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LOYOLA UNIVERSITY CHICAGO

CELL INTRINSIC CHARACTERISTICS OF CORD BLOOD NAÏVE CD4 T-CELLS

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

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CHICAGO, ILLINOIS

AUGUST 2018

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ABSTRACT

The neonatal immune system is functionally distinct from the adult immune system. Neonatal immune responses are less reactive than their adult counterparts, and as such, have an increased susceptibility to infection, resulting in increased duration and severity of disease. There is evidence that T-cells are partially responsible for the observed immunological differences between neonates and adults. Murine studies on neonatal immunity suggest that Th2 like responses predominate the neonatal immune system, an observation not found in the adult immune system. However, studies on human cord blood revealed that human neonates do not exhibit elevated Th2 like cytokines (with the exception of IL-13) when compared to adults, raising the question of whether or not neonatal T-cells are fundamentally different from adult T-cells. We sought to explore potential differences between neonatal and adult T-cells through the comparative analysis of naïve CD4 T-cells derived from cord blood and adult peripheral blood. Our data reveals that cord blood naïve CD4 T-cells have significantly higher expression of CD26. Additionally, cord blood T-cells have a greater capacity to produce Th1/Th2 cytokines than adult T-cells when prompted to differentiate into effector cell types. Additionally, undifferentiated naïve T-cells from cord blood are less responsive to stimulation than adult T-cells, however this decreased activity is likely not due to reduced expression of signaling molecules in neonatal naive T-cells.

CHAPTER ONE

INTRODUCTION

Neonatal Immunity

The perinatal immune system has been demonstrated to be distinct from the adult immune system[1]. The immune system of the perinate is less reactive than their adult counterparts, and as such, has an increased susceptibility to infection, resulting in increased duration and severity of disease[2]. Furthermore, infections are the leading cause of death in newborn babies[3]. This increased susceptibility to infection is attributed to unresponsiveness of the adaptive immune response and deficiencies in the innate response to infection. The adaptive immune response is highly dependent on T-cell function. T helper cells (Th) are responsible for activating cell mediated responses, through CD8 T-cells, and humoral responses, through B cells. Neonatal T-cells have been described as tolerogenic and anti-inflammatory, and as such, the adaptive immune response contributes little to host protective immunity, leaving the neonate heavily reliant on a functional innate response to fight off infection[4].

Despite their reliance on proper function of the innate immune system, neonates have deficiencies in key players, leaving them highly susceptible to prolonged and severe infections. When compared to adults, neonates have deficiencies in neutrophil response to infection through smaller cell numbers, decreased cell mobilization, and functional deficiencies[5,6]. In addition, neonates have reduced levels of major histocompatibility complex (MHC) class II expression on monocytes, CD80 and CD86 (together referred to as B7) ligands on monocytes and dendritic

cells, downregulation of genes in pathways involved with innate activation, and critical innate cytokine production[7]. These deficiencies not only leave them vulnerable to severe infection and sepsis, but they also contribute to reduced activation of T-cells – as evident by the reduction in MHC class II and the co-stimulatory B7 expression.

However, the unresponsiveness observed in the neonate has been revealed to play a crucial role in their development and can be referred to as tolerogenic[8]. Perinatal tolerance was first described by Ray Owen, whose observations about twin fetal bovine red blood cells and their unresponsiveness to previously encountered antigen later in life suggested that immunological tolerance to foreign cells may occur before birth[9]. It soon became apparent that the development of tolerance to an antigen was dependent on the timing of the exposure. Specifically, antigenic exposure during immune development may result in immunological tolerance to that antigen later in life. Medawar *et al*. played a crucial role in this revelation through their research. They demonstrated that mice could accept skin grafts from a different strain if they had previously been inoculated *in utero* with cells from the future donor strain[10]. The development of tolerance is crucial yet delicate concept, as birth presents immense challenges to the newborn child; requiring them to learn and adapt to environmental antigens and commensal bacteria while at the same time forming protective immune responses against potential pathogens[11].

However, the exact mechanisms explaining the tolerogenic behavior of the neonate and how this tolerance evolves into the reactive nature observed in older children and adults are not well understood. There is gathering evidence, primarily with animal models, that suggests there

are intrinsic differences in neonatal and adult T-cells, and that they are a causative agent for the observed immunological differences seen between the two populations[12]. In fact, there are documented differences between adult and neonatal T-cells, with neonates exhibiting deficiencies in their proliferative response to antigenic stimulation, diminished ability to activate B cells, decreased cytokine production, lesser cytotoxic activity, and have an altered cytokine profile[13-18]. Each factor is contributing to the decreased cellular immune response observed in the neonate. Therefore, we seek to explore potential mechanisms contributing to the diminished activity of neonatal T-cells though the analysis of cell intrinsic characteristics. An intrinsic analysis allows for an isolated study of the neonatal T-cell population in the absence of other cells and molecules that may have unknown effects on T-cell activity. A thorough understanding of the T-cell population alone will provide a baseline understanding of the population that is needed for future analysis of the neonatal immune system as a whole.

T-Cell Development and Differentiation

T-cells are crucial for the immune system to protect the host, and as such, they need to be able to distinguish self from foreign antigens through proper activity of the T-cell receptor (TCR)[19,20]. T-cells learn how to distinguish self from non-self during thymic development, which starts with the migration of lymphoid precursor cells from the bone marrow to the thymus[21]. In the thymus, the majority of T-cell progenitor cells differentiate into mature subsets expressing either CD8 or CD4 and a highly specific αβ TCRs. The generation of and selection for a T-cell population with a viable TCR capable of recognizing antigen is crucial to proper immune function. This selection process results in viable T-cells that have TCRs capable of recognizing

and tolerating self-peptide while eliminating those cells incapable of recognizing peptide with sufficient specificity[22]. This process occurs in two main steps during thymocyte development: positive and negative selection. Initially, double positive (DP) thymocytes, expressing both CD4 and CD8 co-receptors, go through positive selection. The ability of DP cells to recognize self-peptide presented on MHC by thymic epithelial cells ensures they will be positively selected for, while those cells incapable of recognition are removed via apoptosis[23-25]. Not only does the recognition of self-peptide promote their survival, but it also establishes MHC restriction through the differentiation of the DP thymocytes into either CD8 or CD4 single positive (SP) cells depending on their relative affinity for class I or class II MHC[26]. Positive selection is a crucial step for proper T-cell function, as they need to be capable of recognizing self MHC in the periphery as a mature T-cell.

After positive selection, the MHC-restricted T-cells undergo negative selection, which is a process that allows for the removal of autoreactive cells[20,27]. Negative selection is a critical step, as it removes thymocytes that recognize and bind to self-peptides too strongly, only allowing those cells capable of recognizing self, but at lower affinity, survive. The autoreactive cells with high affinity for self-MHC/peptide need to be removed prior to thymic emigration, as they are dangerous to the host. Only a small percentage of the original immature thymocyte pool pass the selection process and are released into the blood and secondary lymphoid tissues. Cells initially released from the thymus are known as recent thymic emigrants (RTEs), which transition into mature naive T-cells after establishing residence within secondary lymphoid organs[28-30].

The defining characteristic of naïve T-cells is that they, unlike memory and effector T-cells, have not yet encountered their cognate antigen in peripheral lymphoid tissue. Naïve T-cells are crucial to the initiation of the adaptive immune response by reacting to new pathogens that the immune system has not previously encountered. Phenotypically, naïve T-cells can be characterized by the surface antigen expression of CD45RA in the absence of the memory CD45RO isoform[31]. Additionally, naïve T-cells have been known to express CD28, CD62L, interleukin (IL)-7 receptor α-chain (CD127), and CCR7, although these are not unique to the naïve pheonotype[31,32]. They also lack the traditional activation markers CD25, CD44, and CD69[32].

However, comparisons between the adult and neonatal naïve CD4 T-cell populations have revealed phenotypic and functional differences between the two populations. Phenotypically, human adult and neonatal naïve T-cells both express CD45RA as well as the co-stimulatory CD28 molecule but differ in the expression of CD38 and the IL-7 receptor α-chain, which are both more highly expressed on neonatal naïve T-cells than adult naïve T-cells[12,33]. There are also differences in the initiation and strength of response to antigenic stimulation, with neonatal naïve T-cells having been characterized as hyporeactive when compared to adults[34]. Neonatal and adult peripheral blood naïve T-cells also differ in their susceptibility to apoptosis, as neonatal T-cells have been shown to be more susceptible to apoptosis, and thus, also have a higher rate of cell turnover than their adult counterparts[12,33,35]. However, neonatal naïve T-cell apoptosis can be prevented via IL-2 cytokine signaling through the γ-chain of the IL-2 receptor[35]. IL-4, IL-7, and IL-15 have also been documented to serve anti-apoptotic functions for naïve T-

cells[12,36,37]. There may be differences in other molecules: nuclear factor of activated T-cells (NFAT) and CD154 (also known as CD40 ligand) between cord and adult naïve CD4 T-cells, but there is controversy about their expression. NFAT expression in neonates is widely debated, with evidence suggesting that NFAT is expressed at lower levels in neonates than adults, while other groups have data suggesting that there is no significant difference in NFAT expression[38-40]. Similarly, CD154 has conflicting data indicating that neonates have deficiencies in CD154 when compared to adults while other groups report higher CD154 expression within the neonatal naïve CD4 T-cell population than those from adults[41-45].

After thymic egress and migration to secondary lymphoid tissues, naïve T-cells encounter antigen presented by antigen presenting cells (APCs) and can differentiate into effector and memory cells through stimulation of the TCR in the presence of cytokines[46-48]. The specific cytokine environment helps with the upregulation and expression of different transcription factors that both determine the type of effector cell and the role that it will play in the immune response[49]. Th1 subsets arise from the presence of IL-12 and interferon-γ (IFNγ) leading to the upregulation of the transcription factor T-bet[50,51]. The Th1 effector population produces tumor necrosis factor-β (TNFβ), IFNγ, and IL-12, and is responsible for multiple cell-mediated immune functions including the activation of cytotoxic T-cells and macrophages[52]. Naïve T-cells may also give rise to Th2 effector cell subsets through the presence of IL-4, which triggers a signaling pathway that ultimately upregulates the transcription factor GATA-3[53-55]. Th2 cells are linked with a humoral type immune response and produce a plethora of cytokines including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13[52]. Another T helper cell subset, Th17 cells, are inflammatory T-cells

induced by the presence of transforming growth factor-β (TGFβ), IL-23, and IL-6, leading to the upregulation of the transcription factor RAR-related orphan receptor gamma (RORγt) and ultimately to the production of IL-17, IL-21, and IL-22[56-59]. The last major T helper effector subtype, induced T regulatory cells (iTregs), have important anti-inflammatory effects through their cytokine production and can also directly suppress other T-cells. iTregs do not originate from the thymus, as natural Tregs (nTregs) do; instead, this subset develops from naïve T-cells that are activated in secondary lymphoid tissues in the presence of TGFβ and IL-2, leading to the upregulation of the Foxp3 transcription factor and the production of IL-10 and TGFβ[60,61]. While there are many effector subsets, it has generally been considered that Th2 like responses predominate neonatal immune system, thus helping to explain the decreased inflammatory responses and tolerogenic immune characteristics associated with neonates.

In 1993, Wegmann *et al*. proposed that pregnant woman develop a Th2 biased immune enviornment[62]. While these Th2 conditions leave pregnant woman more susceptible to intracellular pathogens, it is advantageous because it would help create a more tolerant immune system and thus, be able to permit the presence and development a fetal allograft. This proposal was built upon animal models which showed that immune responses to infection or the addition of the Th1 cytokines IFNγ or IL-2 resulted in fetal death - thus demonstrating the importance of Th2 conditions and the dangers of robust immune response during pregnancy[63]. This hypothesis was strengthened by studies on human PBMCs, collected after spontaneous abortions or during preeclampsia, which reported more Th1 than Th2 cytokine production when compared to PBMCs from healthy pregnant women[64-66]. Based off this proposal, the immune system of the

perinate has been hypothesized to also have a Th2 bias due to their consistent exposure to the maternal Th2-inducing environment[67]. This hypothesis has been supported through the observation that neonates have deficits in the production of Th1 inducing cytokines (IFNγ and IL-12) by neonatal monocytes, macrophages, and dendritic cells in addition to a hyporesonsiveness of neonatal monocytes to IFNγ[68-74]. Murine models have also provided strength to this hypothesis by demonstrating that the neonatal T-cells are poised to endogenously produce Th2 like cytokines and have a propensity to secrete elevated levels of Th2 cytokines and reduced levels of Th1 cytokines when compared to adult mice[75-77].

T-Cell Activation and TCR Signaling

T-cells derived from umbilical cord blood have been shown to have reduced activation in response to antigenic stimulation, as evident by their deficiencies in proliferation and decreased cytokine production[13]. T-cell activation is mediated by the TCR and its interaction with the appropriate MHC on an antigen presenting cell (APC)[78-81]. This interaction is facilitated by the specificity of the TCR, which is determined though DNA recombination events during thymic T-cell development[82,83]. The majority of T-cells contain a heterodimer TCR composed of one α-chain and one β-chain, while a small and functionally distinct population consists of γδ chains[84-86]. The transmembrane αβ TCR heterodimer is non-covalently associated with a signaling complex composed of the heterodimer complexes CD3εγ and CD3εδ and with the homodimer CD3ζζ[87-89]. Together, these molecules form the TCR-CD3 complex and are essential for T-cell activation through signal transduction[90,91]. Activation events may occur after T-cell recognition of specific antigen within the context of MHC[92,93].

Signaling events are largely mediated by several proteins and immunoreceptor tyrosine-based activation motifs (ITAM) present on the cytoplasmic tails of the signaling molecules CD3 and ζ chain but can also be found on other molecules not confined to the TCR complex, such as the gamma subunit of the Fc receptor[94-96]. After the tight binding of the T-cell to the MHC-peptide complex, receptor clustering results in the phosphorylation of ITAM tyrosines by Src family kinases such as the proto-oncogene tyrosine-protein kinase Fyn (Fyn) or the T-cell specific lymphocyte-specific protein-tyrosine kinase (LCK)[97-100]. The ability of LCK to phosphorylate ITAM motifs occurs after its recruitment and subsequent activation by the phosphatase CD45[101,102]. The phosphorylated ITAM motifs act as a docking site for ζ-associated protein of 70 kDa (Zap70)[103]. Zap70, a Syk family kinase, is recruited to the site and binds the phosphorylated ITAM sites through its SH2 domains[103,104]. Furthermore, upon Zap70's binding to ITAM sequences, LCK phosphorylates and further activates Zap70, allowing for Zap70 to propagate TCR signaling events[104]. Zap70 phosphorylates the linker of activation of T-cells (LAT) on multiple residues, which serves as a signaling hub for different downstream events[105]. One of the functions of phosphorylated LAT is the recruitment and subsequent activation of phospholipase C gamma-1 (PLCγ1), which is further activated through its phosphorylation mediated by interleukin-2 inducible T-cell kinase (ITK)[106,107]. Once activated, PLCγ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), each serving a different function in T-cell activation[108]. The hydrolysis of PIP_2 into IP_3 leads to downstream calcium mobilization through the release of calcium from the endoplasmic reticulum in addition to the influx of extracellular calcium[109]. Calcium mobilization drives

NFAT into the nucleus, where it regulates the production of several cytokine genes[110]. DAG leads to the activation of both RAS guanyl-releasing protein (Ras-GRP) and protein kinase Cθ (PKCθ), which both contribute to the process of T-cell activation[111,112]. PKCθ leads to the downstream activation of the transcription factor nuclear factor κ-B (NFκB), which activates a number of target genes including those ultimately responsible for the production of IL-2[113,114]. The activation of Ras-GRP, on the other hand, eventually leads to the downstream activation of the transcription factor, activator protein-1 (AP-1), which regulates gene expression and promotes cytokine secretion[115,116].

Co-receptors play a crucial role in T-cell activation, helping to stabilize the interaction between the T-cell and APC. Signaling through the TCR alone, in the absence of co-stimulatory molecules, results in an anergic state in which the T-cell becomes unresponsive to subsequent stimulation. Co-stimulation is necessary for providing additional signals and stabilization that allow for the avoidance of T-cell anergy and allow for full activation of the cell. There are several co-stimulatory molecules, most importantly CD4/CD8 and CD28. T-cell CD4 or CD8 co-receptors bind class I or class II MHC molecules, respectively and help TCR bind its MHC counterpart. CD28, on the other hand, binds the ligands of the B7 family, CD80 (B7-1) and CD86 (B7-2) on APCs to enhance TCR signaling and results in a much more robust response than other co-stimulatory molecules[117]. B7 ligands are only expressed by professional APCs, however dendritic cells appear to constitutively express CD80/86 while macrophages and B cells are able to upregulate B7 expression after activation[118]. Studies involving CD28 deficient mice demonstrated its importance in T-cell activation, revealing that the absence of CD28 resulted in

dampened TCR-mediated immune responses[119]. Another prominent co-stimulatory receptor for T-cells is the inducible co-stimulatory (ICOS), which helps provide positive co-stimulation for T-cell activation similarly to CD28. ICOS differs from CD28 in the fact that it does not bind CD80 and CD86, but instead binds another member of the B7 family, ICOS-ligand (ICOS-L), which is also expressed on APCs[120]. Murine models deficient in ICOS revealed its role in the T-cell response, as there was a reduction in cytokine production and T-cell proliferation in the absence of the receptor[120]. Differences in the expression patterns of ICOS and CD28 suggest that they play distinct roles in T-cell activation. While all T-cells express CD28, the naïve T-cell population, for example, expresses the CD28 co-stimulatory molecule but does not express ICOS, which is only expressed on effector and memory T-cells[121,122]. This may be evidence that suggests initial T-cell activation is largely mediated by CD28 co-stimulation while ICOS has an important function in maintaining the activity of cells that have already differentiated into effector and memory subsets.

While CD28 and ICOS are positive co-stimulatory molecules that serve to enhance T-cell activation, there are also negative costimulatory receptors that serve to inhibit TCR signaling. Negative costimulatory receptors are not expressed by naïve T-cells, which allows them to serve an important role in the initiation of the immune response and become activated in secondary lymphoid tissues. These negative costimulatory receptors are, on the other hand, upregulated in effector cell populations at the end of the immune response, serving to stop cell proliferation when it is no longer needed. There are two main negative costimulatory receptors for T-cells: cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1

(PD-1). CTLA-4, also known as CD152, is structurally similar to CD28 and binds the same B7 family ligands, CD80 and CD86, expressed by APCs[123,124]. CTLA-4 is not constitutively expressed on resting T-cells, but rather is induced within 24 hours after stimulation and binds B7 ligands with much higher affinity than CD28[123]. Studies with CTLA-4 knockout mice demonstrated its function as a negative costimulatory molecule, as mice lacking CTLA-4 presented with lymphadenopathy, splenomegaly, autoimmunity, and ultimately death within 4 weeks after birth as a result of uninhibited T-cell proliferation[125,126]. PD-1, which may be referred to as CD279, also inhibits TCR mediated activation through its binding to other B7 family members, PD-L1 and PD-L2[127,128]. PD-1's role in the inhibition of proliferative T-cell responses was evident *in vivo*, revealing that PD-1 knockout mice developed lupus-like glomerulonephritis and dilated cardiomyopathy[129,130]. Further *in vitro* studies also demonstrated that IFNγ and IL-2 cytokine production and T-cell proliferation were reduced in anti-CD3 stimulated T-cells that were treated with a PD-L1 immunoglobulin[127,131].

CHAPTER TWO

MATERIALS AND METHODS

Mononuclear Cell Isolation and Naïve Cell Purification

Naïve CD4 T-cells were isolated from healthy adult and umbilical cord donors from the National Institute of Health and the Loyola University medical center, respectively. All donors met the outlined exclusion criteria: 1. Usage of glucocorticoids, immunosuppressants, or any other drugs with known effects on the immune system; 2. Evidence or presence of malignancies; 3. Autoimmune disease; 4. Active infection; 5. Uncontrolled hypo or hyperthyroidism. Mononuclear cells were isolated though density gradient centrifugation with Lymphocyte Separation Medium (Corning)and red blood cells were lysed with ACK lysis buffer (Gibco). Recovered mononuclear cells were resuspended in with RPMI 1640 (Hyclone) supplemented with 10% fetal calf serum. Naïve CD4 T-cells were purified via negative selection using an EasySep Human Naïve CD4 T-cell Enrichment Kit (Stem Cell Technologies).

Phenotypic Analysis of Naïve CD4 T-Cells

Purified naïve CD4 T-cells were cultured in a 96 round bottom plate at 0.3×10^5 cells per well in the presence of 20 ng/ml recombinant human IL-7 (PeproTech) and maintained in RPMI 1640 (Hyclone) supplemented with 10% fetal calf serum. Culture media was changed every 2-3 days and concentrations of IL-7 were maintained throughout the duration of the culture. Naïve phenotype was assessed on day 0 and day 7 via flow cytometry (BD FACSCANTO II Flow

Cytometer, BD Biosciences) with the use of anti-CD4, anti-CD45RA, anti-CD26, anti-CD31 (BioLegend), and anti-CD45RO (BD Biosciences). Additionally, 1 x 10^6 naïve cells were stimulated for 4 hours on day 0 with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Fisher Scientific, Hampton, NH) and ionomycin (1 μM; Sigma-Aldrich) in the presence of fresh media. Culture supernatants were collected after stimulation and production of TNFα, IFNγ, IL-2, IL-4, IL-5, and IL-13 was assessed with use of the LEGENDplex Human Th Cytokine Panel (Biolegend) and the BD FACSCANTO II Flow Cytometer (BD Biosciences).

Th1 and Th2 Differentiation Assays

Purified naïve CD4 T-cells were maintained in IL-7 (20ng/ml; PeproTech) for 7 days, then stimulated with plate bound anti-CD3 (OKT3; 5ug/ml; BioLegend) and anti-CD28 (28.2; 5ug/ml; BioLegend) and cultured in the presence of Th1 or Th2 skewing conditions through the use of a Human Th1 or Th2 Differentiation Kit (R&D Systems) according to the manufacturer's protocol. Cells under neutral conditions were cultured in the presence of IL-2 alone. Cells under Th1 conditions were harvested on day 5 while cells under Th2 conditions were harvested on day 13 and washed with RPMI 1640 (Hyclone) supplemented with 10% fetal calf serum. 1 x 10^6 cells from each condition were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Fisher Scientific) and ionomycin (1 μM; Sigma-Aldrich) in the presence of fresh media for 4 hours. After stimulation, culture supernatants were collected and analyzed for production of TNFα, IFNγ, IL-2, IL-4, IL-5, and IL-13 with the LEGENDplex Human Th Cytokine Panel (Biolegend) and the BD FACSCANTO II Flow Cytometer (BD Biosciences).

Naïve CD4 T-Cell Culture for Protein Expression Analysis

A 96 well flat bottom non-tissue cultured plate was coated with 5ug/ml of anti-CD3 (OKT3; 5μg/mL; BioLegend) and anti-CD28 (28.2; 5μg/mL; BioLegend) and incubated at room temperature for 3 hours. Plate was then washed with DPBS twice and blocked with RPMI 1640 (Hyclone) supplemented with 10% fetal calf serum for one hour. Blocking media was removed and $2.5x10^5$ isolated naïve CD4 T-cells were added per well. The plate was then spun down at 700 RPM for 5 minutes, then cultured at 37℃ for either 30 minutes, 3 hours, or 24 hours. Triton X-100 lysates of the soluble fraction were collected.

Western Blot

Cells harvested and lysed in Triton X-100 lysis buffer. Lysates were protein quantified with use of the Pierce BCA protein assay kit (Thermo Fisher Scientific) and measured at A562nm via BioTek Powerwave XS (BioTek). Soluble lysates were loaded into a 10% acrylamide gel at 100μg/well. Proteins were fractionated via SDS-PAGE, which was run at 60V through the stacking gel and at 120V through the resolving gel. Samples were then transferred onto a membrane (EDM Millipore) at 4℃ either overnight at 30V or two hours at 300mA. After which, membranes were blocked in 5% nonfat milk for one hour at 23℃. Membrane was washed and incubated with primary antibody against PLCγ (1:500; Cell Signaling), Zap70 (1:100,000; Novus Biologicals), LCK (1:5000; gifted from Y. Koga, Tokai University), ζ-Chain (1:1000; Santa Cruz Biotechnology), and β-actin (1:10,000; Sigma-Aldrich) for two hours at 23℃ or overnight at 4℃. Membranes were washed three times for 5-7 minutes, then incubated with HRP conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling) in 2.5% nonfat milk

for 1 hour at 23℃. Membranes were then washed three times for 5-7 minutes and visualized with use of chemiluminescent film (GE Healthcare) and Pierce ECL western substrate (Thermo Fisher Scientific). Protein levels were quantitated through the use of ImageJ software. To blot for additional proteins, membranes were stripped using stripping buffer (2% SDS, 50mM Tris, pH 6.8) at 56℃ for 30 minutes, then washed in deionized water (DI) twice for 10 minutes each, washed in TBST, blocked in 5% nonfat milk, and probed with additional antibodies.

Statistical Analysis

Statistical analysis of data was done via GraphPad Prism software. Cytokine and protein expression levels were analyzed with a two-tailed student's unpaired t-test ($p < 0.05$).

CHAPTER THREE

DIFFERENCES BETWEEN CORD AND ADULT NAÏVE CD4 T-CELLS

Introduction

The neonatal immune system is tolerogenic, and as such, is phenotypically and functionally different from adults. Studies with mice have shown that the perinatal immune system has a tendency for T-cells to produce elevated Th2 type cytokines and reduced levels of Th1 cytokines when compared to their adult counterparts[75,76]. This Th2 bias in neonates is partially due to intrinsic properties that make them prone to the production of Th2 associated cytokines – an observation not found in CD4 T-cells from adult blood[77]. However, there has been evidence that gene expression for both Th1 like cytokines (IFNγ) as well as Th2 like cytokines (IL-4) are suppressed in perinatal T-cells[132].

This being said, the majority of our understanding of the neonatal immune response is the product of animal research and the human perinatal T-cell response is largely uncharacterized. There is evidence that suggests that the two systems are not completely similar, and thus reveals a need for species specific analysis of neonatal immunity. For instance, one mouse study demonstrated that neonate memory T-cells had a small Th1 cytokine profile and a robust Th2 type cytokine response to tetanus toxoid vaccination[133]. This is in contrast to some human studies, one of which focusing on T-cells isolated from cord blood and 3-month-old infants that

showed no increased propensity for Th2 cytokine production, except for IL-13[134]. This was also seen with cord blood T-cells *in vitro*, as they also displayed no propensity to produce Th2 cytokines apart from elevated levels of IL-13[135,136]. Together, these data suggest that the human and mouse perinatal immune systems respond differently, thus emphasizing the need for a more detailed analysis of the human perinatal T-cell population. We seek to determine the intrinsic cellular and molecular differences between the perinate and adult immune systems. It is known that the cellular composition of the perinatal immune system differs significantly from the adult system, with naïve T-cells making up the of cord blood cells while effector/memory T-cells predominate the adult population[137]. As such, we isolated our study to the naïve CD4 T-cell population in order to avoid the influences and analytical complexities that may arise from multiple cell populations.

Expression of CD26 By Cord Blood Naïve CD4 T-Cells

T-cells need co-stimulatory molecules, like CD28, for enhanced stimulation and the propagation of a robust immune response. While CD28 elicits a robust immune response, there are other co-stimulatory molecules that aid in the enhancement of T-cell activation. There is evidence that another co-stimulatory molecule may be CD26. CD26 is a glycoprotein expressed on the surface of several cell types including epithelial cells of the liver, kidney, intestine, and T-cells[138]. The exact role that CD26 plays in the immune response is relatively unknown, however. Previous work by a member of our lab showed that T-cells secrete soluble CD26 (data not shown). This knowledge, when combined with the many unknowns about the expression and function within human T-cell populations, prompted us to compare surface CD26 expression

between cord and adult blood naïve CD4 T-cells, hypothesizing that CD26 surface expression may differ between the two groups. This is a distinct possibility, as there are already other documented differences in other surface antigens, such as CD127, between cord and adult blood naïve CD4 T-cells[12].

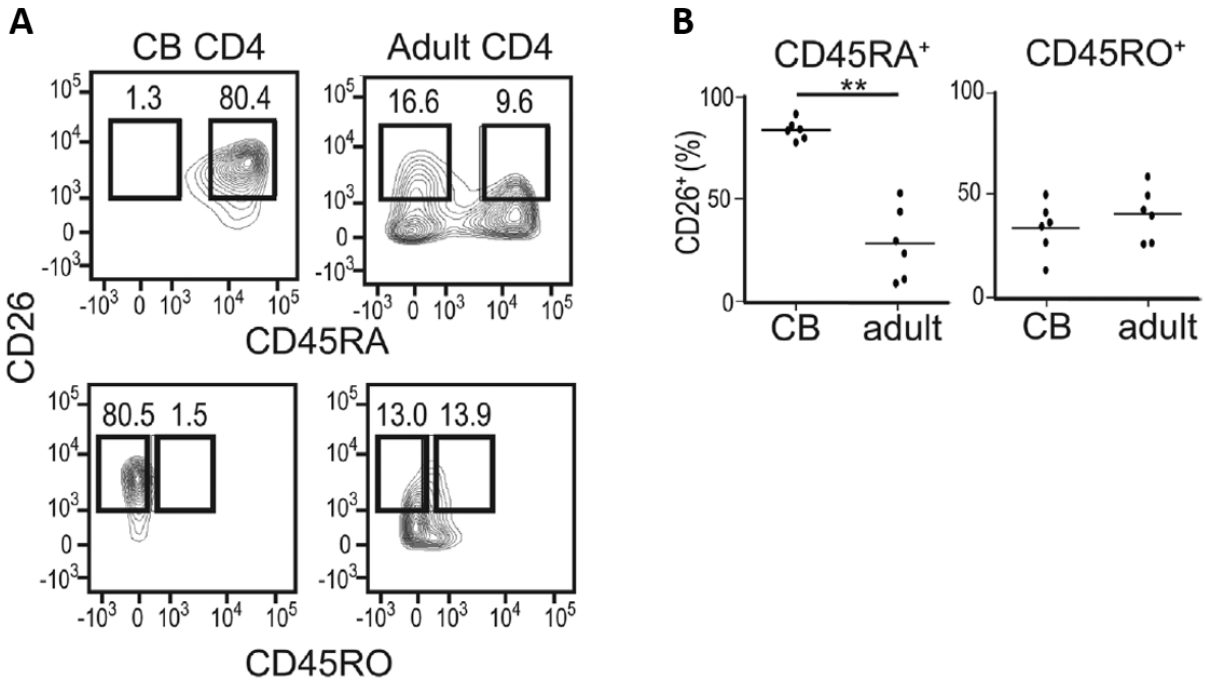

Figure 1. Expression of CD26 by Cord and Adult Naïve CD4 T-Cells. (A) Expression of CD26 by $CD45RA^+$ and $CD45RO^+$ CD4 T-cell populations from cord blood (CB) and adult blood. (B) Percentage of cells expressing CD26 within $CD45RA^+$ and $CD45RO^+$ CD4 T-cell populations from 6 cord blood and 6 adult blood donors. Bars represent mean percentage of $CD26^+$ cells.

In order to determine intrinsic differences between cord and adult blood derived naïve CD4 T-cells, we first defined the naïve population as those cells expressing CD45RA in the absence of CD45RO expression. When the cells were stained for CD26, we found significant

differences in its expression between cord blood and adult peripheral blood naïve CD4 T-cells **(**Fig. 1). In the adult cohort, $CD26^{hi}$ cells were mostly $CD45RA^{-}$ $CD45RO^{+}$ cells, with a fraction of $CD45RA^{+}$ naïve CD4 T-cells expressing low levels of CD26. This was strikingly different than results obtained from cord blood T-cells, which revealed close to 100% of $CD45^{+}$ naïve T-cells expressing high levels of CD26.

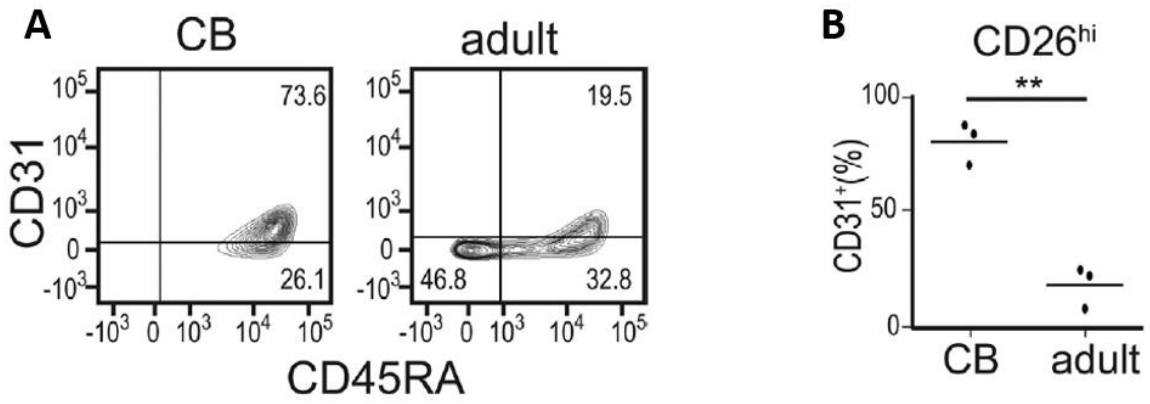

Figure 2. Expression of CD26 and CD31 by Cord and Adult Naïve CD4 T-Cells. (A) Expression of CD31 in the $CD45RA^{+}$ population of cord and adult blood. (B) Percentage of $CD26^{hi}$ cells expressing CD31 in cord blood and adult blood T-cells from 3 cord blood and 3 adult blood donors. Bars represent mean percentage. ** $p < 0.01$.

Elevated levels of CD26 expression by cord blood T-cells could be explained by the high frequency of RTEs in cord blood, as cord blood in known to have much larger RTE population than adult peripheral blood[137]. We can identify cells within the RTE population through the expression of CD31, which is a known marker for RTEs expressed by naïve $CD45RA^{+}$ cells[139]. If CD26 is related to the RTE population, we would expect that all $CD26^{hi}$ T-cells in both cord

blood and adult blood to co-express CD26 and CD31. When looking at $CD45RA^{+}$ populations, both cord and adult blood express CD31 (Fig. 2A). However, when analyzing the $CD26^{hi}$ population of both cord and adult T-cells, only cord blood displayed $CD26^{hi}$ and CD31 co-expression, while adult $CD26^{hi}$ cells did not co-express CD31 (Fig. 2B).

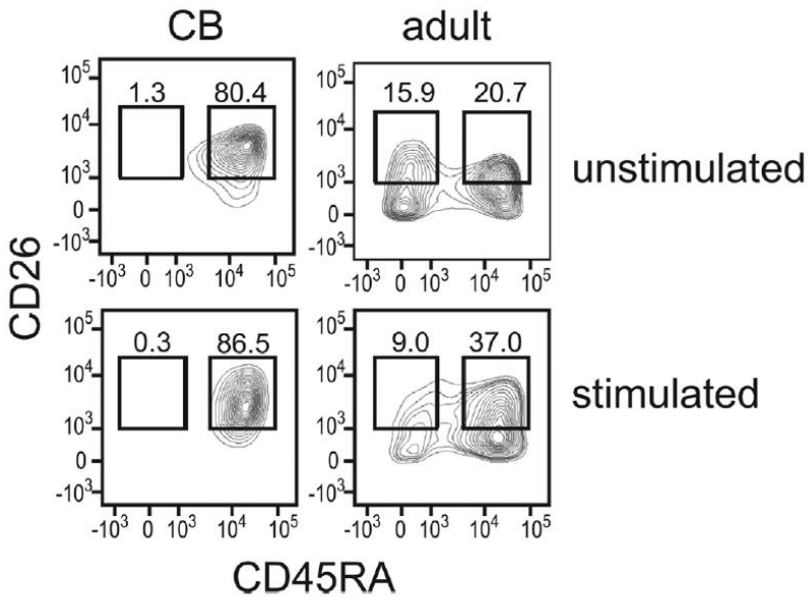

Figure 3. The Effects of Stimulation on CD26 Expression. Expression of CD26 and CD45RA by cord blood and adult blood T-cells with and without stimulation by anti-CD3 for 2 days.

Additionally, CD26 expression has been shown to be upregulated in response to antigenic stimulation, so we wanted to see if TCR stimulation would change surface CD26 expression in cord and adult blood cells[140]. To test this, naïve CD4 T-cells were stimulated with plate bound anti-CD3 and anti-CD28 antibodies, harvested, and stained for CD26 expression. We observed no significant change in CD26 expression in cord blood, with CD26 expression slightly increasing from 80.4% in the unstimulated condition to 86.5% after stimulation. This is in

contrast to adult cells, which showed a larger upregulation in CD26, increasing from 20.7% to 37.0% after stimulation (Fig. 3). Together, these data suggest that CD26 may be a potential cord blood naïve CD4 T-cell marker and that its regulation differs between cord and adult naïve CD4 T-cells.

Effects of IL-7 On the Expansion and Phenotype of Naïve CD4 T-Cells

Continuing our investigation of intrinsic differences between cord and adult peripheral blood naïve CD4 T-cells, we compared the ability of CB and AB naïve CD4 T-cells to respond to IL-7. IL-7 is a critical survival cytokine with anti-apoptotic effects for naïve T-cells and plays a central role in the homeostatic expansion of T-cells[141]. IL-7 signals through its binding to CD127, which is expressed at high levels in cord blood naive T-cells and at reduced levels in $CD4^+$ $CD25^{hi}$ T regs[142]. *Ex vivo*, IL-7 has been shown to be required for the expansion of naïve, memory, and $CD31^+$ T-cells have been shown to proliferate in response to IL-7[141,143,144]. As we observed previously, only a small subset of adult naïve T-cells are $CD31^+$ while the majority of cord blood naïve T-cells are $CD31^+$, and as such, we investigated whether or not cord blood naïve CD4 T-cells and adult peripheral blood naïve CD4 T-cells respond differently to IL-7. To test this, we isolated naïve CD4 T-cells from cord and adult peripheral blood and cultured them in the presence of IL-7 for 7 days. After this time, the cells were harvested and both proliferative responses and surface antigen expression were examined.

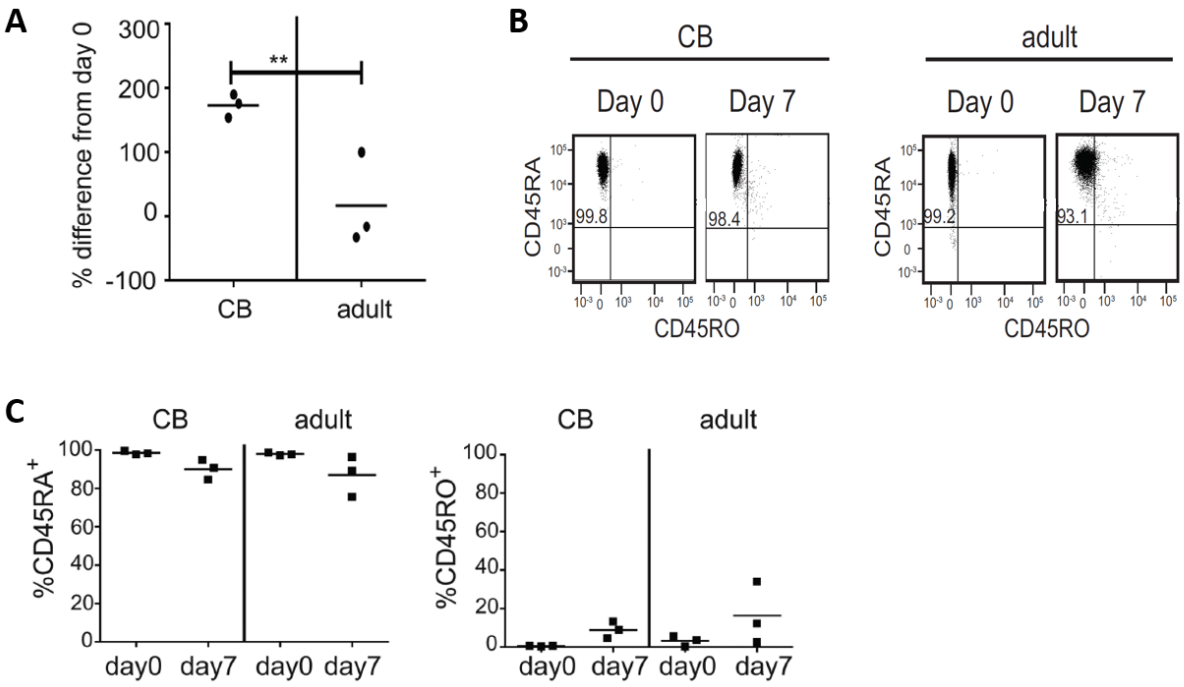

Figure 4. The Effect of IL-7 on Cord and Adult Naïve CD4 T-Cells. (A) Proliferative response as indicated by change in cell numbers from day 0 to day 7 after culture in the presence of IL-7. Bars represent mean percentage difference from day 0. ** $p < 0.01$. (B, C) Phenotypic analysis of naïve CD4 T-cells before and after culture with IL-7 for 7 days. Analysis before IL-7 culture (day 0) was performed directly after naïve cell isolation. Bars represent mean percentage of $CD45RA^+$ and $CD45RO^+$, respectively.

Adult naïve CD4 T-cells had no proliferative response after 7 days of culture with IL-7 while cord blood naïve CD4 T-cells expanded nearly 2-fold under the same conditions (Fig. 4A). This highlights the idea that cord blood naïve T-cells have different requirements for IL-7 induced homeostatic expansion when compared to adult naïve CD4 T-cells. Analysis of surface antigen expression revealed similar results for both cord and adult T-cells after the 7 day culture in IL-7, as both maintained their naïve phenotype, as characterized by the presence of CD45RA in the absence of the CD45RO memory isoform (Fig. 4B and C).

Cytokine Production and Differentiation of Naïve CD4 T-Cells

Previous mouse studies suggest that perinatal T-cells have an elevated propensity to produce Th2 type cytokines when compared to adults[75,76]. Additionally, studies on human T-cells suggest that T-cells expressing CD26 have a propensity to produce IL-17[145]. Therefore, we wanted to investigate which cytokines are produced by human cord blood derived naïve CD4 T-cells and whether human cord blood T-cells also have a propensity to produce Th2 like cytokines. To start, we analyzed cytokine profiles of freshly isolated naïve CD4 T-cells from cord blood and adult peripheral blood with/without stimulation with PMA and ionomycin for 4 hours. Our data shows that, when stimulated, adult peripheral blood naïve CD4 T-cells produce more IFNγ than cord blood naïve CD4 T-cells (Fig. 5A). Both cord blood and adult blood naïve CD4 T-cells produced similar amounts of IL-2 and TNF, while neither produced appreciable levels of IL-17. We also did not see cord blood naïve T-cells produce the Th2 cytokines IL-4, IL-5, or IL-13 (data not shown). Together, our data reveal that fresh cord blood naïve CD4 T-cells are not pre-programmed to produce Th2 like cytokines and do not produce the same level of Th1 or Th2 type cytokines that is observed in adult naïve CD4 T-cells.

We then tested the hypothesis that cord blood naïve CD4 T-cells, rather than adult naïve CD4 T-cells, have a tendency to become Th2 type effector cells. We isolated naïve CD4 T-cells from cord and adult blood, held them in culture for 1 week in IL-7, and then cultured them in the presence of neutral (Th0), Th1, or Th2 skewing conditions. Cells were harvested and re-stimulated with PMA and ionomycin for 4 hours, after which supernatant was collected and cytokine production was analyzed (Fig. 5 B-D). Adult and cord blood cells, under neutral/Th0

conditions, showed no difference in cytokine profile (Fig. 5B). Under Th1 polarizing conditions, cord blood cells produced significantly more IFNγ than those cells from adults (Fig. 5C). However, we did not detect any differences in cytokine production in other Th1 cytokines. Additionally, Th2 skewing conditions resulted in cord blood producing significantly more IL-5 and, while not statistically significant, IL-13, than adult blood cells (Fig. 5D). These data suggest that cord blood naïve CD4 T-cells have a higher capacity to produce both Th1 and Th2 like cytokines than adult naïve CD4 T-cells, as evident by the production of IFNγ, IL-5, and IL-13 when exposed to effector cell differentiation conditions.

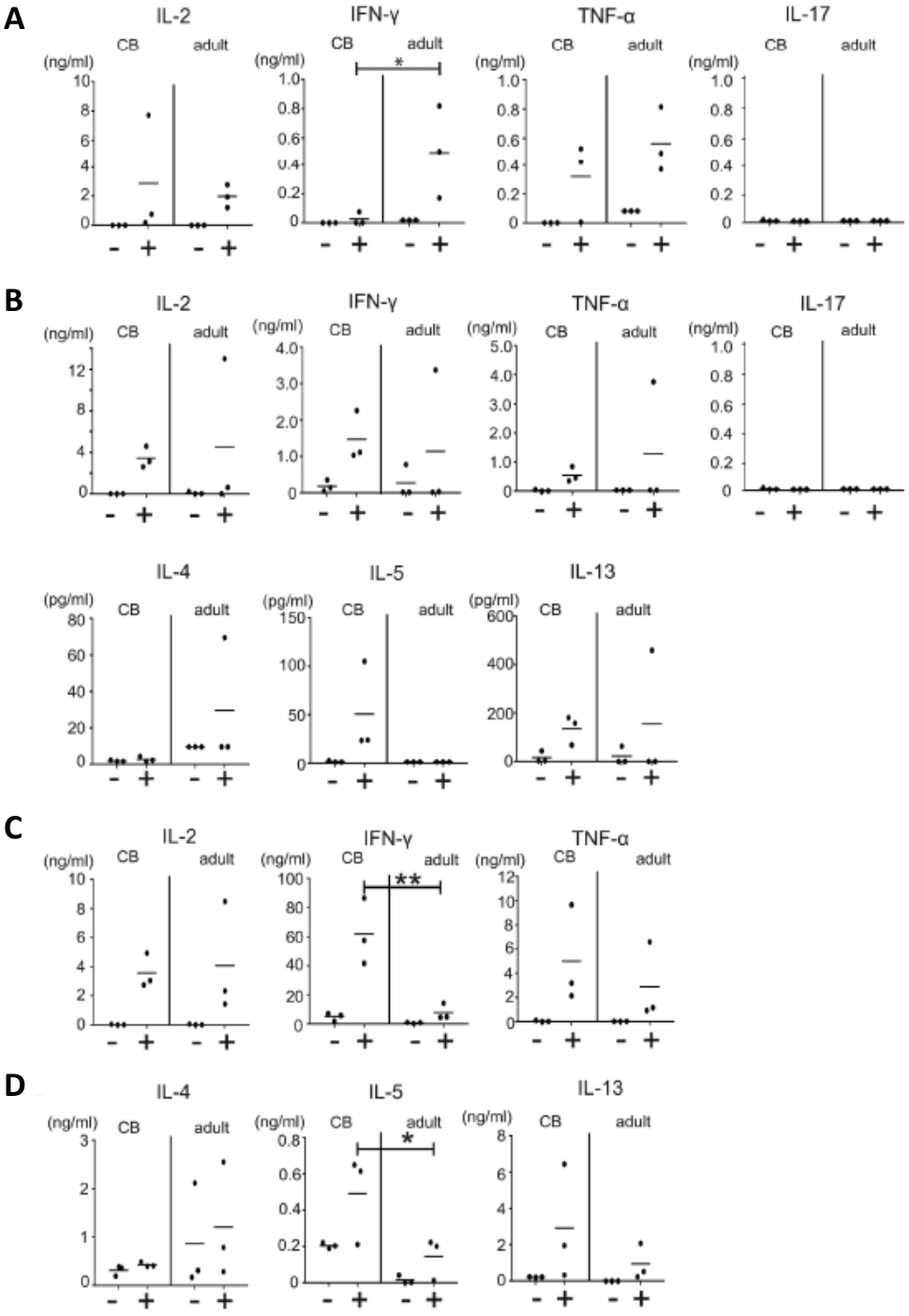
A
IL-2
IFN-γ
TNF-α
IL-17
CB
adult
(ng/ml)
*
B
IL-2
IFN-γ
TNF-α
IL-17
IL-4
IL-5
IL-13
(pg/ml)
C
IL-2
IFN-γ
TNF-α
**
D
IL-4
IL-5
IL-13
*

Figure 5. Cytokine Production by Cord and Adult Blood Naïve CD4 T-Cells. (A) Cord and adult naïve CD4 T-cells were isolated and stimulated (+) with PMA and ionomycin or left unstimulated (-) for 4 hours. Supernatant was collected and cytokine production was assessed. (B) Naïve CD4 T-cells were isolated from adult and cord blood donors and cultured with IL-7 for 7 days. On day 7, cells were collected and stimulated with anti-CD3 and anti-CD28 antibodies for 5 days. Cells were then collected and either stimulated with PMA and ionomycin (+) or left unstimulated (-). (C) Naïve CD4 T-cells were isolated from adult and cord blood donors and cultured for 7 days with IL-7. After 7 days, cells were harvested and stimulated in the presence of Th1 skewing conditions for 5 days. After this time, cells were restimulated and analyzed in the same manner as (B). (D) Naïve CD4 T-cells were isolated from adult and cord blood donors and cultured for 7 days with IL-7. After 7 days, cells were harvested and stimulated in the presence of Th2 skewing conditions for 13 days. After this time, cells were collected and restimulated and analyzed as in (B). Bars represent mean cytokine production for 3 cord and 3 adult donors. *$p < 0.05$. **$p < 0.005$.

Discussion

Taken together, our data suggest that cord blood naïve CD4 T-cells are programmed differently from adult naïve CD4 T-cells. Our data shows that cord blood naïve CD4 T-cells have high levels of CD26 expression that is not uniformly expressed in adult peripheral blood naïve CD4 T-cells. Unique high protein expression in cord blood derived naïve CD4 T-cells could serve as a potential cellular marker for this cell population. There is some literature supporting this result, as previous work has shown that CD45RA expression is positively correlated with CD26 expression in cord blood T-cells[146]. However, other documents have reported CD26 is expressed by memory T-cells, but not by the naïve population[147]. Additionally, we also observed that stimulation of adult naïve CD4 T-cells allows for the induction and increased expression of CD26 while having little effect on the cord blood derived naïve population, whose CD26 expression only slightly increased upon stimulation.

CD26 is an enzyme with known dipeptidyl peptidase 4 (DPPIV) activity in its extracellular domain, allowing it to cleave proline's and alanine's at the N-terminus of chemokines and other natural substrates[148,149]. It is known that CD26 is important for digestion and is a regulator of glucose metabolism through its inactivation of glucagon like peptide 1 (GLP1) and other insulinotropic proteins[150]. As such, the inhibitor for DPPIV is used clinically for the treatment of diabetic patients[151]. In addition to its dipeptidase activity, CD26 is capable of binding the ligand adenosine deaminase (ADA), an essential factor for immune competence in neonates[152]. ADA deficiencies cause severe immune deficiencies in adolescents and neonates; in fact, 15% of all severe combined immunodeficiency (SCID) cases are caused by deficiencies in ADA[153]. ADA is an enzyme involved in purine metabolism and is responsible for the deamination of adenosine into the nucleoside inosine[154]. Endogenous adenosine is sensed by receptors that then prevent the downregulation of the IL-7 receptor α-chain after TCR stimulation, ultimately increasing naïve T-cell survival[155]. Extracellular adenosine has been shown to be inhibitory to T-cell proliferation and the production of pro-inflammatory cytokines like IFNγ, TNFα, and IL-2, but does not affect the production of IL-4 or IL-5[156]. Inhibition of proliferation by adenosine has also been shown to be prevented through the enzymatic activity of ADA[138]. Evidence also suggests that the binding of ADA to CD26 may protect T-cells from the adenosine-mediated inhibition of T-cell proliferation[157-160].

Additionally, CD26 may have a role in T-cell activation, although its exact function is not universally agreed upon. There are studies suggesting that CD26 is not directly linked to T-cell activation, while other studies argue that it has positive/negative co-stimulatory roles[161-163].

There is evidence that CD26 may help provide positive co-stimulation that aids in T memory/effector cell activation[164,165]. Additional studies suggest that CD26 is more efficient at promoting activation after stimulation in $CD45RO^+$ effector/memory T-cells than in $CD45RA^+$ naïve T-cells[162,166]. CD26 may also enhance the formation of Th1 effector functions, as clinical analysis of Multiple Sclerosis (MS) disease severity revealed that $CD4^+$ $CD45RO^+$ $CD26^{hi}$ T-cell subsets correlated with more severe MS presentation with enriched Th1 activity[167]. However, there is also evidence, primarily with animal models, suggesting that CD26 may have inhibitory roles in T-cell activation. Murine models with CD26 knockout mice exhibited more severe experimental autoimmune encephalomyelitis (EAE), with elevated T-cell activation as assessed through IFNγ and TNF cytokine production and robust T-cell proliferation[163]. Taking this all into account, the function of CD26 remains in question; as there appear to be functional differences that vary within species and within cell type. Our data, combined with data collected from several human studies, suggest that CD26 has positive co-stimulatory effects in $CD45RO^+$ cells while having negative co-stimulatory functions in $CD45RA^+$ cells.

When looking at the effects of IL-7, we found that both cord and adult naïve CD4 phenotypes, defined by the surface antigens $CD45RA^+$ and $CD45RO^-$, can be maintained in IL-7 after 7 days in culture. This was an expected fining, as IL-7 is known to have survival and anti-apoptotic effects[141]. Additionally, while there were no differences in the maintenance of naïve phenotype, we did see differences in proliferative responses between the two groups. CB naïve T-cells were able to proliferate approximately 2-fold while the AB naïve T-cells were not able to proliferate in response to IL-7. The different proliferative responses to IL-7 further demonstrates

the notion that the two groups are intrinsically different. Differences in proliferative responses could likely be attributed to either due to their short lifespan after thymic emigration or due to the disproportionate expression of the IL-7 receptor CD127, which has been shown to be more highly expressed in cord blood T-cells[12,33,37].

Under neutral conditions we found that cord blood naïve CD4 T-cells produce lower levels of cytokines than adult peripheral blood naïve CD4 T-cells, yet they are capable of producing elevated levels of Th1 (IFNγ) and Th2 (IL-5 and IL-13) when stimulated under conditions promoting cell differentiation. This was an unexpected finding, as previous studies with mice suggested that neonates have a Th2 bias culminating in a reduced capacity to produce IFNγ and TNFα[77,168]. However, an epidemiological study with PBMC T-cells isolated from over 400 children revealed that there was no significant bias towards Th2 type cytokine production except for IL-13[169]. Our data aligns with these outcomes, demonstrating no bias in Th2 cytokine production from freshly stimulated naïve CD4 T-cells from human cord blood. However, we did observe significantly elevated levels of IFNγ and IL-5 in cord blood naïve T-cells when they were expanded under Th1 or Th2 stimulating conditions, respectively. The Th2 polarizing conditions also did not, however, produce elevated levels of IL-4. IL-5 does not affect T-cell differentiation into Th2 effector cells, but instead promotes B cell and eosinophil differentiation and growth.

When isolated naïve CD4 T-cells were stimulated immediately without IL-7 culture, we saw adult blood produce significantly higher levels IFNγ, yet this difference in cytokine expression was eliminated when naïve CD4 T-cells were cultured in the presence of IL-7 prior to

stimulation. This may be evidence that cord blood naïve CD4 T-cells are held in a relatively unreactive state, and that IL-7 may be removing this inhibition of activation. Additionally, in future research, we would like to examine whether CD26 is playing a role in the observed IFNγ production in freshly isolated naïve CD4 T-cells. As mentioned previously, we found that cord blood naïve CD4 T-cells highly express CD26, and there are conflicting opinions regarding the effect that CD26 has on T-cell activation. If CD26 has inhibitory effects towards $CD45RA^{+}$ T-cell activation then it may explain why we observed that freshly isolated cord blood naïve CD4 T-cells have decreased IFNγ production when compared to adult blood peripheral naïve CD4 T-cells[138].

CHAPTER FOUR

PROXIMAL TCR SIGNALING PROTEIN ANALYSIS IN CORD AND ADULT NAÏVE CD4 T-CELLS

Introduction

T-cell functions in neonates, such as cytokine production and proliferative responses, are downregulated in response to antigenic stimulation[14]. Our data supports this notion, with fresh naïve CD4 T-cells from cord blood displaying significantly lower IFNγ production after stimulation than adult peripheral blood naïve CD4 T-cells. Although surrounded by controversy and conflicting reports, some literature partially attributes the decreased immune responses in the neonatal population to lower numbers of neonatal lymphocytes and that the observed deficiencies in T-cell activation may be due to decreased expression of molecules associated with T-cell activation, such as CD40L and NFAT[38,44]. We wanted to determine if there are other factors contributing to the decreased T-cell activity exhibited by cord blood naïve CD4 T-cells.

T-cell activation has been extensively studied and well characterized in adults, where optimal activation requires TCR stimulation in addition to the presence of a co-stimulatory molecule, like CD28. TCR signaling utilizes several adaptor proteins and kinases to propagate signal down its signal transduction cascade that ultimately result in activation of the cell. Once bound to its cognate antigen, LCK is recruited to the signaling complex, activated by CD45, and phosphorylates ITAM residues on the cytoplasmic tails of CD3 and ζ-chain of the TCR complex[97-100,102]. These phosphorylated ITAMs serve as binding sites for Zap70, which is then

further activated by the kinase activity of LCK and may propagate further signaling events through the phosphorylation of LAT, which leads to the recruitment and activation of PLCγ and ultimately resulting activation of the cell[170].

Deficiencies and limitations in protein expression or molecular activity within the TCR signaling pathway may contribute to the hyporeactive nature observed in neonatal T-cells. Furthermore, there has been little research conducted comparing the expression of proximal signaling proteins of naïve CD4 T-cell populations between cord and adult blood. We hypothesize that observed differences in activation responses between cord blood and adult blood naïve CD4 T-cells are due to differences in the expression levels of proteins involved in the TCR signaling pathway.

Expression of Proximal TCR Signaling Proteins

To compare protein expression levels between cord and adult blood T-cells, purified naïve CD4 T-cells were either unstimulated or stimulated with plate bound anti-CD3 and anti-CD28 antibodies for 30 minutes, 3 hours, or 24 hours. After stimulation, cells were lysed and the expression of PLCγ, Zap70, LCK, and ζ-chain were assessed via western blot. PLCγ levels decreased after initial stimulation in both cord blood and adult blood naïve CD4 T-cells and protein levels rebounded after 24 hours stimulation (Fig. 6A). Protein expression levels of cord blood and adult peripheral blood were compared, and there was no significant difference in PLCγ expression in the unstimulated condition ($p = 0.3463$) or after 30 minutes ($p = 0.1584$), 3 hours ($p = 0.4599$) or 24 hours ($p = 0.0782$) stimulation (Fig. 6B). Levels of Zap70 showed a similar trend, with protein levels initially decreasing with stimulation and an increase in expression upon 24 hours in both CB and AB samples (Fig. 6E,K). However, there was no

statistically significant difference observed between cord or adult without stimulation ($p = 0.1719$) or with 30 minutes ($p = 0.7334$), 3 hours ($p = 0.1974$), or 24 hours ($p = 0.4421$) stimulation (Fig. 6F). Expression of LCK also decreased upon stimulation in both CB and AB samples and returned to baseline levels after 24 hours of stimulation (Fig. 6C,J). Much like PLCγ and Zap70, LCK did not show any statistically significant differences between the two groups in the unstimulated condition ($p = 0.7615$) or with stimulation for 30 minutes ($p = 0.1381$), 3 hours ($p = 0.4265$), or 24 hours ($p = 0.9467$) (Fig. 6D). Lastly, when looking at the expression of ζ-chain, a difference can be seen between the two groups at 24 hours post stimulation, with adult blood naïve CD4 T-cells maintaining a slightly higher level of protein expression than those from cord blood, however this difference was not statistically significant ($p = 0.0679$), nor were there significant differences found at the other stimulation conditions of 30 minutes ($p = 0.9668$) or 3 hours ($p = 0.6494$) or in the absence of stimulation ($p = 0.5451$) (Fig. 6H).

Interestingly, unlike the other signaling molecules we investigated, we see protein levels decline with stimulation for both cord and adult blood samples and these levels continued to decrease through 24 hours of stimulation (Fig. 6G,L). Additionally, unlike LCK, PLCγ, or Zap70, ζ-chain displayed statistically significant differences in protein expression between the unstimulated condition and 24 hours stimulation for both cord and adult samples (CB $p = 0.0028$, adult $p = 0.0075$). Together, these data suggest that differences in T-cell activity between naïve cord and adult T-cells is not due to expression level of proximal TCR signaling proteins, as we observed no significant differences in protein expressions, yet also show decreased expression of ζ-chain in both CB and adult samples.

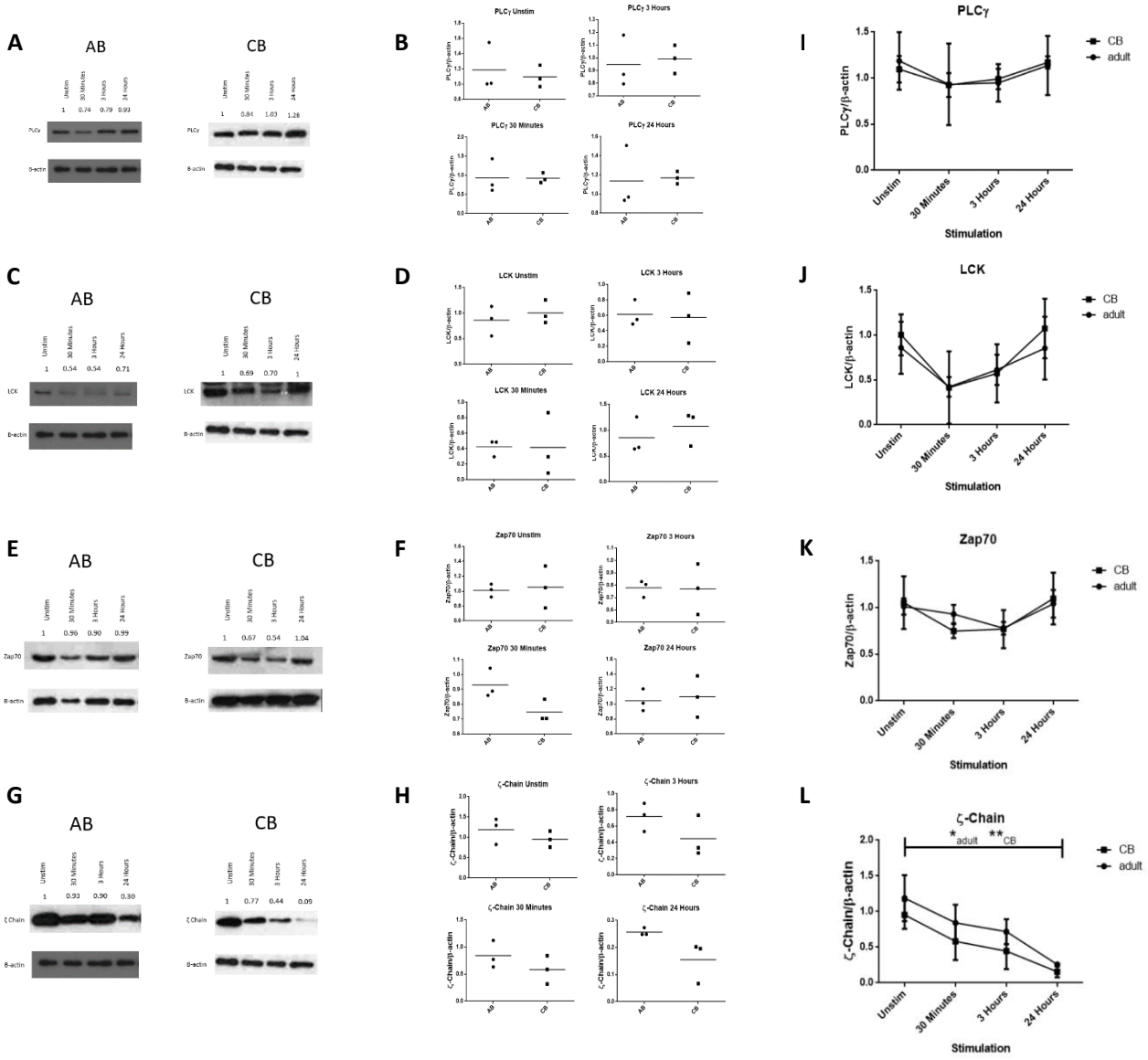
A
AB
CB
Unstim
30 Minutes
3 Hours
24 Hours
1 0.74 0.79 0.93
1 0.84 1.03 1.28
PLCγ
β-actin
B
PLCγ Unstim
PLCγ 3 Hours
PLCγ 30 Minutes
PLCγ 24 Hours
PLCγ/β-actin
AB
CB
I
PLCγ
CB
adult
PLCγ/β-actin
Unstim
30 Minutes
3 Hours
24 Hours
Stimulation
C
AB
CB
1 0.54 0.54 0.71
1 0.69 0.70 1
LCK
β-actin
D
LCK Unstim
LCK 3 Hours
LCK 30 Minutes
LCK 24 Hours
LCK/β-actin
J
LCK
CB
adult
Stimulation
E
AB
CB
1 0.96 0.90 0.99
1 0.67 0.54 1.04
Zap70
β-actin
F
Zap70 Unstim
Zap70 3 Hours
Zap70 30 Minutes
Zap70 24 Hours
Zap70/β-actin
K
Zap70
CB
adult
Stimulation
G
AB
CB
1 0.93 0.90 0.30
1 0.77 0.44 0.09
ζ Chain
β-actin
H
ζ-Chain Unstim
ζ-Chain 3 Hours
ζ-Chain 30 Minutes
ζ-Chain 24 Hours
ζ-Chain/β-actin
L
ζ-Chain
* adult ** CB
CB
adult
ζ-Chain/β-actin
Unstim
30 Minutes
3 Hours
24 Hours
Stimulation

Figure 6. Signaling protein expression in cord and adult blood naïve CD4 T-Cells. Naïve CD4 T-cells were purified from cord and adult donors and stimulated with anti-CD3 and anti-CD28 antibodies for 30 minutes, 3 hours, 24 hours, or left unstimulated. Western blot analysis of protein expression was quantified via ImageJ. (A,C,E,G) Representative data of PLCγ, LCK, Zap70, and ζ-chain expression, respectively. Numerical values represent protein expression relative to the unstimulated condition and β-actin loading control. (B,D,F,H) Comparison of PLCγ, LCK, Zap70, and ζ-chain expression levels between cord and adult naïve CD4 T-cells at each time point, respectively. Numerical values are derived from the ratio of protein expression to β-actin loading control at each time point. Bars represent mean protein expression of 3 cord and 3 adult donors. (I,J,K,L) Average protein expression overtime of PLCγ, LCK, Zap70, and ζ-chain as expressed by ratio of protein expression to β-actin loading control. Statistical analysis between unstimulated and 24 hours stimulation was conducted. * $p < 0.05$. ** $p < 0.005$.

Discussion

Our results suggest that the decreased T-cell activity observed in neonates is not due to decreased expression of proximal signaling proteins. These results were somewhat surprising, however, as we expected there to be significant differences between the cord blood and adult peripheral blood naïve CD4 T-cell populations. There is data suggesting differences in the level of PLC activation, and both phosphorylation and expression of LCK and Zap70 in adult and neonatal total T-cell populations that has previously been reported[171-173]. Our data suggests that there is no difference between the two populations, and that differences observed in T-cell reactivity are not due to the level of proximal signaling protein expression. However, Miscia's work, while showing differences in proximal signaling molecules, examined total T-cell populations[172]. This is different from our work, which is a direct comparison of the naïve CD4 T-cell populations derived from umbilical cord blood and adult peripheral blood. Naïve T-cells compose a large portion of the umbilical cord blood T-cell population, while the adult cellular

composition is mainly effector and memory cells, with a much smaller portion of naïve T-cells than cord blood. As such, a direct comparison of total T-cell populations contains influences from multiple cell types and adds a significant level of complexity during data analysis. Taking this into account, it is possible that the naïve cell populations between adult and cord do not differ in protein expression, as evident by our results, while the differences previously observed in the levels of LCK and Zap70 seen in previous studies of total T-cell populations may be stemming from the larger effector and memory T-cell populations in adult samples.

If the hyporeactive nature of neonatal naïve CD4 T-cells is not due to reduced expression of proximal signaling proteins, then what could be other factors contributing to the differences in T-cell activation between neonates and adults? As mentioned previously, there is some literature supporting the hypothesis that neonates exhibit lower levels of NFAT and CD40L, which would help explain their hyporeactive nature. Deficiencies in CD40L would impact the interaction T-cells have with APCs, thus lowering the strength of the cell/cell interaction and decreasing T-cell stimulation[44]. Deficiencies in NFAT would contribute to decreased calcium influx, a crucial step in the production of cytokines needed for cell proliferation[174]. It is unlikely that the differences in T-cell activity are due to surface expression of the αβTCR, as there has been no significant difference in TCR expression detected between adult and umbilical cord blood T-cell populations[175].

While there was no statistically significant difference in protein expression levels between cord and adult T-cells, we did see a decrease in ζ-chain expression upon stimulation that continued to decrease with increased stimulation. This is an intriguing finding, as the expression

of ζ-chain differs from the activity observed in the other proximal signaling proteins we probed for. PLCγ, Zap70, and LCK each showed reduced expression after initial stimulation, however by 24 hours protein levels had risen back up. The return of protein expression level would allow for re-stimulation and further expansion after the initial signaling event had occurred. By this logic, we expected that all proximal signaling molecules would be fully re-expressed by the 24 hour time marker. However, we found that ζ-chain expression does not return to the levels observed in the unstimulated condition by 24 hours. In fact, levels continued to decrease with prolonged stimulation in both the cord blood naïve CD4 s and in the adult peripheral blood naïve CD4 T-cells. This continued decrease in ζ-chain expression has been observed previously in T-cells, however our findings are novel in the fact that this ζ-chain activity has not been shown in the human naïve cell population in any literature we have encountered thus far[176,177]. Additionally, while not reaching the level of statistical significance, there were observable differences in the expression of ζ-chain between cord and adult blood cells. Cord blood appeared to have lower expression by 24 hours than adult blood, suggesting that potential cord blood hyporeactiveness to simulation may be in part due to ζ-chain expression. This being said, there was observable decreases in ζ-chain expression for both cord and adult blood naïve CD4 T-cells with stimulation. While protein degradation occurs frequently after initial stimulation, the low abundance of ζ chain after 24 hours begs the question of how cell signaling occurs again in the absence of the ζ-chain.

ζ-chain is a critical mediator of signaling events through its interaction within the TCR and with Zap70 and LCK. After the initiation of the signaling pathway, proteins may undergo

lysosomal degradation prior to their re-expression after transcription and translational events. The ability of LCK, PLCγ, and Zap70 to return to appreciable levels by 24 hours stimulation suggests that cells are capable and ready for future signaling events. However, this trend is not observed with ζ-chain, as in both cord and adult there is a continued decrease in expression. The cause of this continued decrease in expression, and why this profile is isolated to that of the ζ-chain has not, to our knowledge, yet been characterized.

Furthermore, should ζ-chain not be re-expressed for future stimulation events, how then, can T-cells propagate future signaling events? A potential answer could be an accessory molecule that could replace CD3ζ and allow for TCR complex surface expression and facilitate future cell signaling events. This molecule would have to serve the structural and functional role that ζ-chain plays in the TCR complex. There is evidence that suggests that this molecule could be the γ subunit of the Fcε receptor (FcεRIγ). It was found through molecular cloning that the FcεRIγ was a member of the CD3ζ gene family and that they likely derived from the same common ancestral gene. FcεRIγ is found in a multitude of cell types including T-cells, basophils, macrophages, and natural killer (NK) cells. The FcεRIγ has also been shown to have structural homology to CD3ζ and can associate with components of the TCR to help upregulate surface expression of the TCR complex[178]. Thus, FcεRIγ, at least structurally, replace ζ-chain and allow for the proper assembly of the TCR complex. Additionally, FcεRIγ contains ITAM motifs, which are key components of the signaling response, likely allowing for T-cell re-stimulation in the absence of ζ-chain due to the functional replacement with FcεRIγ. However, studies using CD3ζ

knockout mice reconstituted with FcεRIγ, revealed that while FcεRIγ was capable of replacing CD3ζ structurally, the capability for T-cells to signal with FcεRIγ was greatly reduced[179].

CHAPTER FIVE

GENERAL DISCUSSION

Our results demonstrate that cord blood naïve CD4 T-cells have cell intrinsic differences than adult peripheral blood naïve CD4 T-cells and that the two populations are programmed differently. We found that CD26 is expressed at high levels in naïve CD4 T-cells from cord blood while not found at high levels in naïve CD4 T-cells from adult peripheral blood, suggesting that it could serve as a potential marker for cord blood naïve T-cells. Additionally, we found that surface expression of CD26 increased in response to TCR stimulation in adult T-cells but was relatively unaffected with stimulation in cord blood T-cells. This is suggestive that CD26 may serve different purposes in cord and adult naïve CD4 T-cells.

We also found that both cord blood and adult blood naïve CD4 T-cells can be maintained in a naïve phenotype, as characterized by $CD45RA^{+}$ $CD45RO^{-}$. However, their proliferative responses differ when cultured with IL-7, with cord blood derived naïve CD4 T-cells expanding nearly 2 fold while adult naïve CD4 T-cells did not exhibit any proliferative response, thus further demonstrating the concept that neonatal and adult T-cells are functionally distinct.

Surprisingly, when stimulated to differentiate in to effector Th1/Th2 populations, cord blood derived naïve CD4 T-cells had a higher capability to produce elevated levels of both Th1 (IFNγ) and Th2 (IL-5 and IL-13) like cytokines. This differs from the general dogma, which suggested that cord blood naïve CD4 T-cells had a propensity to form Th2 like responses[75,168]

Furthermore, our data also demonstrated that, when stimulated under neutral conditions, fresh cord blood naïve T-cells produced significantly less IFNγ than adult peripheral blood naïve CD4 T-cells. This deficiency in IFNγ exhibited by cord blood naïve CD4 T-cells is similar to previously reported data, and the differences between cord and adult T-cells in the response to stimulation led us to hypothesize that there were differences within the TCR signaling pathway that could account for the differences in cytokine production by the two cell populations. Our results showed that there were no statistically significant differences in the expression of any proximal signaling proteins involved in the TCR signaling pathway. PLCγ, Zap70, and LCK each showed similar trends throughout stimulation, with protein expression decreasing with initial stimulation and returning to appreciable levels by 24 hours stimulation. The expression pattern of ζ-chain, however, displayed a unique pattern, with reduced expression with initial stimulation that continued to decrease with prolonged stimulation.

Our findings have implications on the current understanding of T-cell immunity and the functions of the neonatal immune system. Notably, our data suggests that the neonatal naïve CD4 T-cells do not have an intrinsic propensity to form Th2 like effector T-cell responses. They are equally as capable of forming Th1 cytokine responses as well as Th2 effector responses, even at a greater capacity when compared to naïve T-cells from adult peripheral blood. This demonstrates that the neonate is capable of forming both humoral and adaptive immune responses to infection. However, further research needs to be conducted to see if these intrinsic naïve CD4 T-cell characteristics are similar *in vivo*, where they are exposed to a more diverse cellular and biochemical environment.

Additionally, our data combined with known immunodeficiencies observed in ADA deficient newborns, outline the importance of CD26 to the functioning of the neonatal immune system[153]. The high expression of CD26 in cord blood naïve CD4 T-cells not only serves a function as a marker we can use for cellular identification, but physiologically it may serve to enhance proliferative responses to antigenic stimulation through its binding to ADA and subsequent cleavage of extracellular adenosine, thus eliminating adenosine's inhibition of proliferation. This deamination of adenosine may be a systemic effect, as ADA may bind both membrane bound and soluble CD26. We have previously found that soluble CD26 is increased in activated cord blood T-cells, thus allowing for systemic T-cell proliferative responses. This increased T-cell proliferative responses may help to compensate for decreases in T-cell activation observed in neonatal T-cells. Observed decreases in T-cell activation exhibited by neonates helps to explain why they are susceptible to sever and prolonged infections. Less responsive T-cells leave them with deficiencies in both humoral and cellular mediated immune responses. Furthermore, their decreased activation profiles likely have large implications on their ability to clear established infections.

Our data revealed that the decreased responsiveness to TCR stimulation exhibited by neonatal T-cells, when compared to those derived from adult peripheral blood, is not due to deficiencies in proximal TCR signaling components. While protein expression levels do not differ between adult and neonatal naïve T-cells, future research is needed to investigate the activation cascades between the two populations. It is possible that neonates have deficiencies in phosphorylation events responsible for the propagation of TCR signals, which would have no

effect on total protein expression but could have effects on proliferative response and cytokine production in response o stimulation. Additionally, our data focused on soluble fractions of cell lysates, however failed to investigate the insoluble fractions. There may be observable differences in insoluble components between adult and neonatal naïve CD4 T-cells.

Unexpectedly, however, we saw a significant decrease in the expression of ζ-chain between the unstimulated and 24 hours stimulation conditions in both cord and adult blood naïve CD4 T-cells. ζ-chain, a critical component of the TCR complex, contains ITAM motifs that are crucial for the propagation of signal necessary for proper T-cell activation. While we saw decreases in other signaling components after initial stimulation, only ζ-chain failed to be re-expressed by 24 hours stimulation. Further research needs to be conducted regarding the reduction in expression of ζ-chain and the ability of cells to propagate future signaling events. Perhaps the observed reduction is due to ubiquitination and subsequent lysosomal degradation, however fails to explain why this trend is only observed in ζ-chain. It is possible that ζ-chain expression could be deleterious to future signaling events and that cells require a separate mechanism to propagate signal.

In summary, our work suggests that human cord blood naïve CD4 T-cells are intrinsically distinct from adult naïve CD4 T-cells, which may provide insight into observed differences in the immune responses between the two populations. They differ in the expression of CD26, which may further amplify functional differences between the two populations. Additionally, we found that cord blood naïve CD4 T-cells are not pre-programmed to produce Th2 like cytokines yet are able to differentiate into effector subsets with a higher capacity for both Th1 and Th2

cytokine production than adult T-cells. Furthermore, we found that freshly isolated cord blood naïve CD4 T-cells display a reduced response to antigenic stimulation in comparison to adult naïve CD4 T-cells that is not due to deficiencies in signaling molecules. Lastly, we found that prolonged stimulation of both cord blood and adult blood naïve CD4 T-cells results in a continued decrease in ζ-chain expression, which differs from expression dynamics exhibited by other signaling molecules. This is suggestive that there are ζ-chain independent mechanisms of propagating stimulatory signals in naïve T-cells.

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VITA

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