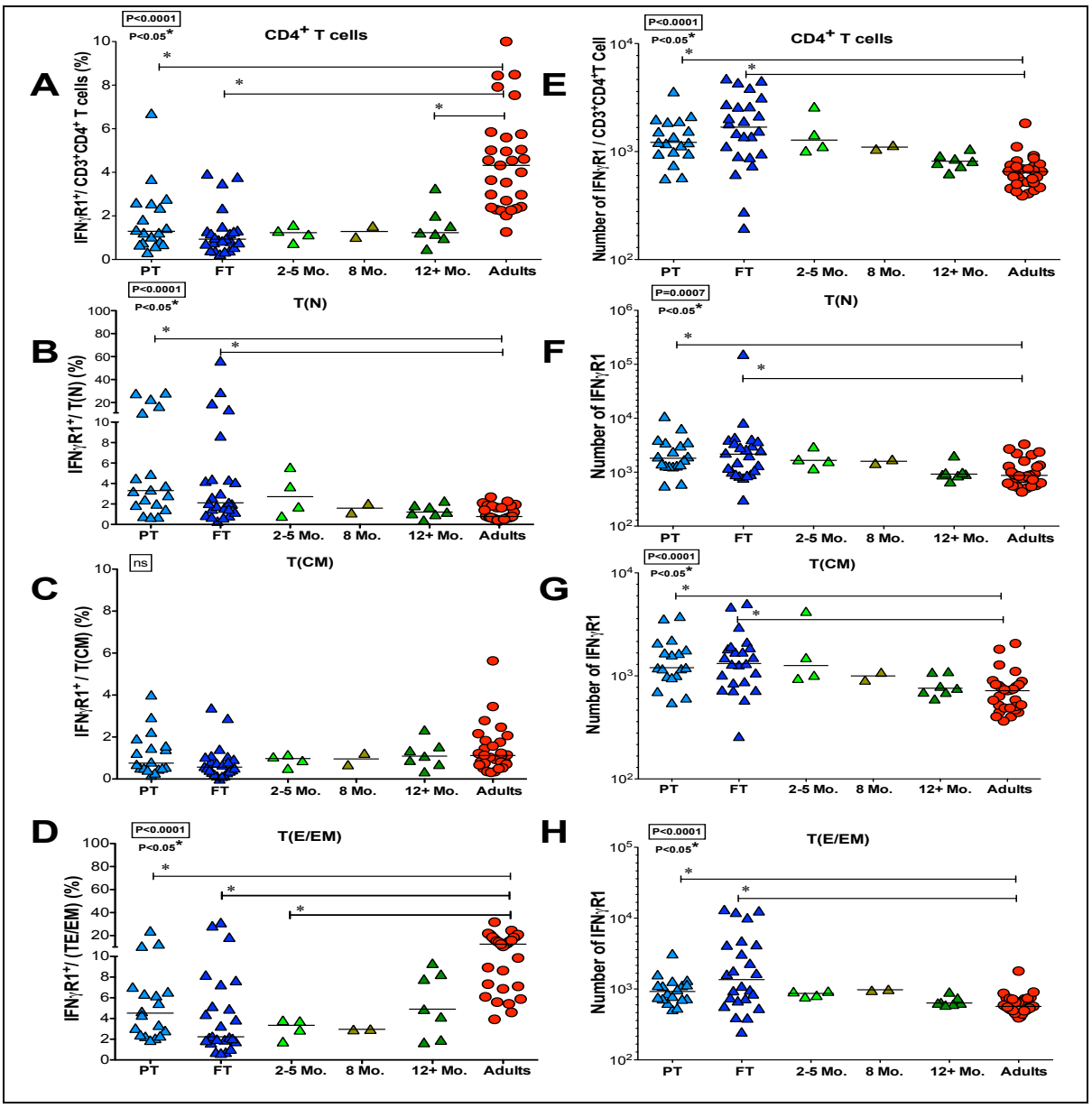

Figure 1: Frequencies and absolute numbers of IFNγR1 on CD4$^+$ T cell subsets. Panels A-D show the frequencies of IFNγR1- expressing CD4$^+$ T cells. Panels E-H show the absolute number of IFNγR1 per CD4$^+$ T cell. Each symbol represents an individual. Red circles represent adults and colored triangles represent different infant age groups. Peripheral blood mononuclear cells from preterm (PT, n=18, light blue), full term (FT, n=24, blue), 2-5 Mo. (n=4, light green), 8 Mo. (n=2, dark yellow), 12+ Mo. old infants (n=7, green), and adults (n=29, red circles) were isolated and stained for flow cytometric analysis. Horizontal lines represent median values. Statistical differences among all groups were determined by

Kruskal-Wallis test and the P- value is listed in the top left corner. Capped lines show statistical significant differences between two groups as determined by Dunn's comparison test.

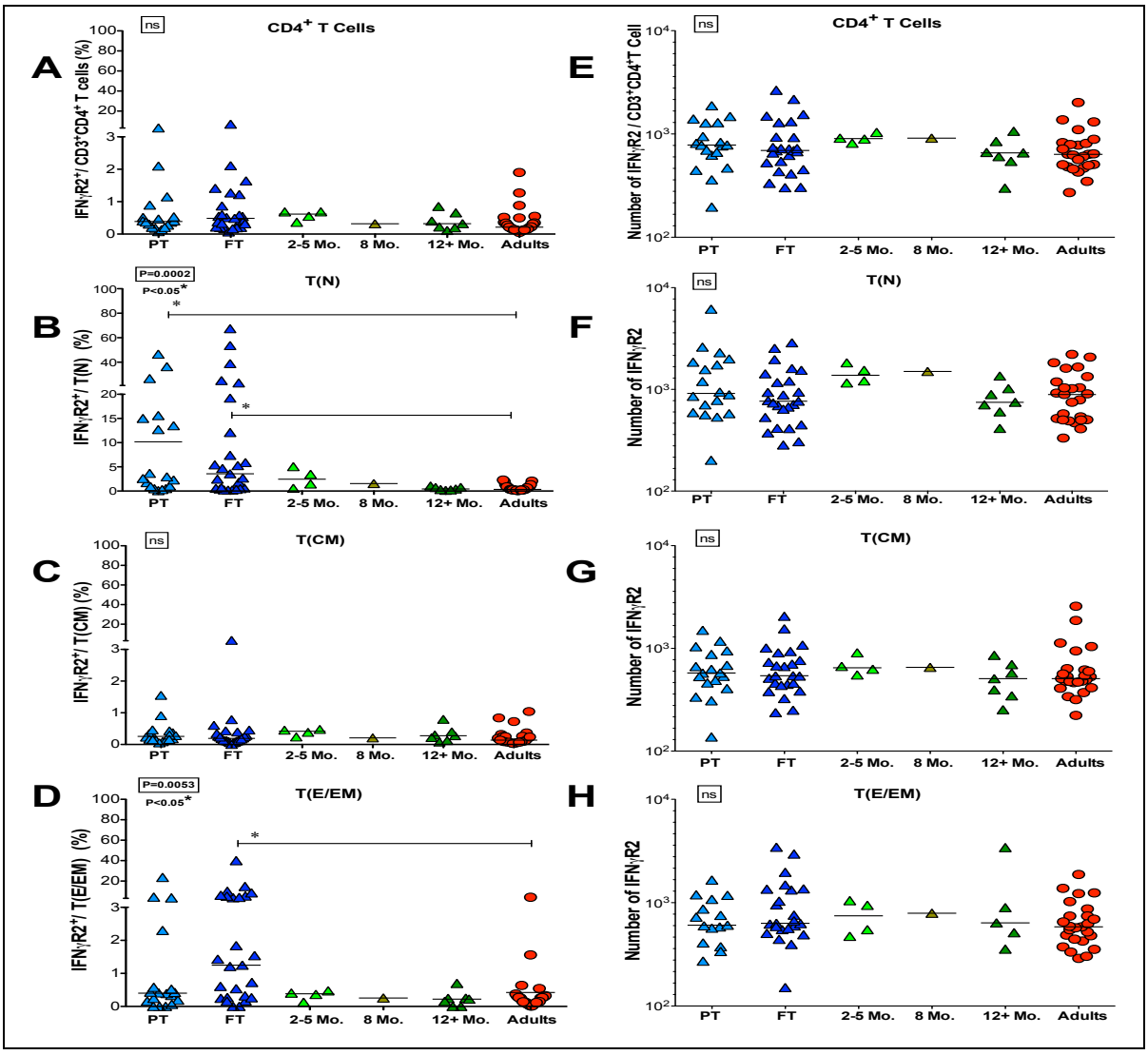

Figure 2: Frequencies and absolute numbers of IFNγR2 on CD4$^+$ T cell subsets. The legend is as described in Figure 1 with the following sample numbers: PT, n=18; FT, n= 25; 2-5 Mo., n= 4, 8 Mo., n=1, 12+ Mo., n=7; and adults, n=25.

Since cytokine receptors lack intrinsic kinase activity, we next determined if the activation of relevant receptor-associated JAKs and corresponding STAT molecules differed between the various age groups. As our main focus was on the evaluation of IFNγ responses

in infants, we focused on the evaluation of JAK2 activation in response to IFNγ treatment in FT infants at birth, 12+ month old infants and adults. We found that the phosphorylation of JAK2 (pJAK2) in response to IFNγ in T(N) $CD4^+$ T cells of FT infants was significantly lower compared to adults (Figure 3A-B). Though not statistically significant, it appeared that adult T(CM) had higher frequencies of $CD4^+$ T cells expressing activated JAK2 than FT infants at birth. There was no difference in JAK2 phosphorylation in the T(E/EM) compartment (Figure 3C-D). Moreover, there was a significant increase of pJAK2 in 12+ month old infants compared to FT infants in the both T(N) and T(CM) compartments except in the T(E/EM) (Figure 3A-C). Interestingly, 12+ month old infants also have higher frequencies of pJAK2 compared to adults both within the T(CM) and T(E/EM) compartments (Figure 3C-D). Consistent with lower JAK2 activation, STAT1 phosphorylation (pSTAT1) in $CD4^+$ T cells after IFNγ treatment was also significantly lower in FT infants compared to adults in all $CD4^+$ T cell subsets except in the TE/EM compartment (Figure 4A-C). Surprisingly, PT infants seemed to respond to IFNγ treatment better than FT infants as higher pSTAT1 was observed in both T(N) and T(CM) compartments (Figure 4B&C). Additionally, $CD4^+$ T cells from FT infants at birth had considerably lower pSTAT1 expression compared to older infants that were 2-5 months and 12+ months, consistent with lower JAK2 phosphorylation. There appeared to be an age-dependent increase of IFNγ responsiveness assessed by STAT1 phosphorylation reflected in all $CD4^+$ T cell subsets (Figure 4A-D).

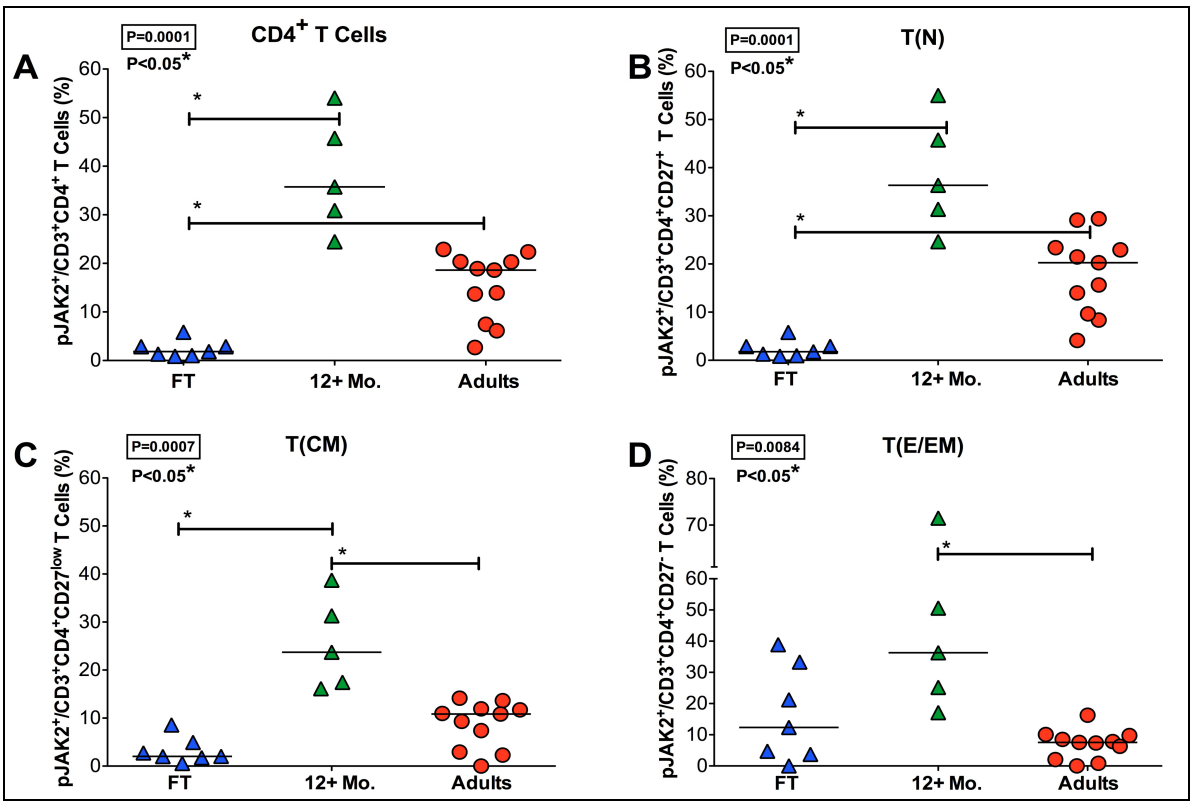

Figure 3: JAK2 phosphorylation (pJAK2) after IFNγ stimulation is increased with age. Shown are percentages of pJAK2 positive cells within the A. Total $CD4^+$ T cell population, B. T(N) $CD4^+$ T cells. C. T(CM) $CD4^+$ T cells and D. T(E/EM) $CD4^+$ T cells. Each symbol represents an individual. Red circles represent adults and colored triangles represent different infant age groups. Whole blood from full term (FT, n=7, blue), 12+ Mo. old infants (5, green), and Adults (11, red circles) were treated with or without recombinant human IFNγ (10μg/ml) for 10 min, fixed, permeabilized and stained for Phosflow. Statistical differences among all groups were determined by Kruskal-Wallis test and the P- value is listed in the top left corner. Horizontal lines represent median values. Capped lines show statistical significant differences between two groups as determined by Dunn's comparison test.

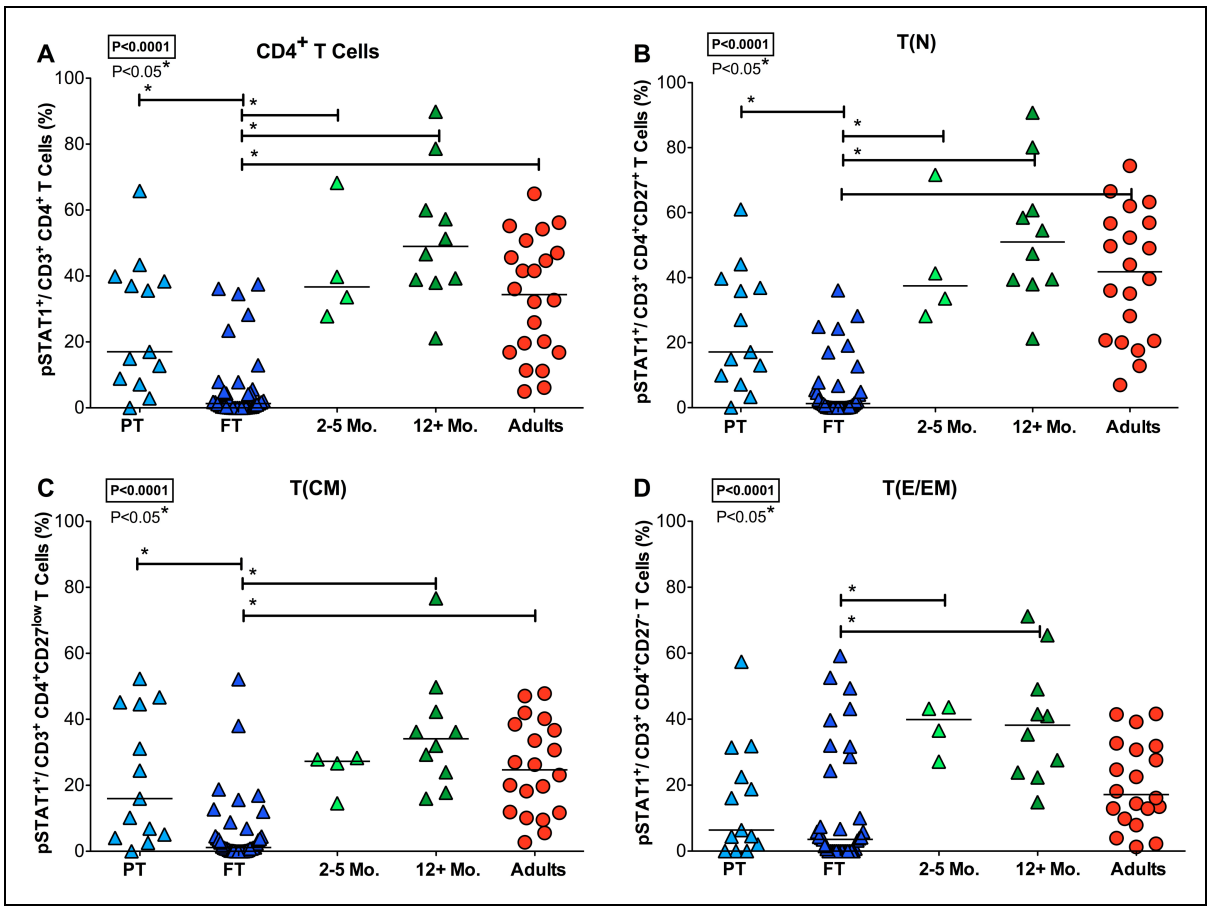

Figure 4: STAT1 phosphorylation (pSTAT1) is increased with age. Shown are percentages of STAT1 positive cells within the A. Total $CD4^+$ T cell population, B. T(N) $CD4^+$ T cells. C. T(CM) $CD4^+$ T cells and D. T(E/EM) $CD4^+$ T cells. Each symbol represents an individual. Red circles represent adults and colored triangles represent different infant age groups. Whole blood from preterm (PT, n=13, light blue), full term (FT, n=38, blue), 2-5 Mo. (n=4, light green), 12+ Mo. old infants (n=10, green), and Adults (n=22, red circles) were treated with or without recombinant human IFNγ (10µg/ml) for 15 min, fixed, permeabilized and stained for Phosflow. Statistical differences among all groups were determined by Kruskal-Wallis test and the P- value is listed in the top left corner. Horizontal lines represent median values. Capped lines show statistical significant differences between two groups as determined by Dunn's comparison test.

Propensity of infant compared to adult CD4$^+$ T cells to express Th2-associated receptors and cytoplasmic transcription factors

Given that infants have a Th2-biases response, we tested whether infant CD4$^+$ T cells preferentially express Th2-associated cytokine receptors (IL4Rα) and respond to the canonical Th2 cytokine, IL4, via the phosphorylation of the cytoplasmic transcription factor, STAT6 similar to adult CD4$^+$ T cells. In general, we found that the frequencies of CD4$^+$ T cells positive for the IL4Rα were not significantly different between CB (PT & FT), older infants or adults (Figure 5A). Although the frequencies of IL4Rα expressing T(N) CD4$^+$ T cells differed significantly among all groups by Kruskal-Wallis analysis, only TCM and not T(E/EM) from FT and PT infants at birth contained higher frequencies of IL4Rα positive cells than adults (Figure 5B-D). Assessing the number of receptors on a per cell basis, both PT and FT infants had significantly more IL4Rα per CD4$^+$ T cell within both T(N) and T (E/EM) compartments while only PT and adults were different in the T(CM) (Figure 5E-H). Interestingly, we also observed that within T(E/EM) compartment, FT infants had higher IL4Rα numbers than 12+ month old infants (Figure 5H). To determine whether cytokine receptor expression could influence the Th2-bias, we compared the ratio of IL4Rα:IFNγR2 on CD4$^+$ T cells. Although we had access to only few blood samples between birth and 1 year of age, the ratios of IL4Rα and IFNγR2 within the different compartments were statistically different across the various age groups (Figure 6A-D). Specifically, we found that FT infants at birth compared to 2-5 month olds and to adults, showed a bias towards higher IL4Rα expression as opposed to IFNγR2 expression within the T(N) compartment (Figure 6B).

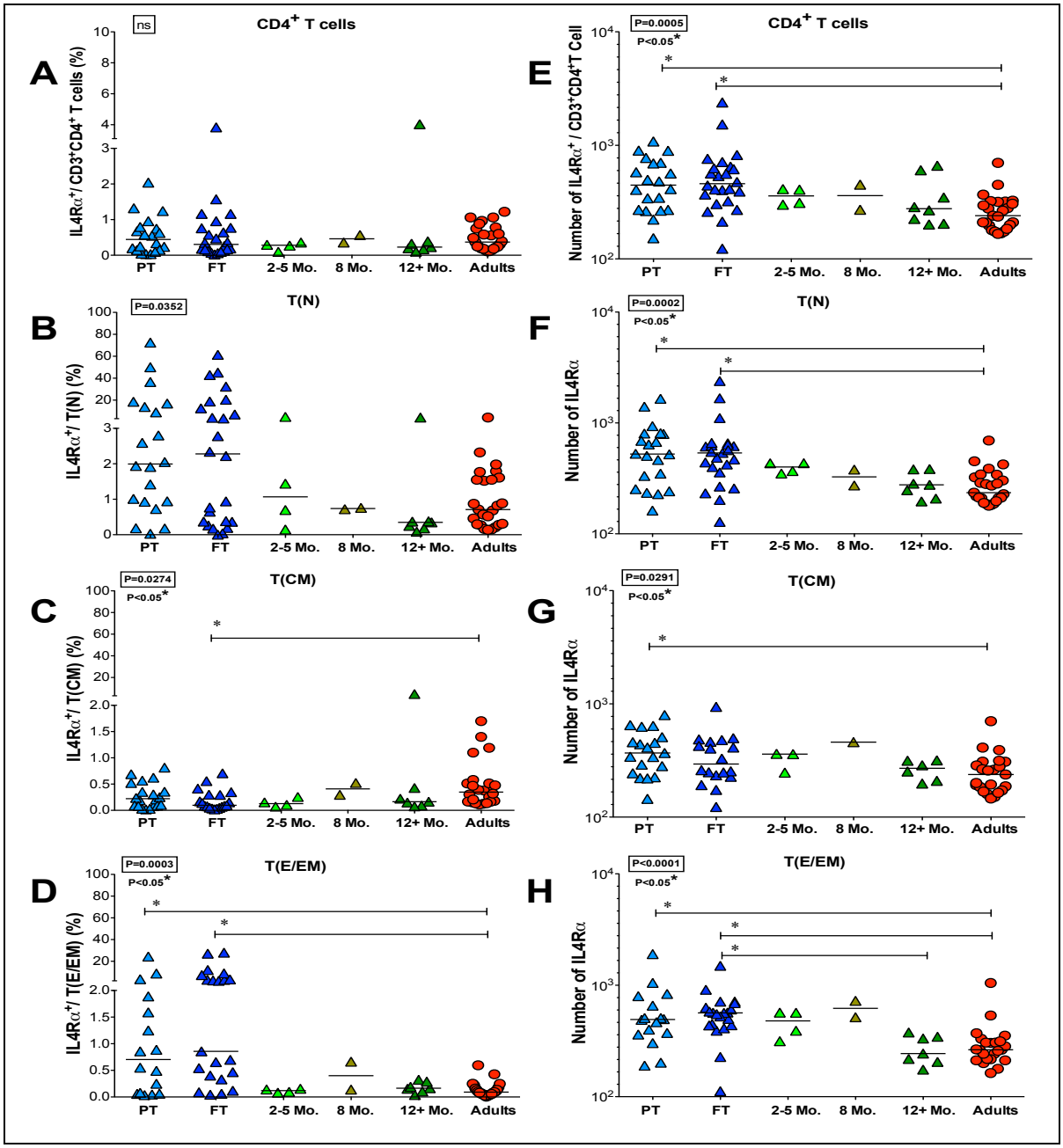

Figure 5: Frequencies and absolute numbers of IL4Rα on CD4$^+$ T cell subsets. The legend is as described in Figure 1 with the following sample numbers: PT, n=20; FT, n=24; 2-5 Mo., n=4; 8 Mo., n=2; 12+ Mo, n=7; and adults, n=25.

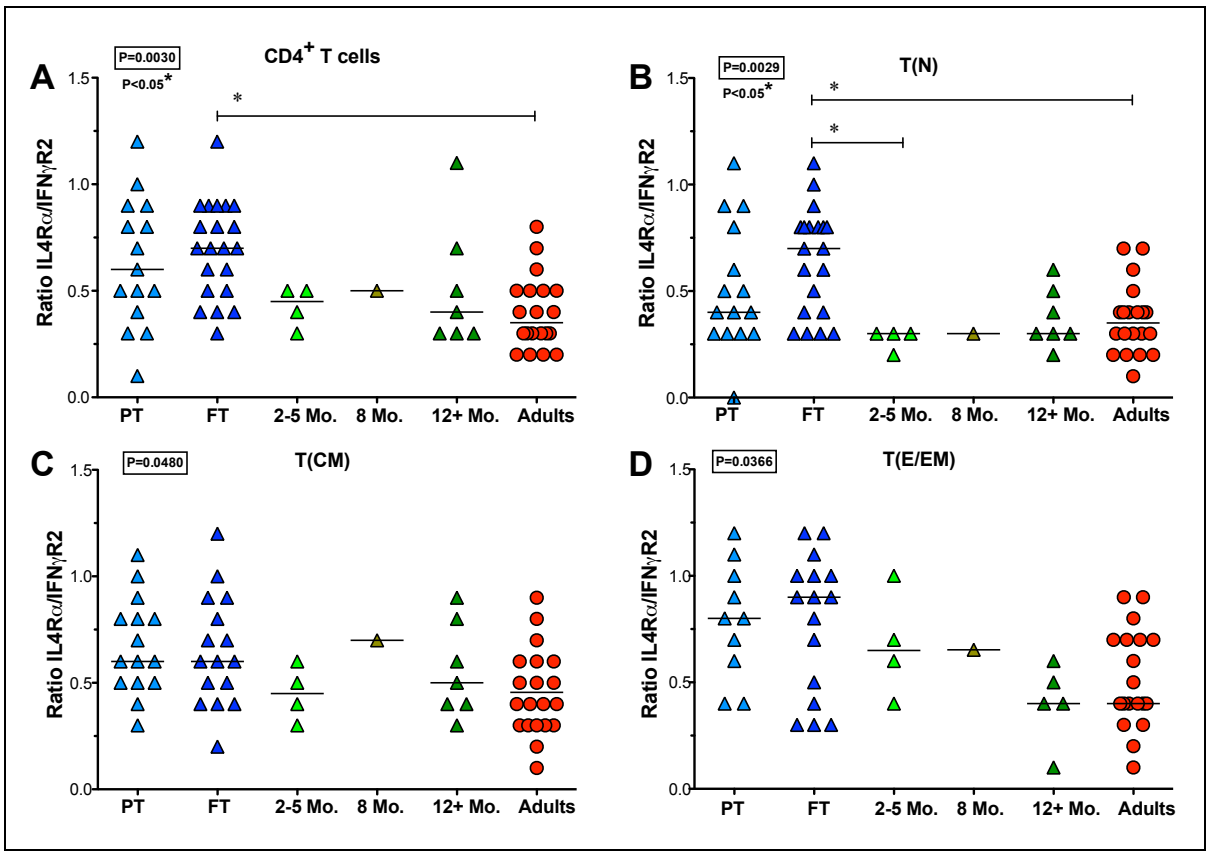

Figure 6: The ratio of IL4Rα/IFNγR2 within CD4$^+$ T cells and subsets shows a Th2-bias. Shown are the ratio of receptors within the A. Total CD4$^+$ T cell population, B. T(N) CD4$^+$ T cells, C. T(CM) CD4$^+$ T cells, D. T(E/EM) CD4$^+$ T cells of (PT, n=15; FT, n=21; 2-5 Mo., n=4; 8 Mo., n=1; 12+ Mo., n=7, green; and adults, n=20. Statistical analysis and labels are as described in Figure 1.

In contrast to IFNγ stimulation, a much higher percentage of infant CD4$^+$ T cells seemed to have the ability to respond to IL4, as assessed by STAT6 phosphorylation (pSTAT6) (Figure 7A-D). However, the responses of FT infant CD4$^+$ T cells at birth showed significantly less pSTAT6 compared to adults and older infants that were 12+ Mo. of age. Analogous results were also detected in the T(N) and memory subsets (T(CM) and T(E/EM)) of CD4$^+$ T cells (Figure 7B-D). Furthermore, gestational age didn't seem to make a difference in affecting the ability of PT or FT infant CD4$^+$ T cells to respond to IL4. A higher

percentage of $CD4^+$ T cells at birth was able to respond to IL4 not IFNγ, as both the percentage of IL4Rα-expressing $CD4^+$ T cells and the number of the IL4Rα on a per cell basis were higher in the infants compared to adults. Thus, these data revealed that infants readily responded to IL4 at birth and continuing on, at least in the first year of life, showing the propensity of infant $CD4^+$ T cells to develop towards Th2.

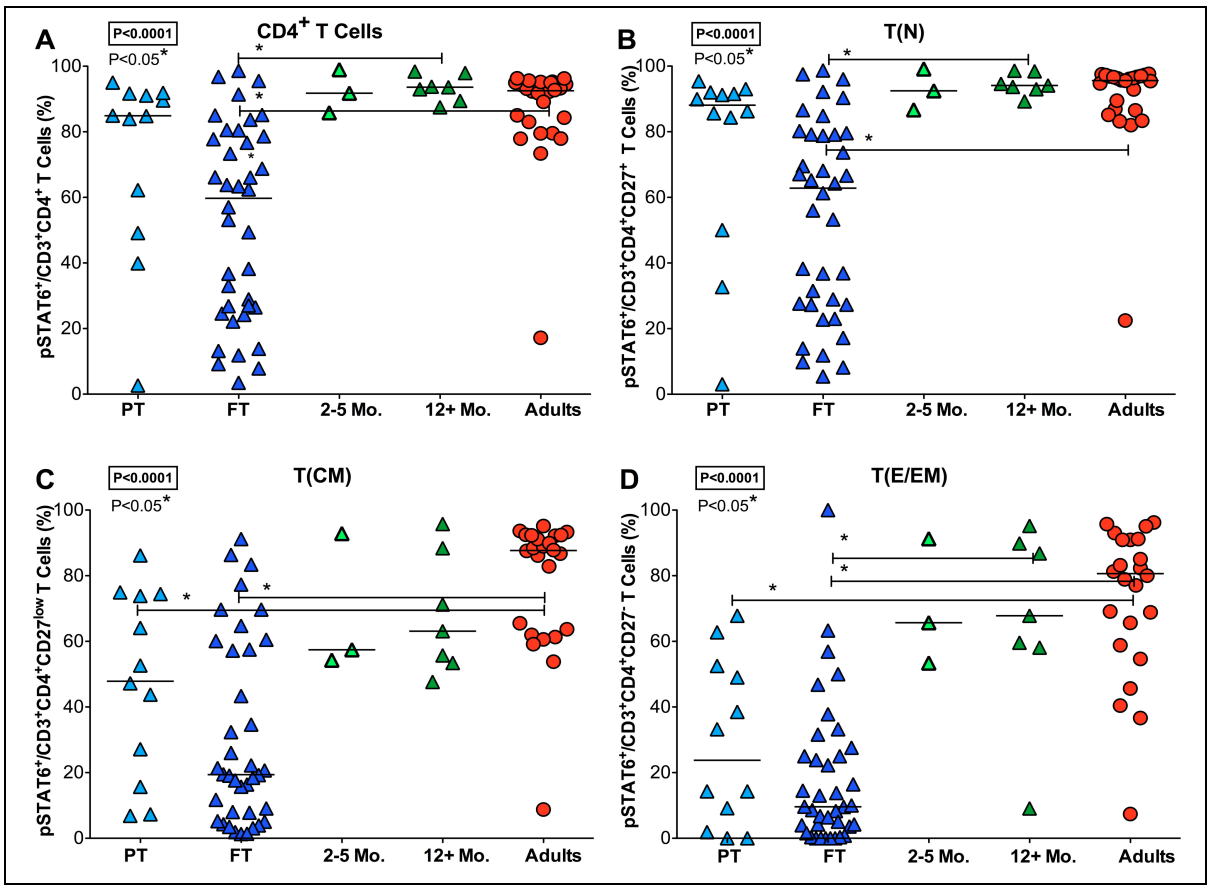

Figure 7: Detection of STAT6 phosphorylation (pSTAT6) at birth. Shown are percentages of STAT6 positive cells within the A. Total $CD4^+$ T cell population, B. T(N) $CD4^+$ T cells. C. T(CM) $CD4^+$ T cells and D. T(E/EM) $CD4^+$ T cells of PT, n=12; FT, n=38; 2-5 Mo., n=3; 12+ Mo., n=7; and adults, n=26. Whole blood from these samples were treated with or without recombinant rhesus IL4 (0.1μg/ml) for 15 min, fixed, permeabilized and stained for Phosflow. Statistical analysis, legends and other labels are as described in Figure 4.

Responsiveness to IL2 is age-dependent

Interleukin 2 is a growth factor required for the differentiation and expansion of both Th1 and Th2 cells. We sought to determine whether the deficiency to develop adequate Th1 responses might also be due to the reduced ability of infant $CD4^+$ T cells to respond to IL2. We evaluated 2 of the 3 subunits of the IL2 receptor complex, α and β chains, respectively corresponding to the binding and signaling chains. We found that the frequency of IL2Rα-expressing $CD4^+$ T cells was significantly higher in the adults compared to FT infants at birth (Figure 8A). Interestingly, $CD4^+$ T cells at birth from PT infants had even higher frequencies of IL2Rα-expressing $CD4^+$ T cells than FT infants (Figure 8A-D). Furthermore, there was a significant difference in IL2Rα expression in PT infants at birth and adult $CD4^+$ T cells within the T(CM) but not in the T(N) and T(E/EM) compartments. Based on the number of receptors on the $CD4^+$ T cell surface, we observed significantly higher numbers of IL2Rα in T(N) and T (CM) $CD4^+$ T cells at birth of PT infants compared to adults (Figure 8F&G) whereas, only within the T(E/EM) compartment that we observed higher numbers of IL2Rα in FT infants compared to adults (Figure 8H). Interestingly, infants that were 2-5 months of age had significantly more IL2Rα compared to adults in T(N) and T(CM) compartments. The frequency T(N) $CD4^+$ T cells expressing the signaling chain, IL2Rβ, in the PT infants appeared higher than in adults (Figure 9B). However, the absolute number of IL2Rβ on a per cell basis was not significantly different across the different age groups (Figure 9E-H).

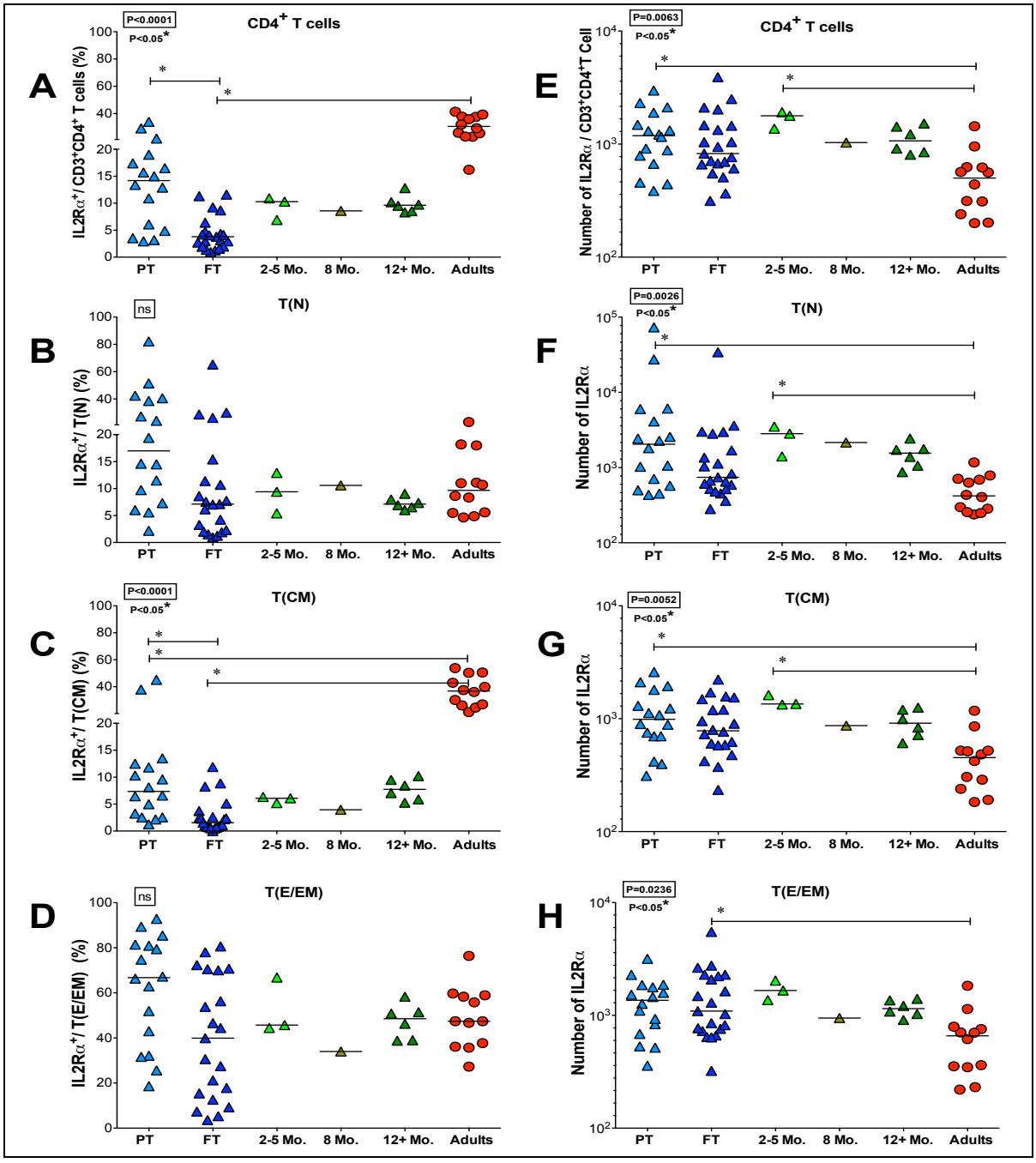

Figure 8: Frequencies and absolute numbers of IL2Rα on CD4$^+$ T cell subsets. The legend is as described in Figure 1 with the following sample numbers: PT, n=16; FT, n=21; 2-5 Mo., n=3; 8 Mo., n=1; 12+ Mo., n=6; and adults, n=12 were isolated and stained for flow cytometric analysis.

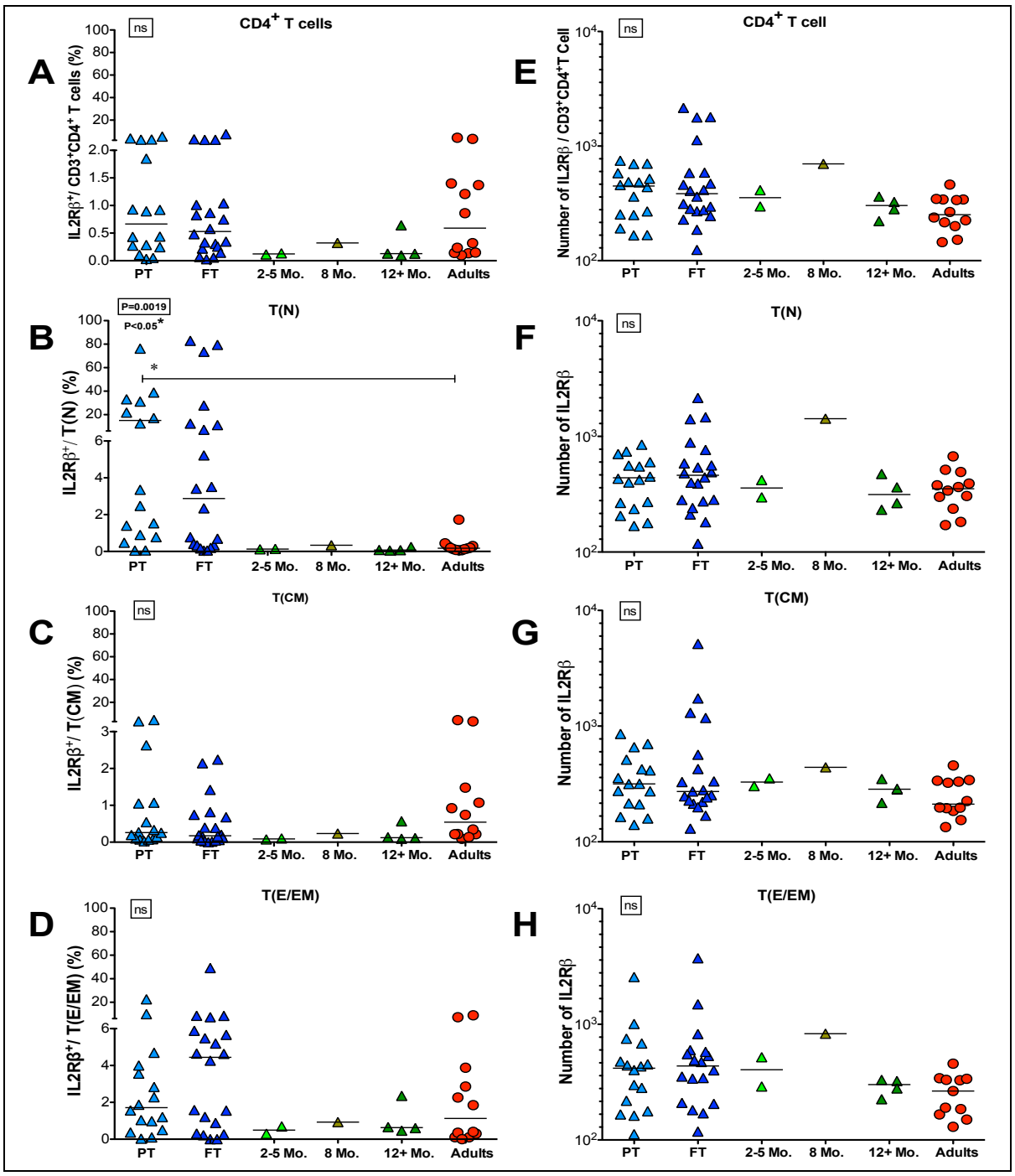

Figure 9: Frequencies and absolute numbers of IL2Rβ on CD4$^+$ T cell subsets. The legend is as described in Figure 1 with the following sample numbers: PT, n=16; FT, n=20; 2-5 Mo., n=2; 8 Mo., n=1; 12+ Mo., n=4; and adults, n=12 were isolated and stained for flow cytometric analysis.

Unexpectedly, infants at birth (CB) had a significantly lower ability to phosphorylate STAT5 (pSTAT5) in response to IL2 even though infants had more IL2Rα on the cell surface and similar frequencies of IL12Rβ positive $CD4^+$ T cells and similar numbers of IL2Rβ per $CD4^+$ T cell. In particular, both PT and FT infants at birth had significantly lower pSTAT5 compared to adults in total $CD4^+$ T cells and within the different $CD4^+$ T cell subsets (Figure 10-A-D). Moreover, we found that STAT5 activation across the different subsets was significantly higher in 12+ month old infants compared to FT infants at birth. Similar to IFNγ responses, the ability to respond to IL2 seemed to increase with age as 2-5 month old infants started to gain the ability to respond to IL2. Although not statistically significant, even at 12+ months of age, infant $CD4^+$ T cells still showed a trend towards lower ability to activate STAT5 compared to adult $CD4^+$ T cells. Together with our receptor data, our findings indicated differential activation of the IL2 associated transcription factor STAT5 on infants and adult $CD4^+$ T cells and subsets, suggesting a correlation of $CD4^+$ T cell maturity and age. The decreased ability to respond to a potent T cell growth factor may direct the infant's immune responsiveness away from Th1.

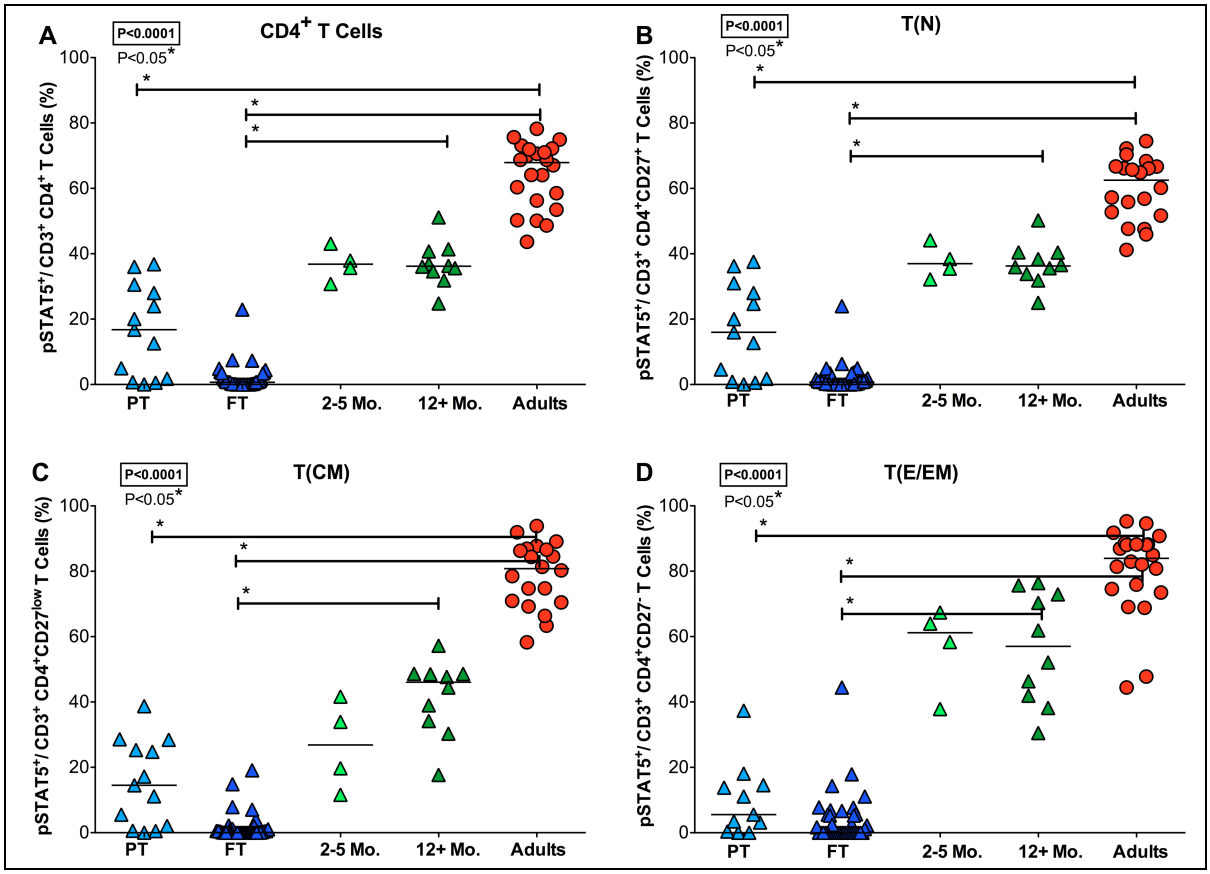

Figure 10. STAT5 phosphorylation (pSTAT5) is increased with age. Shown are percentages of STAT5 positive cells within the A. Total $CD4^+$ T cell population, B. T(N) $CD4^+$ T cells. C. T(CM) $CD4^+$ T cells and D. T(E/EM) $CD4^+$ T cells of PT, n=13; FT, n=38; 2-5 Mo., n=4; 12+ Mo., n=10; and adults, n=22. Whole blood from these samples were treated with or without recombinant human IL2 (0.1µg/ml) for 15 min, fixed, permeabilized and stained for Phosflow. Statistical analysis, legends and other labels are as describe in Figure 4.

Addition of exogenous IFNγ resulted in unique gene and protein expression between TCR stimulated infant and adult CD4$^+$ T cells

The combined effect of TCR and cytokines will result in the activation of various signaling pathways and transcription factor networks which will act in synergy to promote cellular differentiation and specialized patterns of gene expression in differentiated cells (8), (104). Thus, our last attempts to unravel the reasons for the diminished Th1 activity in infants were to determine the resulting gene and protein expression between infant and adult CD4$^+$ T cells after *in vitro* stimulations. We activated CD4$^+$ T cells from FT infants at birth and adults under 2 conditions: i) directly through the TCR only by using anti CD3 and CD28 antibodies and ii) a combination of stimulation through the TCR and exogenous IFNγ (added immediately at the start of culture). Our data showed that the combination of the stimuli seemed to have an additive effect, especially in the induction of genes encoding IFNγ, IL2 and CXCL9. Notably, the combined stimulation induced a broad range of genes related to the JAK/STAT signaling pathway including STAT-induced genes and negative regulators (Figure 11). Moreover, genes involved in the immune response and cell growth/maintenance were also differentially expressed in FT infant and adult CD4$^+$ T cells. Statistical analysis was not performed due to low numbers.

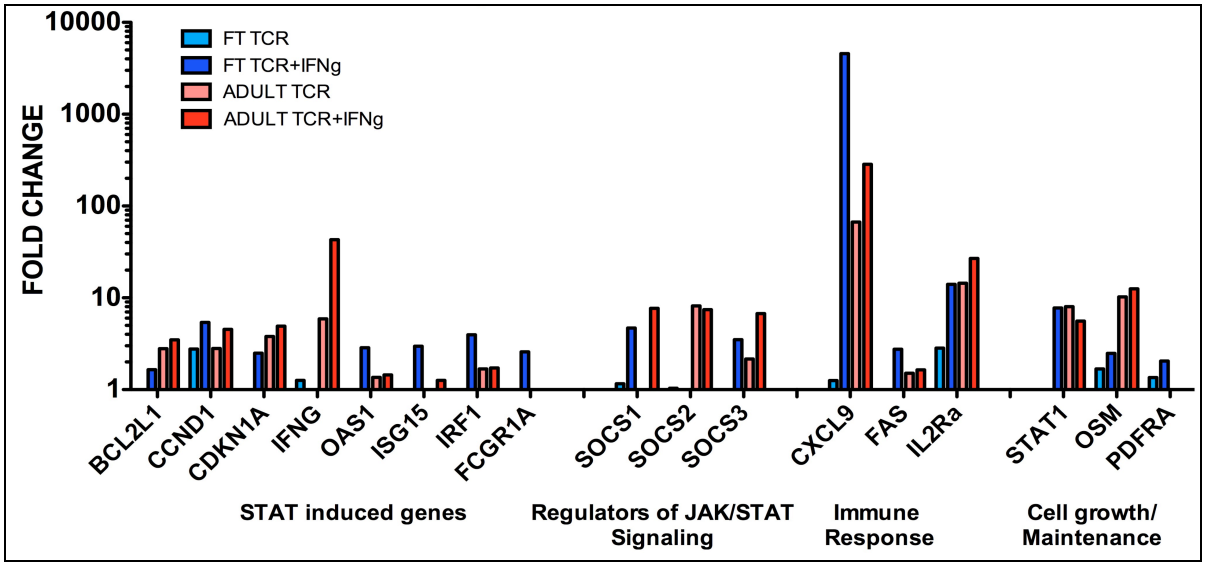

Figure 11. Infant and adult purified CD4$^+$ T cells induced distinctive genes related to JAK/STAT signaling. Purified CD4$^+$ T cells from full term (FT) and adults were stimulated through the TCR using plate bound anti CD3/28 only (FT n=2, light blue and adult n=1, light red) and TCR with exogenous recombinant human IFNγ (FT, n=3; dark blue or adults, n=2; dark red) and cultured for 24 hr. RNAs were extracted, processed and analyzed as described in Materials and Methods.

Supernatants from TCR only and TCR+IFNγ stimulated infant and adult CD4$^+$ T cell cultures were tested for a panel of cytokines and chemokines to determine changes in cytokine production. Infant CD4$^+$ T cells generally produced lower amounts of cytokines than adults (Figure 12). Upon stimulation through the TCR only, every marker analyzed was expressed at lower levels in infant CD4$^+$ T cell cultures compared to adults, except PDGF-AA (Figure 12A-B). The addition of IFNγ to TCR stimulation resulted in the up-regulation of most cytokines and chemokines in the infant CD4$^+$ T cell cultures compared to TCR only stimulation. The combined stimulation through the TCR and IFNγ overcame some differences in the production of certain cytokines or chemokines (e.g. IL8, IP-10) by infant and adult CD4$^+$ T cells while others (e.g. IL15, TGFα) were still produced at lower levels

(Figure 12A&B). Moreover, the exogenous addition of IFNγ with TCR stimulation resulted in the up-regulation of some Th1 and Th2 –associated cytokines like IL2, and IL10, Th17-associated cytokine IL17A, and pro-inflammatory cytokines such as TNFα, IL1α and IL1β. However, statistical analysis was not performed due to small number sizes and data on IFNγ only stimulations were not available for comparison. Our data did reveal that the combination of TCR and exogenous IFNγ stimuli on infant and adult $CD4^+$ T cells resulted in a broad up-regulation of distinctive genes and protein productions. The resulting cellular responses may have implications in the preferential development of certain $CD4^+$ T helper cell subsets in infants.

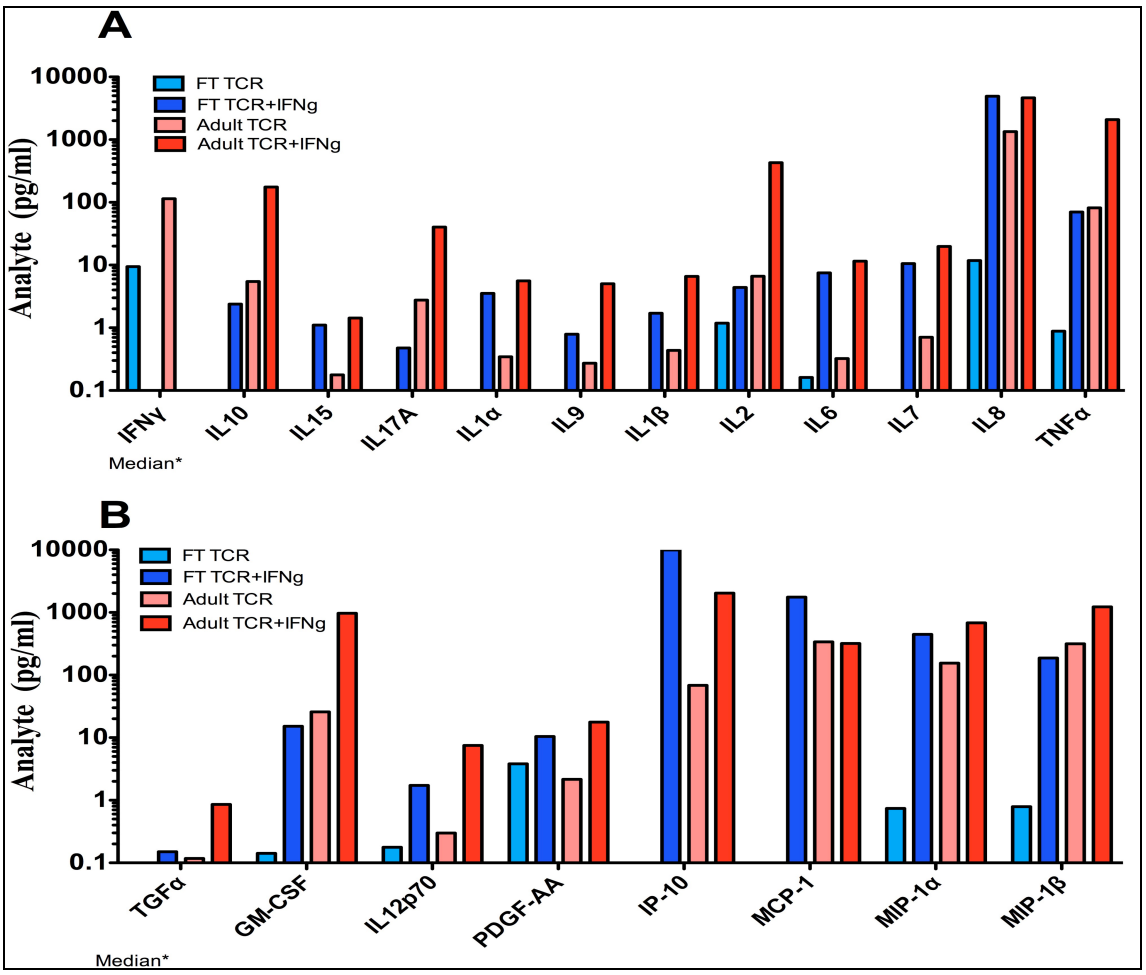

Figure 12: Purified infant and adult CD4$^+$ T cells induced a unique blend of cytokines and chemokines after *in vitro* stimulation. Purified CD4$^+$ T cells from FT infants and adults were stimulated through the TCR using plate bound anti CD3/28 only (FT n=3, light blue and adults n=4, light red) and TCR with exogenous recombinant human IFNγ (FT, n=4; dark blue or adults, n=4; dark red) and cultured for 24 hr. Supernatants were assayed using Luminex technology to measure A. cytokines and B. Chemokines as described in Materials and Methods.

DISCUSSION

In this study, we tested whether IFNγ, IL4 or IL2 -receptor mediated signaling through the JAK/STAT pathway could provide novel insights into the preferential Th2 development and diminished Th1 response of infant $CD4^+$ T cells. Reduced ability to respond to IFNγ, IL4, and IL2 may have broader impact as these cytokines and their respective transcriptional network are involved in epigenetic modifications affecting $CD4^+$ T cell differentiation. We postulated that the preferential development of Th2 response in infants is due to reduced activation of the components of IFNγ-receptor mediated JAK/STAT signaling machinery to appropriately induce Th1 development. We have now shown that the diminished Th1 activity and Th2-biased in infants could be attributed to the differential processing of signals initiated by IFNγ and IL4. IL4 responsiveness and to a lesser degree IFNγ, was readily detected at birth. Additionally, our data on IL2 responsiveness indicated that infant $CD4^+$ T cells have reduced ability to respond to a potent T cell growth factor, potentially affecting the expansion of naïve and differentiated $CD4^+$ T cells. Approximately at 2-5 months of age, infants gained the ability to respond to both IFNγ and IL2. Our findings could be summarized as follows: 1) Cytokine signaling is reduced in infant compared to adult $CD4^+$ T cells, 2) Altered cytokine signaling is due to multiple factors, including receptor expression, activation of kinases (JAKs) and phosphorylation of cytoplasmic transcription factors (STATs), 3) Gradual cytokine responsiveness is associated with age and differentiated status of $CD4^+$ T cells, 4) Kinetics of cytokine responsiveness is cytokine specific and 5) Gestational age influences the ability of infant $CD4^+$ T cells to respond to cytokines. Figure 13 schematically illustrates these findings.

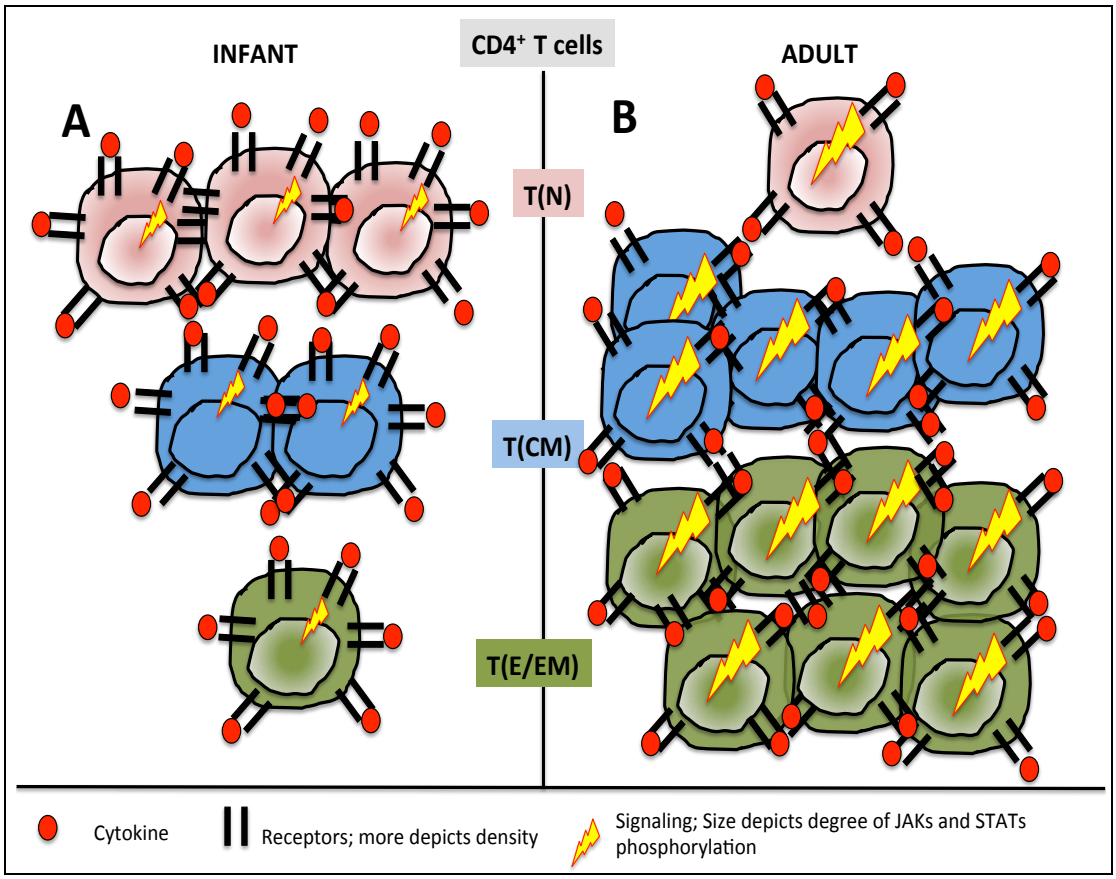

Figure 13: Altered cytokine-receptor signaling in infant and adult CD4$^+$ T cells affect the generation of Th1 immunity. A. The pool of infant CD4$^+$ T cells predominantly consist of naïve T cells (T(N)) compared to higher presence of differentiated or memory (T(CM) and T(E/EM)) CD4$^+$ T cells in B. adults . When cytokines like IFNγ (red circles) bind to receptors, infants CD4$^+$ T cells have higher density of Th1-associated receptors (depicted as 2 black lines) compared to adults but have reduced signaling capabilities (JAKs and STATs) (depicted as a lightning bolt). Together with the lower frequency of memory and differentiated CD4$^+$ T cells, the net cytokine responsiveness in infants is weak compared to adults affecting the development of Th1. Gradual increase in cytokine responsiveness is associated with increasing age.

One caveat of our study was that frequencies and absolute number of receptors were assessed on resting $CD4^+$ T cells. It is likely that Th1 or Th2-associated receptors will have differential kinetics under polarized conditions. Other studies had determined that IFNγR1 expression and distribution is ubiquitous, but within T cells, IFNγ-STAT1 signaling is detected only in naïve and polarized Th2 cells as they express the signaling chain IFNγR2 (120), (109). Moreover, Th1 cells do not express IFNγR2 due to reduced gene transcription but these cells can re-express IFNγR2 after TCR stimulation. Interestingly, there are also reports on IFNγ mediating the death or proliferation of T cells and other IFNγR2 expressing cells (17). Though expression of IFNγR2 may result in death of T cells, IFNγ derived from $CD4^+$ T cells is sufficient to induce the development of Th1 cells (151). In our study, it was difficult to determine whether infant or adult $CD4^+$ T cells and subsets lost IFNγR2 expression as the receptor was immediately assayed after cell isolation. Additionally, our data are still incomplete, as we still need to show that PT and older infants have lower or higher JAK2 activation. Acquiring samples from these age groups and increasing our numbers are currently underway in the lab.

Studies looking at cytokine signaling in other immune cells showed that neonatal macrophages (MΦ) had a deficient ability to respond to IFNγ (89). Their data were not attributed to the lower IFNγ receptor expression on MΦ, or total STAT 1 protein levels, but instead due to defective phosphorylation of STAT1. The reason of lower STAT1 phosphorylation was not determined but our data suggested that a possible defect is upstream of STAT1. We found that JAK2, one of the JAK kinases associated with the IFNγ receptor complex, showed lower phosphorylation in $CD4^+$ T cells of FT infants than adults. Our data showed that within 10 min and 15 min, lower activation of JAK2 and STAT1 by IFNγ

treatment, respectively, was observed in infants, demonstrating that though infants have the necessary receptors present on the $CD4^+$ T cell surface or even higher compared to adults, the frequency of cells able to respond to IFNγ might not be sufficient to exhibit a detectable response. Indeed, infants compared to adults have much lower frequencies of differentiated $CD4^+$ T cells (unpublished observations).

We confirmed in our studies through IL4-signaling that infants preferentially develop a Th2-biased response. We have shown that the ratio between IL4Rα and IFNγR2 distinctly favored Th2 in infants. Moreover, after IL4 treatment, phosphorylation of downstream signaling component, STAT6, was readily detectable in PT and FT infants in all $CD4^+$ T cell subsets, consistent with having higher numbers of IL4Rα per $CD4^+$ T cell. Interestingly, research showed that IL4 receptor expression could be modulated by intracellular signaling pathways and by the cytokine itself at both the transcriptional and post-transcriptional level (40), (97). The propensity of infant $CD4^+$ T cells to respond to IL4 by pSTAT6 supports their bias towards a Th2 response, as IFNγ treatment did not result in similarly high frequencies of pSTAT1 positive $CD4^+$ T cells. However, STAT6 activation was highly variable with some FT infants having much lower responses compared to older infants and adults. There might be other underlying mechanisms controlling these variable responses in the FT infants at birth. Future experiments need to determine the relevant JAK kinase (JAK1) involved to completely define this IL4-STAT6 signaling axis in infants. We expect to find variable degrees of JAK1 phosphorylation after IL4 treatment in infant $CD4^+$ T cells regardless of gestation.

Observations in our lab showed that infant $CD4^+$ T cells are able to produce IL2 and initial expansion was evident as assessed by Ki67 expression during the first weeks of life

(unpublished results). These observations suggested to us that infant $CD4^+$ T cells should have similar IL2 responsiveness as adults. Thus, we wanted to determine the extent of IL2 involvement in Th1 development. Unexpectedly, infant $CD4^+$ T cells showed reduced responsiveness to IL2 compared to adults. We found that IL2Rα but not IL2Rβ expressing $CD4^+$ T cells were higher in the adults compared to infants. However, infants had significantly higher absolute numbers of IL2Rα per cell, but no difference was observed in the IL2Rβ chain. When stimulated with IL2, infant $CD4^+$ T cells showed drastically less STAT5 phosphorylation compared to adults. However, there was a trend of increasing IL2 responsiveness because older infants had more pSTAT5 positive $CD4^+$ T ells than FT infants. Research also showed that infants have a higher frequency of regulatory T cells and these cells are phenotypically recognized to express $CD25^{high}$, or also known as IL2Rα (52), (160), (56). Early studies in the field also demonstrated that IL2 is a strong IFNγ-inducing cytokine in human T cells (69), (148). Our gene and protein expression data showed that after stimulation through the TCR and IFNγ, slightly higher expression of *Il2ra* gene and protein was observed in purified adult than in infant $CD4^+$ T cells. Moreover, IL2 has a broader role in T helper differentiation as it has the ability to modulate expression of key cytokine receptors involved in both Th1 and Th2 specificity and maintenance (81), (80). Reduced responsiveness to both IL2 and IFNγ cytokines could result in an additive effect and may lead to the Th1 diminished response in infants.

In conclusion, our findings have demonstrated a role of cytokine-receptor mediated signaling in infant immune development and maturation especially in the $CD4^+$ T cell response. Nupponen et al had described multiple aberrations in signaling profiles in CB lymphocytes but cytokine signaling data in specific $CD4^+$ T cell subsets in the developing

infant are still lacking (102). We showed that receptiveness of infant and adult $CD4^+$ T cells and subsets to cytokines resulted in a distinct activation of the JAK/STAT signal transduction network. Such transduction networks act in synergy with other signals such as those experienced by naïve $CD4^+$ T cells after encountering their cognate antigens. Differentiation of these cells to Th1 or Th2 will involved epigenetic changes at the chromatin level that may affect the *Ifng,* and *Il4* locus making them accessible to number of Th1 or Th2 lineage-specifying transcription factors (158), (12). The findings in our study provided additional molecular mechanisms that elucidated the unique immune status of infants, especially in regards to the altered function of infant $CD4^+$ T cells. Delineating the immune mechanisms that control or modulate $CD4^+$ T cell responsiveness and function in the unique infant environment will be important in both biological and immunological processes. Our findings may be of value in formulating pediatric vaccines and therapeutics that are protective early in life.

CHAPTER 5: SUMMARY, SIGNIFICANCE, FUTURE DIRECTIONS AND CONCLUSIONS

SUMMARY OF FINDINGS

The research in our lab is centered on pediatric infectious diseases. Our goal is to determine why disease outcome is often more severe in infants compared to adults. During the window of time when passive immunity from the mother wanes and the infant's immune cells and their functions still develop, infants are especially vulnerable to many diseases. My research project was aimed at defining the role of infant $CD4^+$ T cells in viral pathogenesis. The infant immune system is generally described as being immature. I examined the term "immature" by assessing what functional properties of $CD4^+$ T cells are different in infant and adults and determined whether differences in $CD4^+$ T cells function could impact viral pathogenesis. Our primary question was: When do infant immune cells reach maturity and function equally well as adult cells? Toward this objective, I pursued several different projects covered in Chapters 2, 3 and 4.

The study described in Chapter 2 was part of a larger collaborative effort to define the potential impact of chronic RhCMV infection on the host immune system. My role was to investigate the immune development during the first year of life. Access to infant human samples in infants is extremely limited, so our lab took advantage of the rhesus macaque model to define normal immune development from birth to 1 year of age. The NHP model enabled us to collect longitudinal blood samples and document changes in frequencies and maturation of various lymphocyte populations. In this study, I was able to document $CD4^+$ T cell development during the first year of life. I showed that although IFNγ responses were

drastically reduced in $CD4^+$ T cells at birth, they gradually increased during the first year, consistent with the transition of naïve $CD4^+$ T cells to effector and effector memory T cells. Interestingly, frequencies of $CD8^+$ T cells producing IFNγ were similar from birth throughout the first year of life. Therefore, my data indicated that IFNγ production by infant $CD4^+$ T cells is perhaps restricted by $CD4^+$ T cell-intrinsic factors. In the fight against infectious diseases, it is important to know when $CD4^+$ Th1-IFNγ responses mature because such responses are critical in the clearance of most intracellular pathogens. In fact, it is this lack of Th1 responses that results in the Th2 bias of the infant immune response. Thus, determining the time frame during which Th1 responses mature in infants will have great implications in vaccine designs, implementation strategies and immunization schedules. Our longitudinal studies were a first step in this process.

The significance of these findings was underlined by an *in vivo* RhCMV-infection study. Chapter 3 described the data we found on a smaller longitudinal study following experimentally RhCMV-infected infant and adult animals. Our goal was to establish an oral infection model of RhCMV in infant macaques to model oral HCMV acquisition by breast milk or by contact with saliva in human infants. My data showed that infant monkeys were easily infected after a single exposure, whereas 2 of 3 adult macaques required multiple exposures of RhCMV via the oral route. Moreover, RhCMV-infected infant monkeys had decreased ability to control viral replication as they frequently shed RhCMV in saliva and urine while adult monkeys only transiently shed the virus in the same compartments. Lack of viral control might be the result of insufficient immune response in infants as best illustrated by the rapid progression of HIV-infected infants compared to adults (Prendergast Andrew 2012). Our work in the NHPs made it possible to study the interplay of age and a chronic

viral infection in the development of the immune system and allowed us to study viral pathogenesis in infants from birth to 1 year of age.

In the same study, Chapter 2 also evaluated juveniles and adult macaques of SPF and non-SPF status, where non-SPF animals were naturally infected with RhCMV. We wanted to determine how the function of the immune system is perturbed by the presence of RhCMV. We found that age-related changes in immune cell populations in the peripheral blood were common in animals regardless of SPF status. Moreover, frequent shedding of RhCMV in the bodily fluids was more readily detected in non-SPF juvenile compared to adult monkeys. RhCMV infection appeared to influence the maturation of both T and B cells and altered T cell responsiveness. Compared to adult monkeys, non-SPF juveniles showed a propensity towards higher inflammatory cytokine production after specific and non-specific stimulation. Lifelong exposure to inflammatory cytokines due to a chronic viral infection like RhCMV or HCMV in humans could affect the generation of effective immunity against novel pathogens and increase the host's susceptibility to tissue damage. Rhesus macaques could serve as a valuable tool to model certain immunological alterations such as immunosenescence and "inflammaging" in relation to the host's age, health and disease status.

I have determined that IFNγ response in infant rhesus monkeys increase throughout the first year of life (Chapter 2) and I indirectly showed the importance of Th1- IFNγ response in RhCMV control in infants (Chapter 3). Given these findings, I wanted to know whether comparable and supporting data regarding potential mechanisms could be observed in human infants. Specifically, I was interested in determining the mechanisms that control IFNγ responses and the factors important for Th1 differentiation. Chapter 4 followed up on the above findings and took them a step forward by asking whether infant $CD4^+$ T cells could

respond to exogenous IFNγ. These studies were done with human infant blood samples. I performed a detailed analysis of the components of the IFNγ signaling pathway to evaluate both IFNγ responses and the factors involved in Th1 differentiation. My results clearly show that activation of the IFNγ signaling pathway in infant $CD4^+$ T cells is reduced at multiple levels and is significantly lower compared to adult $CD4^+$ T cells. This reduced response was not due to lack of IFNγ receptor expressing $CD4^+$ T cells, but was due to reduced activation of the JAK2 kinase and the transcription factor STAT1. I was able to demonstrate though that signaling defects can be overcome by 2-5 months of age. In contrast, I could show that IL4 signaling was not impaired. Unexpectedly, I also determined that infant $CD4^+$ T cells show a drastically reduced response to IL2. In fact and in contrast to IFNγ, IL2 signaling still resulted in lower activation of the relevant transcription factor STAT5 by 1 year of age. I obtained similar data for IL7, another important T cell growth factor.

Though not covered in this thesis work, I contributed significantly to a project that tested the safety and immunogenicity of a novel pediatric combination vaccine with the goal to prevent HIV/SIV and TB infection in infants. The vaccine was found to induce both SIV and TB-specific immune responses in infant rhesus macaques. My work was acknowledged by being a 2^{nd} author on a resulting publication (64).

SIGNIFICANCE

Research data regarding cytokine signaling in the developing infant have been limited and the data available have not thoroughly explored such signaling pathways in infant $CD4^+$ T helper cells and subsets (102). The data from this work provided evidence that during infancy, there are intrinsic factors (e.g. cytokine signaling) underlying the reduced

performance of infant immune cells (e.g. $CD4^+$ T cells) in generating effective immunity against intracellular pathogens. From birth, adulthood and to aged, the host's immune system has to strictly balance pro and anti-inflammatory cytokines to effectively deal with infectious pathogens and avoid detrimental damage to the host's tissues. Identifying and targeting the elements that contribute to the reduced immune responsiveness of infants are important to protect these young hosts from infectious diseases. When the host is aged, the overall performance of the immune system is changed leaving the host susceptible to novel pathogens and tissue pathologies. At these extreme stages of life, understanding the intricate network of factors that shape the immune responses in the infant and aged environment will be imperative in optimizing vaccines and targeted therapeutic strategies.

REMAINING QUESTIONS AND FUTURE DIRECTIONS

More longitudinal and comparative studies are still needed in defining the exact mechanisms leading to distinct immune responses of infants compared to adults. Specifically, in Chapter 2, some of the questions worth examining are (i) Do chronic infections such as HCMV/RhCMV result in T and B cell changes at the molecular level? (ii) Can these changes be detected early and corrected through therapeutic means? (iii) Importantly, in humans, are there genetic markers that can identify aged people that are at risk for "inflammaging"? And lastly, (iv) what triggers RhCMV reactivation in the young and old? In Chapter 3, we described that infants were more susceptible to RhCMV infection via the oral route. What host immune factors in the infant oral cavity are involved in increasing RhCMV susceptibility? Similarly, what immune responses or lack thereof are responsible for the persistent viral replication in certain body compartments (e.g. oral and urogenital tract)? Such questions are still open for exploration. We also expect that increasing our animal numbers

and choosing a more appropriate age group that best represent adult humans will offer significant differences in the quality and magnitude of RhCMV-specific responses. The findings in cytokine signaling presented in Chapter 4 are incomplete because differences in phosphorylation of the relevant JAK kinase for IL4 and IL2 in infant and adult $CD4^+$ T cells have not been evaluated. Efforts in measuring JAK1 phosphorylation after IL4 or IL2 treatment are underway in the lab. Increasing our sample numbers between birth and 1 year of age will be critical in defining time frames when infant $CD4^+$ T cells will be functionally competent as adults.

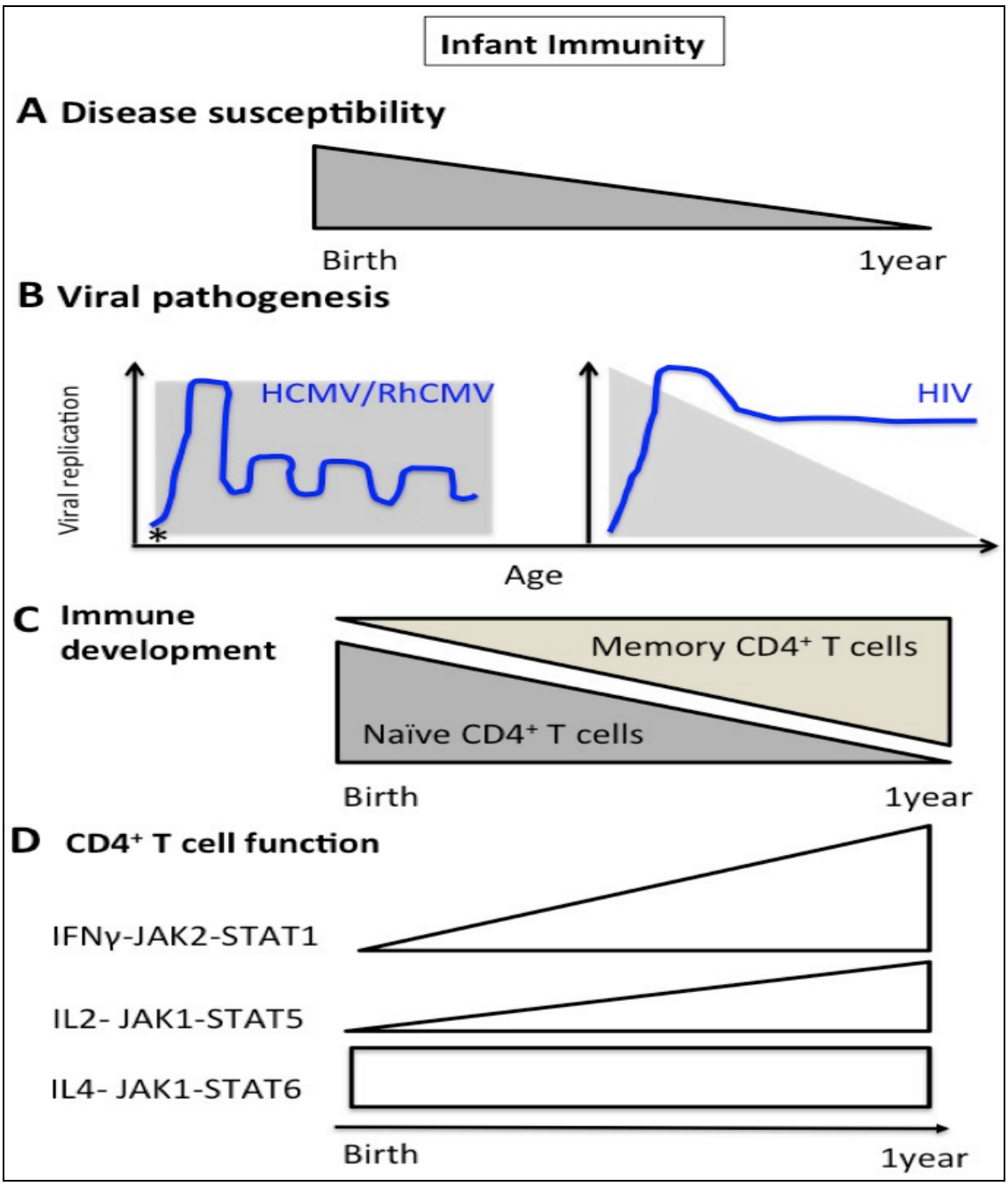

Figure 1: Graphical representation of infant immunity. This picture highlights the dynamic relationship of age and the maturity of the immune system in infectious disease control. A. Disease susceptibility of infants decreases with age as immune function increases. B. Distinct viral pathogenesis are observed between HIV and HCMV/RhCMV because the latter is rarely fatal and overall infant health is preserved (depicted by the shaded area) unless infection was acquired in *utero* or under immune suppression* C. As infants become older,

the frequency of naïve $CD4^+$ T cells decreases overtime with concurrent increase of memory $CD4^+$ T cells, consistent with increasing antigen exposure with age. D. Age-dependent maturity of the components of IFNγ, IL2, and IL4 signaling are critical for the proper development and function of effector $CD4^+$ T cells to effectively combat infectious diseases.

CONCLUDING REMARKS

Fine-tuning in the components of the immune system must be achieved to successfully face the unique challenges encountered at different stages of life. My thesis work focused on evaluating the functional properties of $CD4^+$ T cells early in life and how their function changes in relation to age (Figure 2). My studies found that age-related differences are detected in the infant and adult $CD4^+$ T cell development, maturation, function and signaling in normal setting. Perturbations, such as caused by viral infections, will likely affect these processes. It is of great interest to understand the dynamic relationship between age and $CD4^+$ T cell immunity to identity which immune parameters in infants can be used as "immune signatures" to evaluate vaccines and therapeutics as means to develop appropriate and effective immune function.

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