EQUINE B LYMPHOPOIESIS AND $CD5^+$ B LYMPHOCYTES

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The development of the humoral immune system starts during gestation with the production of two major types of B cells: B1 and B2. B2 cells are the classic players in the adaptive humoral immunity, while B1 cells are considered components of innate immunity and have important developmental, phenotypic, and functional differences with B2 cells. B1 cells are predominant early in ontology in mice and humans; therefore, my studies of B cell development in the horse began in the primary lymphoid organs of the fetus.

In the first part of my study, I tested how primary lymphoid tissues of the equine fetus were equipped to support B cell hematopoiesis and immunoglobulin (Ig) diversity of the pre-immune repertoire. I found that the equine fetal liver and the bone marrow are active sites of hematopoiesis based on the expression of signature mRNA (c-KIT, CD34, IL7R, CXCL12, IRF8, PU.1, PAX5, NOTCH1, GATA1, CEBPA) and protein markers (CD34, CD19, IgM, CD3, CD4, CD5, CD8, CD11b, CD172A) of hematopoietic development and leukocyte differentiation molecules, respectively at approximately 100 days of gestation (DG). Immunoglobulin V(D)J segments were sequenced in primary lymphoid organs of the equine fetus and adult horse, revealing that some aspects of Ig diversity in the adult were established in the fetus (similar heavy chain VDJ segment utilization, CDR3 lengths), while others were developmentally programmed (lambda light chain segment utilization, sequence diversity). These data suggest that the B cells

produced in the liver and bone marrow of the equine fetus generate a wide repertoire of pre-immune Igs for protection, and the more diverse use of different lambda variable gene segments in fetal life may provide the neonate an opportunity to respond to a wider range of antigens at birth.

In the second part of my study, I tested aspects of ontogeny, phenotype, and function of equine $CD5^{hi}$ B cells, putative B1-like cells. The expression of CD5 can distinguish murine B1a from B2 cells, and my study tested how this marker could distinguish B1-like cells in the horse. I learned that $CD5^{hi}$ B cells were more frequent early in development and in the peritoneal cavity. $CD5^{hi}$ cells more frequently co-expressed CD2, CD21, and CD11b than $CD5^{lo}$ B cells in the peripheral blood when measured with flow cytometry, but had similar mRNA expression of B1 and B2 signature genes including DGKA, FGL2, PAX5, and IGHM when measured with qRT-PCR. Sequencing of lambda light chain segments revealed that $CD5^{hi}$ and $CD5^{lo}$ B cells generate similar levels of immunoglobulin diversity; however, $CD5^{hi}$ B cells more frequently bound to fluorescence-labeled phosphorylcholine and secreted IL-10 in vitro. $CD5^{hi}$ B cells also represented a greater proportion of remaining B cells in horses with common variable immunodeficiency. Taken together, the results of my study show that equine $CD5^{hi}$ B cells share some of the same characteristics attributed to B1 cells in other species, and may be explored for immune protection in patients with humoral immunodeficiency.

BIOGRAPHICAL SKETCH

Jennifer grew up in Homer, NY, a small town in Central New York State. Her appreciation for the human-animal bond began in childhood, participating in 4-H, horseback riding, and showing her Shetland Sheepdog, Abbi, in agility and obedience through the American Kennel Club. Jennifer was fortunate to be introduced to research early in her career and worked in the Laboratory of Dr. Peter Jeffers in the Chemistry Department at SUNY Cortland during the summers of her Junior and Senior Years of High School. She graduated from Homer High School where she graduated Salutatorian of her class.

Jennifer was a Biological Sciences major in the Cornell College of Agriculture and Life Sciences (CALS) and continued to be involved in research as a Rawlings Presidential Research Scholar. She first worked with Dr. Jan Scarlett, studying the behavior of cats in single verses colony housing; and then completing an Honor's Thesis in Dr. Lisa Fortier's Lab, studying the effect of a matrix metalloproteinase on equine chondrocytes. As an undergraduate, Jennifer quickly decided that Veterinary Medicine would be the ideal career to combine her love for biology and animals.

To fulfill a requirement for the Biology major, Jennifer enrolled in Communication 101 in the Spring Semester of 2005. It was in this class that she met her husband, Adrian, who later proposed in Milan, Italy the day she presented at the International Veterinary Immunology Symposium in August of 2013. Jennifer graduated from CALS summa cum laude and was accepted to the combined DVM/PhD program at the Cornell Veterinary College. Jennifer joined Julia Felippe's Equine Immunology Lab and was excited to have the opportunity to be trained in basic and clinical immunology. She

graduated from the DVM program in May of 2013, and following completion of the PhD program will begin a position at the Veterinary Medical Center of Central New York as a rotating small animal intern.

To my father, Joseph, and husband, Adrian, for their love and support in the brightest and darkest of days.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Hematopoiesis in fetal life

Hematopoiesis begins early in development in the aorta-gonad-mesonephros/para-aortic splanchnopleura region (AGM/P-SP) and yolk sac (YS) of the mammalian embryo (Butler et al. 2011, Ivanovs et al. 2011, Medvinsky and Dzierzak 1996, Moore and Metcalf 1970, Weissman et al. 1977). Hematopoiesis can be detected as early as 16 to 19 days of gestation (DG) in the human fetus (Luckett 1978, Peault et al. 2002). In the mouse, an initial wave of cells with B lymphoid potential arises at 8.5 to 9.5 DGs (Godin et al. 1993, Yoshimoto et al. 2011) just before definitive hematopoietic stem cells (HSCs) can be identified at 9 to 10 DG (Medvinsky and Dzierzak 1996, Yoder et al. 1997). A primitive wave of murine fetal erythrocytes also develops independent of definitive HSC at 7 DG (Palis et al. 1999). Hematopoietic stem cells proliferate and migrate to the fetal liver, where they further expand and differentiate into the erythroid, myeloid or lymphoid lineages (Houssaint 1981, Johnson and Moore 1975, Yokota et al. 2006). In human fetuses, definitive HSCs appear around 27 DG and seed the fetal liver beginning at 30 DG (Tavian et al. 1999, Tavian et al. 2001). B cell progenitors first appear in the human fetal liver by 7.5wks of gestation (Gathings et al. 1977). The first definitive B-lymphoid restricted c-kit^{+}AA4.1^{+}CD19^{+} cells in the mouse can be identified in the AGM, YS, and fetal liver at 10 to 11 days post coitus (de Andres et al. 2002). Yoshimoto et al. (2011) showed that these B cell progenitors arise independently in the AGM and YS, and both subsequently seed the fetal liver using mice that lack a heart beat and functional systemic circulatory system due to a deletion in the Na^{+}/Ca^{2+}

exchanger *Ncx1* (Koushik et al. 2001, Yoshimoto et al. 2011). The liver becomes a primary hematopoietic organ during fetal life and supports a second expansion of B cells until the bone marrow takes over near the end of gestation (Dzierzak and Speck 2008, Yokota et al. 2006). In humans, the fetal liver functions as the main hematopoietic organ from 6 to 22 weeks of gestation; subsequently, the bone marrow becomes the main hematopoietic organ for the remainder of gestation and throughout adult life (Gathings et al. 1977, Kamps et al. 1989, Timens and Kamps 1997).

Hematopoiesis has also been studied in ruminants such as sheep (150 day gestation) and cattle (280 day gestation). In sheep, the first evidence of hematopoiesis is detected by 17 to 18 DG with the fetal liver producing definitive erythrocytes by 27 DG (Alsalami and Filippich 1999). B cells are observed in the fetal liver, but the spleen appears to be an important organ for B cell expansion during fetal life, where they are first observed by 40 DG (Press et al. 1993). Gut associated lymphoid tissue (GALT) is an important site of B lymphopoiesis in postnatal sheep, and the Peyer's patches in the ileum are colonized by lymphoid progenitors between 68 to 70 DG (Reynolds and Morris 1983). In cattle, B cell progenitors are detected in the fetal liver by 60 DG and in the fetal bone marrow by 90 DG (Ekman et al. 2010, Schultz et al. 1973). B cells are also observed in other lymphoid tissues such as the spleen and lymph nodes between 60 to 90 DG (Ishino et al. 1991, Schultz et al. 1973). Though seemingly less so than sheep, GALT is also important in postnatal B cell development in cattle. Lymphocytes begin to infiltrate the lamina propria of the ileocecal region by 120 DG, with IgM^+ B cells and distinct Peyer's patches observed in this location by 180 DG (Ishino et al. 1991).

In the rabbit, B cells are primarily produced during fetal and neonatal life. B cell progenitors are detected in the fetal liver by 14 DG (of an approximately 30 day gestation) and in the bone marrow by 21 DG (McElroy et al. 1981, Tunyaplin and Knight 1995, Zhu et al. 1999). Rabbit GALT includes the Peyer's patches, the sacculus rotundus, and the appendix, and in contrast to other species discussed, B cell progenitors do not colonize these tissues until birth (Mage et al. 2006). B cell production peaks at 3 to 4wks of age and then steadily declines; few B cell progenitors are detectable after 3 to 4mths of age as bone marrow and GALT production of B cells is very limited in the adult (Crane et al. 1996, Hayward et al. 1978, Lanning et al. 2000, Mage et al. 2006).

Hematopoiesis during fetal life has also been extensively studied in swine. B cell progenitors are detected with molecular markers in the yolk sac at 20 DG, fetal liver at 30 DG, and then BM at 45 DG (Sinkora et al. 2000, Sinkora et al. 2003). B lymphopoiesis does not occur in the Peyer's patches of pigs, but these structures are formed by 70 DG (Butler et al. 2013). The yolk sac involutes at 24 DG, so the fetal liver is the main hematopoietic organ from approximately 24 to 45 DG, after which hematopoiesis occurs primarily in the bone marrow (Sinkora et al. 2005).

Differentiation of B cell progenitors

B cells develop from hematopoietic precursors in a tightly controlled manner by gene regulatory networks, chromatin remodeling, cytokine signaling, and selection through BCR affinity reviewed in (Busslinger 2004, Hardy et al. 2007, Herzog et al. 2009, Johnson et al. 2005a, Nagasawa 2006, Nutt and Kee 2007, Singh et al. 2005).

HSCs are a heterogeneous population of cells (Christensen and Weissman 2001). The earliest HSCs are capable of both self-renewal and multi-lineage differentiation (Christensen and Weissman 2001, Prashad et al. 2015). Activation of the c-KIT tyrosine kinase receptor by its ligand stem cell factor (SCF) promotes survival of the long-term reconstituting HSCs (LT-HSCs) (Busslinger 2004). The capacity for self-renewal is progressively diminished, while the potential for multi-lineage differentiation remains, as cells differentiate from LT-HSCs to short-term reconstituting HSCs (ST-HSCs) to multipotent progenitors (Table 1.1) (Benveniste et al. 2010, Christensen and Weissman 2001, Morrison and Weissman 1994).

In mice, ST-HSCs produce lymphoid-primed multipotent progenitors (LMPPs) and common myeloid progenitors (CMPs) (Adolfsson et al. 2005). LMPPs generate lymphoid and myeloid, but not erythroid, megakaryocyte, or granulocyte lineages, while CMPs generate myeloid, erythroid, and megakaryocyte lineages (Adolfsson et al. 2005, Doulatov et al. 2010). In mice, the combined expression of the transcription factors Ikaros and PU.1 induce the expression of the tyrosine kinase receptor Flt3, and down regulate erythroid lineage genes such as GATA1 and EpoR (Adolfsson et al. 2005, Medina et al. 2004, Yoshida et al. 2006). LMPP cells also down-regulate HSC-related transcription factors (TAL1, GATA2), and up-regulate lymphoid (E2A) and myeloid (MPO, CSF1R) associated genes (Kohn et al. 2012).

Within the LMPP population, a subpopulation of cells referred to as early lymphoid progenitors (ELPs), identified by the expression of the B cell specific gene RAG1, have increased lymphoid potential with decreased myeloid potential, and

Table 1.1 Phenotype of hematopoietic progenitors in the B lymphoid lineage

Progenitor	Phenotype in Mouse	Phenotype in Human
Long-term reconstitution HSCs (LT-HSCs)	$Lin^-Sca\text{-}1^{hi}c\text{-}kit^{hi}CD34^-Flt3^-CD49b^{lo}$ (Benveniste et al. 2010, Christensen and Weissman 2001, Doulatov et al. 2012, Morrison and Weissman 1994,)	$CD34^+CD38^{lo/-}CD90^+GPI\text{-}80^+$ (Prashad et al. 2015)
Intermediate-term HSCs (IT-HSCs)	$Lin^-Sca\text{-}1^{hi}c\text{-}kit^{hi}CD34^{lo}Flt3^-CD49b^{hi}$ (Benveniste et al. 2010)	not defined
Short-term reconstituting HSCs (ST-HSCs)	$Lin^-Sca\text{-}1^{hi}c\text{-}kit^{hi}CD34^{hi}Flt3^+$ (Benveniste et al. 2010, Christensen and Weissman 2001)	not defined
lymphoid-primed multipotent progenitors (LMPPs)	$Lin^-Sca\text{-}1^{hi}c\text{-}kit^{hi}Flt3^+IL\text{-}7R\alpha^+$ (Adolfsson et al. 2005, Doulatov et al. 2012)	$Lin^-CD34^+CD38^{lo}CD45RA^+$ $Flt3^+CD62L^+CD10^-$ (Kohn et al. 2012)
Common myeloid progenitor (CMP)	$Lin^-Sca\text{-}1^-c\text{-}kit^{hi}CD34^+Flt3^-$ (Adolfsson et al. 2005, Doulatov et al. 2012)	$CD45RA^-Flt3^+CD10^-CD7^-$ (Doulatov et al. 2012)
Common lymphoid progenitor (CLP)	$Lin^-Sca\text{-}1^{lo}c\text{-}kit^{lo}IL\text{-}7R\alpha^+$ (Mebius et al. 2001)	$CD34^+CD38^+CD10^+CD19^-$ (Galy et al. 1995)
Pre-pro B cell (Early B in human)	$AA4.1^+CD45RA^+CD43^+HSA^-CD19^-$ (Hardy et al. 1991, Li et al. 1996)	$CD34^+CD38^+CD10^+IL7R^+CD19^-$ (LeBien 2000)
Pro B cell	$CD45RA^+CD43^+BP\text{-}1^+HSA^+CD19^+$ (Hardy et al. 1991)	$CD34^+CD38^+CD10^+IL7R^+CD19^+$ (LeBien 2000)
Pre B cell	$CD45RA^+CD43^-BP\text{-}1^+HSA^+CD19^+Ig^-$ (Hardy et al. 1991)	$CD34^-CD10^+CD19^+Ig^-$ (LeBien 2000)
Progenitor	**Phenotype in Cattle**	**Phenotype in Pigs**
Pre-B cell	$CD79a^+CD21^-IgM^-$ (Ekman et al. 2010)	$CD2^{lo}sIgM^-CD3\varepsilon^-$ (Sinkora et al. 1998)

differentiate into common lymphoid progenitors (CLPs) (Igarashi et al. 2002). The CLPs have the potential to differentiate into B, T, NK, and DC cells (Galy et al. 1995, Medina et al. 2004). CLPs in the murine fetal liver have greater plasticity than those in the adult bone marrow, and they retain the capacity to differentiate into macrophages (Borrello et al. 2001, Davidson et al. 1988, Mebius et al. 2001). Signaling through Flt3 in addition to PU.1 and FOXO1 activity induce the expression of the IL-7R (Dengler et al. 2008, Kondo et al. 1997). The IL-7R is also upregulated in human cells (Kohn et al. 2012); in contrast to mice (Erlandsson et al. 2004), IL-7 is not absolutely required for human B cell development (Noguchi et al. 1993, Noguchi et al. 2008, Prieyl and LeBien 1996). PU.1, E2A, and IL-7R signaling in the CLP induce the expression of EBF (Kee and Murre 1998, Roessler et al. 2007). EBF and E2A act synergistically to specify B cell differentiation with the induction of B cell-specific genes, including enzymes important in immunoglobulin rearrangement (RAG1 and RAG2), and components of the surrogate lambda chain (VPREB, IGLL1) (Kohn et al. 2012, Medina et al. 2004, O'Riordan and Grosschedl 1999, Sigvardsson et al. 1997). These B cell specification genes are expressed in the earliest B cell specific progenitor, the pre-pro B cell (Li et al. 1996). EBF and E2A also induce the expression of the B cell specific transcription factor PAX5, which commits specified progenitors to the B cell lineage at the pro-B cell stage (Fuxa and Busslinger 2007).

PAX5 controls commitment to and maintenance of B cell identity by promoting transcription of B cell specific genes and repressing lineage inappropriate genes (Schebesta et al. 2007). Genes activated by PAX5 include pre-BCR and BCR signaling components (CD19, CD21, CD79a, VPREB, BLNK), and transcription factors (EBF1,

E2A, IRF8, IRF4, IKZF3, LEF1) that further reinforce B cell differentiation (Horcher et al. 2001, Pridans et al. 2008, Schebesta et al. 2007). PAX5 down-regulates the expression of many cell surface receptors and intracellular signaling molecules associated with early progenitors (FLT3) or other hematopoietic lineages, including myeloid cells (CSF1R*)* and T lymphocytes (NOTCH1) (Pridans et al. 2008). The transcription factor SOX4 (Schilham and Clevers 1998) and IL-7 signaling are also important at the pro-B cell stage for differentiation and proliferation, though the dependence of pro-B cells on IL-7 varies with ontogeny and by species. Pro-B cells in the murine adult bone marrow require IL-7 signaling through the IL-7R receptor (a heterodimer of IL-7Rα and IL2R-γc) for survival and proliferation (Carvalho et al. 2001, Namen et al. 1988), while pro-B cells in the fetal liver can be supported in the absence of IL-7 by thymic stromal-derived lymphopoietin (TSLP), the receptor of which is also composed of the IL-7Rα chain along with at least one other protein named TSLP receptor (Carvalho et al. 2001, Vosshenrich et al. 2003). Human pro-B cells also develop in the absence of IL-7R signaling, but their proliferation in vitro is augmented with IL-7 supplementation (Johnson et al. 2005b, Noguchi et al. 2008).

B cell commitment and immunoglobulin rearrangement

Critical to B lymphopoiesis from the pro-B cell through the immature B cell developmental stages is the generation of a functional immunoglobulin (Ig) molecule, which requires somatic recombination of the heavy chain variable (IGHV), diversity (IGHD), and joining (IGHJ) gene segments, and lambda or kappa light chain variable (IGLV or IGKV) and joining (IGLJ or IGKJ) gene segments. Rearrangement of the

immunoglobulin genes occurs only in the B lymphoid lineage and in a highly ordered and developmentally regulated pattern (Fuxa et al. 2004, Fuxa and Busslinger 2007). Heavy chain recombination occurs first in the pro-B cell, with IGHD to IGHJ followed by IGHV to IGHD-IGHJ rearrangement. The enzymes RAG1 and RAG2 are the main components of the recombination machinery responsible for V(D)J rearrangement (McBlane et al. 1995). RAG1/2 recognize recombination signal sequences (RSSs), consisting of a conserved nanomer and heptamer sequence separated by either 12 or 23bp that flank each V(D)J segment (Hiom and Gellert 1998, McBlane et al. 1995). Segments with different length spacers (one 12bp and one 23bp) are recombined ensuring that only IGHD to IGHJ, or IGHV to IGHD-IGHJ recombination occurs according to the 12/23 rule (McBlane et al. 1995). RAG1/2 induce double stranded breaks at RSSs, which are repaired by the cell's DNA repair machinery (primarily the non-homologous end-joining pathway) (McBlane et al. 1995). The excised genomic DNA between each V(D)J segment is circularized and not replicated with subsequent cell division. RAG1/2 is expressed at the ELP and CLP stage and, accordingly, IGHD-IGHJ rearrangements can be detected in these progenitors (Allman et al. 2003, Igarashi et al. 2002).

In ELP and CLP progenitors, the immunoglobulin loci chromatin is maintained in a repressive state preventing recombination machinery access to the RSSs and, consequently, further recombination from occurring, reviewed in (Johnson et al. 2005a). Histone modifications associated with active chromatin increase specifically at the pro-B cell stage in a step-wise manner first in the regions of the IGH locus spanning the IGHD and IGHJ gene segments, and then at the promoters and RSSs flanking IGHV gene

segments, as a result of indirect actions of transcription factors (e.g. EBF, E2A, PAX5) and IL-7R signaling (Chowdhury and Sen 2001, Corcoran et al. 1998, Fuxa et al. 2004, Johnson et al. 2003, Johnson et al. 2004, Kosak et al. 2002). IGHV, IGHJ, and IGHD segments are distantly spread, spanning more than 3Mb in the mouse, and must be brought in close proximity for recombination (Kosak et al. 2002). PAX5 expression in the pro-B cell induces contraction of the IGH locus, increasing IGHD to IGHJ and initiating IGHV to IGHD-IGHJ recombination (Fuxa et al. 2004). During rearrangement of the heavy chain, the chromatin including the IGL loci are maintained in a repressive state to prevent recombination of the light chain segments during the pro-B cell stage (Morshead et al. 2003).

Rearrangement of the IGH locus marks the transition from the pro-B cell to pre-B cell stage, and also works as an important checkpoint in B cell development. The productively rearranged heavy chain (Igμ) must associate with the surrogate light chain (VpreB and λ5), and be expressed on the cell surface as the pre-B cell receptor (pre-BCR) along with CD79a and CD79b (Karasuyama et al. 1990, Nagata et al. 1997). The inability to express membrane bound Igμ in mice (Kitamura et al. 1991, Yel et al. 1996) or λ5 in humans (Minegishi et al. 1998) results in a complete block in B cell development beyond the pre-B cell stage. Pre-BCR expression induces proliferation of pre-B cells in both the fetal liver and adult bone marrow environments (Rolink et al. 2000). This proliferation is suggested to occur in a ligand independent manner, as neither stromal cells nor cytokines are required in vitro (Rolink et al. 2000). Ikaros expression is necessary for effective downstream signaling through the pre-BCR, and for repressing negative regulators. In large pre-B cells, pre-BCR signaling also down-

regulates RAG1/2 expression (Anbazhagan et al. 2013, Grawunder et al. 1995) preventing immunoglobulin recombination of the second IGH allele (ten Boekel et al. 1998), and consequently, double-stranded DNA breaks in these proliferating cells.

In the more mature small pre-B cell, pre-BCR signaling results in a BLNK-dependent upregulation of transcription factors such as IRF4, IRF8, and Aiolos, which down-regulate surrogate light chain components and promote exit from the cell cycle (Lu et al. 2003, Nodland et al. 2011, Parker et al. 2005, Thompson et al. 2007). Pre-BCR signaling and the activity of transcription factors including IRF4, IRF8, PU.1, E2A, EBF, FOXO1, and FOXO3a promote RAG1 expression and rearrangement of the light chain variable and joining gene segments (Grawunder et al. 1995, Nodland et al. 2011, Romanow et al. 2000). Traditionally, kappa light chains were believed to rearrange before lambda light chains (Hieter et al. 1981); however the order of light chain rearrangement may be species-dependent as lambda rearrangement precede kappa rearrangement in the pig (Sun et al. 2012). This is consistent with the varying ratios of kappa and lambda light chains in circulating antibodies between species (Hood et al. 1967).

Similar to the recombination of IGH gene segments, rearrangement of the IGK or IGL gene segments is associated with chromatin modifications making RSSs more accessible to the recombination machinery and cis-regulatory elements promoting transcription (Johnson et al. 2003). In contrast to heavy chain rearrangement, IL-7 suppresses light chain rearrangement (Nodland et al. 2011). IRF4 induces expression of the CXL12 receptor (CXCR4) on pre-B cells, and promotes migration away from IL-7-producing to CXCL12-producing bone marrow stromal cells (Nodland et al. 2011). B cell

precursors similarly migrate to different microenvironments in the fetal liver during development (Tsuneto et al. 2013).

Pre-B cells that successfully rearrange the kappa or lambda light chain become immature B cells, which express the newly rearranged IgM on the cell surface, and form the BCR with signaling components CD79a and CD79b (Fuentes-Panana et al. 2006, Torres et al. 1996). Basal signaling through the BCR is necessary to suppress recombinase machinery and maintain allelic exclusion at the light chain loci (Tze et al. 2005). V(D)J rearrangement is not an efficient process, and it is estimated that 75% of cells do not survive differentiation from the pro-B to immature B cell stage (Osmond 1991).

In humans, mice, and pigs, B cells are produced in the primary lymphoid organs, which include the fetal liver, fetal bone marrow, and adult bone marrow. In some mammalian species such as rabbits (Vajdy et al. 1998) and sheep (Reynolds and Morris 1983), GALT is considered a primary lymphoid organ. In both species the GALT has been shown to be important sites of B cell expansion and diversification (Gerber et al. 1986, Lanning et al. 2000, Reynaud et al. 1991, Vajdy et al. 1998). Intestinal microflora is required for GALT development in rabbits (Rhee et al. 2004) but not sheep (Reynolds and Morris 1983). Others challenge the idea of GALT being considered a primary lymphoid tissue, pointing in part to studies showing that surgical removal of these structures does not completely ablate the B cell compartment (Butler and Sinkora 2013).

Generation of the pre-immune repertoire

The pre-immune immunoglobulin repertoire develops in the absence of exogenous antigens in the primary lymphoid tissues, and is often more specifically defined as developing during fetal life (Liljavirta et al. 2014, Sun et al. 1998). Diversity of the pre-immune repertoire is generated primarily by combinatorial and junctional diversities. Combinatorial diversity is produced by joining different heavy chain and light chain gene segments. The number of gene segment used to construct immunoglobulin molecules varies by species: for example, the mouse has more than 90 functional IGHV segments, while the chicken has only one (Das et al. 2008). Different species utilize lambda or kappa light chain sequences in different ratios. For example, mice use primarily the kappa chain, while the horse predominantly the lambda chain, and humans have comparable use of lambda and kappa chains (Butler et al. 2006, Ford et al. 1994, Hood et al. 1967, Kelus and Weiss 1977). In some species, including humans (Zemlin et al. 2001), mice (Yancopoulos et al. 1984), pigs (Butler et al. 2000), cattle (Koti et al. 2010), and sheep (Gontier et al. 2005), combinatorial diversity differs during phases of development, and certain Ig segments are preferentially utilized in fetal or adult life (e.g. IGHJ-proximal IGHV genes in early in ontogeny of the mouse).

Junctional diversity is created by the deletion and addition of base pairs at the junctions of the Ig gene segments during recombination events. Addition of base pairs is created by the enzyme terminal deoxynucleotidyl transferase (TdT) that adds non-template nucleotides, or N-nucleotides, to the regions flanking Ig gene segments (Desiderio et al. 1984). P-nucleotides, are template dependent dinucleotide additions palindromic to the flanking joining ends (Feeney 1990). Species with more limited

combinatorial diversity than mice or humans, including the pig and some ruminants, depend more on junctional diversity and post-recombination processes such as somatic hypermutation (Butler et al. 2006, Butler et al. 2011, Kaushik et al. 2009, Koti et al. 2010, Reynaud et al. 1995, Verma and Aitken 2012). Rabbits use both somatic hypermutation and gene conversion to diversify their immunoglobulin repertoires (Becker and Knight 1990, Pinheiro et al. 2011).

The structure of the antibody molecule includes four framework sequences separated by three complimentary determining regions (CDRs). The CDRs are the sequences that come in contact with antigen, and are the most diverse regions of the immunoglobulin molecule. CDR3 contains the greatest degree of sequence variability and spans the junction of the IGHV, IGHD, and IGHJ gene segments in the heavy chain, and IGLV and IGLJ gene segments in the light chain. Diversity of the CDR3 region is influenced by both combinatorial and junctional diversity. CDR3 length differs in fetal compared to adult life in certain species including humans (Zemlin et al. 2001), mice (Bangs et al. 1991), and sheep (Gontier et al. 2005). The pre-immune repertoire that develops during fetal life is, therefore, primarily germline encoded with elements of combinatorial and junctional diversity that help prepare the neonate for pathogen antigen encounter after birth.

Early development of different B cell populations

In mice, three main populations of B cells arise during fetal life: B1, B2, and marginal zone B cells. B cells that predominantly arise in the early phases of B lymphopoiesis in the fetus are termed B1 cells (Godin et al. 1993), and have distinct

developmental, phenotype, tissue distribution, and functional characteristics that differ from B2, or follicular B cells, which are produced in greater abundance near the end of fetal life and in the adult (Hayakawa et al. 1983) and reviewed in (Berland and Wortis 2002, Montecino-Rodriguez and Dorshkind 2012). B1 cells play important roles in innate immunity, while B2 cells are the conventional players in adaptive humoral immunity (Choi and Baumgarth 2008). Marginal Zone (MZ) B cells reside mainly in the marginal zone between the red and white pulp of the spleen, and share characteristics with both B1 and B2 cells (Casola et al. 2004, Martin et al. 2001, Song and Cerny 2003, Yoshimoto et al. 2011) reviewed in (Cerutti et al. 2013).

B1 cells were identified in the mouse in the early 1980s but their developmental origin remained unresolved for nearly three decades. Two theories emerged and were debated: the lineage hypothesis and the selection model, reviewed in (Montecino-Rodriguez and Dorshkind 2012). The lineage model follows that B1 and B2 cells develop from distinct B cell precursors based on the observation that B cell progenitors in the fetal liver repopulated both the B1 and B2 compartments of an immunodeficient host, while adult bone marrow precursors only efficiently reconstituted the B2 population (Hayakawa et al. 1985, Herzenberg and Herzenberg 1989). Later, Montecino-Rodriguez et al. (2006) identified the $Lin^{-}CD45R^{-/lo}CD19^{+}$ B cell progenitors as precursors that preferentially generate B1 but not B2 cells; subsequent studies have confirmed the bias for generating B1 and MZ B cells, but not B2 cells (Esplin et al. 2009, Montecino-Rodriguez et al. 2006, Yoshimoto et al. 2011). $CD45R^{-}CD19^{+}$ cells in the adult bone marrow were previously reported to be bipotential B cell-macrophage precursors (Montecino-Rodriguez et al. 2001); indeed, mature B1 cells share similarities with

macrophages, such as an increased adherence to glass or plastic in comparison to B2 cells (Hayakawa et al. 1986a), and the expression of the integrin CD11b (Herzenberg et al. 1987). The $Lin^{-}CD45R^{-/lo}CD19^{+}$ cells are observed in the AGM and fetal liver by 11 DG, the fetal bone marrow by 15 DG, and then steadily decline in frequency to low numbers in adult bone marrow (Montecino-Rodriguez et al. 2006). It has been proposed that the sequential timing of generation of first B1 then B2 cells is due to an "ontological switch" in development, as described in T cell development transitioning from $\gamma\delta$T cells to $\alpha\beta$T cells, and the transition from fetal to adult hemoglobin in erythrocytes (Enver et al. 1990, Hardy and Hayakawa 1991, Ikuta et al. 1990, Lee et al. 2013).

There are a few notable differences in early B1 and B2 cell development in mice. Cells with B1 potential are detected earlier than those that have B2 potential in the fetus; B1a and MZ progenitor potential is detected in the para-aortic splanchnopleura region at embryonic days 8.5 to 9 (Godin et al. 1993), and B1 but not B2 potential is observed in the 13 DG omentum (Solvason et al. 1991). PU.1 is absolutely required for B2 B cell development but B1 cells can differentiate from $PU.1^{-/-}$ progenitor cells in vitro (Ye et al. 2005). Wild type B1 cells may have a decreased dependence on the PU.1 transcription factor in vivo as well, as the B1 progenitor is $CD45R^{-/lo}$ and PU.1 has been shown to induce CD45R expression (Medina et al. 2004). Some investigators have found that PAX5 expression is lower in B1 cells relative to B2 cells (Tumang et al. 2005), while others report comparable expression (Fuxa and Busslinger 2007). Following development during fetal life, B1 cells in the mouse are primarily maintained

by self-renewal shown in vivo with adoptive transfer experiments (Hayakawa et al. 1986b, Kantor et al. 1995), and in vitro with long-term culture experiments (Braun 1983).

The alternative theory to the lineage hypothesis, the selection or induced differentiation model, proposed that B1 and B2 cells differentiate from a common progenitor; this theory involves particular signals received during development and selection, such as strength of signaling through the B-cell receptor (BCR) and response to T cell-independent (TI) antigens determining fate (Haughton et al. 1993, Lam and Rajewsky 1999, Watanabe et al. 1999) reviewed in (Berland and Wortis 2002). Strong BCR signaling favors the development of B1 cells, while weaker signaling promotes the development of B2 cells (Casola et al. 2004). This model explains that the difference in precursors from fetal liver and adult bone marrow sources to repopulate an immunodeficient host is due to the difference in BCR specificities; the fetal repertoire is biased for immunoglobulins reactive to frequently encountered TI-antigens, such as self-antigens abundant during fetal life, while the adult repertoire rarely includes these specificities (Berland and Wortis 2002). Further support of this hypothesis included studies showing that, when B cells were transgenic for particular immunoglobulin heavy and light chains commonly utilized by B1 cells, the transgenic B cells developed a B1 phenotype (Arnold et al. 1994, Arnold et al. 2000) reviewed in (Berland and Wortis 2002). In vivo ablation of PU.1 with Cre-recombinase under the control of the endogenous CD19 locus resulted in a decreasing population of B2 cells, as these cells acquire a B1b-like phenotype and accumulate in the peritoneal cavity (Ye et al., 2005).

These two models are not mutually exclusive, however, and Baumgarth (2011) suggests that both developmental pathways occur in the two-pathway model. This

model proposes that B1 cells develop initially, and primarily, from B1 restricted precursors during fetal life and undergo positive selection for reactivity to self-antigens and strong BCR signaling. The B1 cells are maintained in the adult mainly through the process of self-renewal; however, B1 precursors in the adult bone marrow contribute to the B1 pool (particularly B1b cells) over time (Baumgarth 2011). Alterations in the steady state, such as whole body irradiation, seem to increase the B1 generation from bone marrow precursors by unknown mechanisms (Baumgarth 2011).

Peripheral B cell development

Immature B cells emigrate from the fetal liver or bone marrow and further differentiate into mature, naïve, and then into follicular B cells that inhabit germinal centers, marginal zone B cells in the spleen, or B1 cells as reviewed in (Carsetti et al. 2004, Casola 2007). The majority of immature B cells egressing from the bone marrow do not survive to become mature B cells: immature B cells in mice have a life span of about 4 days and only 10 to 20% become mature B cells (Rolink et al. 1998). Mature B2 cells have a life span of 15 to 20wks (Rolink et al. 1998), while B1 cells are longer lived. In mice, B1 cells populate mainly the peritoneal and pleural cavities with a small population in the spleen, and are infrequently found in peripheral blood, bone marrow, and lymph nodes (Baumgarth 2011, Montecino-Rodriguez and Dorshkind 2006).

The expression of a BCR in the immature B cell controls a second important checkpoint during development. B cells with strong specificity to self-antigens undergo receptor editing or are clonally deleted in the bone marrow before egress (central tolerance), or become anergic and are eliminated by apoptosis in the periphery

(peripheral tolerance) (Hartley et al. 1991, Nemazee and Burki 1989, Spanopoulou et al. 1994). Immature B cells in the bone marrow are more likely to undergo receptor editing, while mature B cells in the periphery are more likely to undergo apoptosis (Melamed et al. 1998, Tiegs et al. 2011). Receptor editing involves upregulating RAG1/2 expression, and rearranging the remaining IGK or IGL alleles in an effort to generate a new BCR with decreased specificity to self-antigens (Tiegs et al. 2011).

Not all self-reactive B cells are eliminated; B1 and MZ B cells are positively selected for their BCR engagement with self-antigen (Casola et al. 2004, Wen et al. 2005). Hayakawa and colleagues developed transgenic mice expressing immunoglobulin specific for the Thy-1 glycoprotein, and demonstrated that high levels of antigen positively selected for B1 cells, while low levels positively selected for MZ B cells, and no antigen favored follicular B cell development (Hayakawa et al. 1999, Hayakawa et al. 2003, Wen et al. 2005). Further evidence that the strength of BCR signaling is important in B1 development comes from mice transgenic for immunoglobulin genes specific for phosphatidylcholine (PtC) (Lam and Rajewsky 1999) and the red blood cell antigen 4C8 (Watanabe et al. 1999). In these mice, reduction of surface expression of BCR molecules significantly reduced the number of B1 cells present (Lam and Rajewsky 1999, Watanabe et al. 1999). Mutations that inhibit or disrupt BCR signaling, such as CD19 (Rickert et al. 1995), CD21 (Ahearn et al. 1996), BTK (Hayakawa et al. 1986a), and BLNK (Jumaa et al. 1999), severely impair the development of B1 cells and, to a lesser extent, B2 cells reviewed in (Berland and Wortis 2002). In contrast, increased BCR signaling with mutations of negative BCR signaling regulators, including Lyn (Chan et al. 1997), CD72 (Pan et al. 1999), SHP-1

(Sidman et al. 1986), or overexpression of molecules that promote BCR signaling, such as CD19 (Haas et al. 2005), promote the development of B1 cells and inhibit that of B2 cells reviewed in (Berland and Wortis 2002).

The splenic environment is critical for the development and maintenance of B1a cells: asplenic mice deficient of the Hox11 gene have a severe reduction in B1a cells, although $Hox11^{-/-}$ fetal liver cells transplanted into wild-type mice reconstitute all B cell compartments (Wardemann et al. 2002). When adult mice were splenectomized, the B1a numbers were reduced by 75% (Wardemann et al. 2002). The cytokine BAFF, in addition to low levels of tonic signaling through the BCR, is necessary for the survival and differentiation of immature follicular and MZ B cells in the spleen, but not murine B1 cells (Rowland et al. 2010, Sasaki et al. 2004, Schiemann et al. 2001).

Mature B cells circulate through the blood and lymph visiting secondary lymphoid organs, such as lymph nodes and mucosa-associated lymphoid tissues. In a T cell-dependent antigenic response, B2 cells that recognize their cognate antigen interact with activated T cells, generate germinal centers, and undergo somatic hypermutation (Jacob et al. 1991, Pascual et al. 1994) and class switch recombination (Liu et al. 1996) to produce high-affinity antigen-specific antibodies reviewed in (De Silva and Klein 2015). The enzyme activation-induced cytidine deaminase (AID) that deaminates cysteine residues converting them to uracil residues, is instrumental in both somatic hypermutation and class switch recombination (Muramatsu et al. 2000, Petersen-Mahrt et al. 2015). During somatic hypermutation, DNA mismatches could be replicated, excised by uracil-DNA glycosylase (UDG), or repaired by mismatch repair (MMR) proteins with error prone polymerases (e.g. DNA polymerase η) (Pavlov et al. 2002,

Rada et al. 2004, Schrader et al. 2007) reviewed in (Peled et al. 2008). A subset of the selected cells becomes long-lived plasma cells that home back to the bone marrow or memory B cells reviewed in (Benson et al. 2007).

B1 cells are rarely observed in germinal centers (Choi and Baumgarth 2008) but have been shown, upon influenza infection or intravenous LPS injection, to migrate from body cavities to secondary lymphoid tissues, and differentiate into plasma cells (Yang et al. 2007). B1 cells spontaneously producing immunoglobulin express low levels of BLIMP1, the transcription factor responsible for terminal plasma cell differentiation, while B1-derived plasma cells produce levels comparable with B2-derived plasma cells (Tumang et al. 2005, Yang et al. 2007). While B2 cells class switch to all immunoglobulin isotypes, B1 cells primarily produce IgM and preferentially class switch to IgA (Kaminski and Stavnezer 2006, Tarlinton et al. 1995). A subset of peritoneal B1 cells (B1b) more frequently express IgA mRNA transcripts and secrete more IgA than B2 cells (Roy et al. 2009). B1 cells are major contributors to the overall immunoglobulins secreted at mucosal sites, including the intestinal and respiratory tracts (Choi and Baumgarth 2008, Kroese et al. 1989). Peritoneal B1 cells that home to the intestinal lamina propria and differentiate into long-lived IgA plasma cells can secrete up to half of the total intestinal IgA (Kroese et al. 1989).

B1 cell phenotype and function in mice

B1 cells in the mouse were initially identified based on the expression of CD5 (Ly-1, a traditional T cell marker at the time) and the unique functional characteristic of spontaneous secretion of IgM reactive to self-antigens (Hardy et al. 1989, Hayakawa et

al. 1983, Hayakawa et al. 1984, Hayakawa et al. 1990). CD5 expression on B cells is lower than that of T cells: when measured with flow cytometry, the CD5 mean fluorescence intensity (MFI) in B cells is 10% of the MFI on T cells (Hippen et al. 2000, Manohar et al. 1982). The CD5 molecule associates with the antigen receptor complexes of T and B cells (Lankester et al. 1994), and negatively modulates signaling through these receptors raising the threshold for activation (Bikah et al. 1996). A second population of murine B1 cells that lacked the expression of CD5 but otherwise was identical for the other phenotypic markers was subsequently identified (Stall et al. 1992); therefore B1a, B1b, and B2 cells are best distinguished with a combination of different markers including $CD5^{+}CD11b^{+}$(Mac-1)$CD45RA^{lo}$(B220)$IgM^{hi}IgD^{lo}CD23^{-}CD43^{+}$ for B1a; $CD5^{-}CD45RA^{lo}IgM^{hi}IgD^{lo}CD23^{-}CD43^{+}$ for B1b; and $CD5^{-}CD11b^{-}CD45RA^{hi}IgM^{lo}IgD^{hi}$ $CD23^{+}CD43^{-}$ for B2 cells (Herzenberg et al. 1987, Tung et al. 2004, Yenson and Baumgarth 2014). B1 and B2 cells also have distinct gene expression profiles with differential mRNA expression of genes, including those coding for surface molecules, signaling molecules, molecules important in metabolism, and transcription factors (Diehl et al. 2011, Mabbott and Gray 2014, Tumang et al. 2005, Yamagata et al. 2006).

B1 cells are crucial in host defense against certain pathogens and for maintaining homeostasis, largely by their production of natural immunoglobulins that have specificities for many evolutionary conserved pathogen-associated antigens reviewed in (Baumgarth et al. 2005, Berland and Wortis 2002, Panda and Ding 2015). Natural antibodies in both humans and mice are polyreactive, recognize both foreign and self-antigens (Bhat et al. 1992, Haury et al. 1997, Masmoudi et al. 1990), have weak affinities (Nakamura et al. 1988), can be produced in the absence of exogenous

antigen stimulation (Boes et al. 1998b, Hooijkaas et al. 1984), and are often germline encoded (Briles et al. 1982, Naparstek et al. 1986). Spontaneous antibody secretion by B1 cells has been measured by methods including radial immunodiffusion (Hayakawa et al. 1983), ELISA (Hayakawa et al. 2003), and ELISPOT (Griffin et al. 2011a). B1 and MZ B cells are the primary antibody producers against TI-antigens; B1 cells in the body cavities are poised to respond to organisms that translocate from the gut or lungs, while MZ B cells are positioned to respond to blood-borne infections (Berland and Wortis 2002, Martin et al. 2001).

Unlike B2 cells, which undergo somatic hypermutation and have diverse immunoglobulin repertoires specific to a variety of antigens, B1 immunoglobulin repertoires are more restricted and reactive to TI antigens reviewed in (Berland and Wortis 2002, Hayakawa and Hardy 2000). Limited diversity of B1 cell repertoires was initially described based on cloning V(D)J sequences from hybridomas specific for PtC, a phospholipid frequently detected by B1 BCRs (Hardy et al. 1989, Mercolino et al. 1989, Pennell et al. 1989), and anti-T cell antigen (ATA) (Hayakawa et al. 1990). These studies showed that B1 derived immunoglobulins preferentially used certain V(D)J gene segments that were essentially germline sequences with few mutations, and none to very few N-nucleotide additions. Though there is a bias use by B1 cells of certain IGHV segments such as V_H11 and V_H12 for PtC reactive antibodies (Mercolino et al. 1989), these IGHV segments are not necessarily J-proximal IGHV segments preferentially used in the murine fetal immunoglobulin repertoire (Hardy et al. 1989, Pennell et al. 1989, Yancopoulos et al. 1984).

Subsequent studies measuring combinatorial, sequence, and junctional diversity in B1 cells that were unbiased for antigen specificity have shown that B1 repertoires, particularly those of the B1b subset, are more diverse than originally described. Kantor et al. (1997) showed that FACS-sorted peritoneal B1 cells used a variety of IGHV, IGHD, and IGHJ gene segments similar to peritoneal B2 cells, though B1 cells use certain IGHD and IGHJ gene segments more frequently than B2 cells (Kantor et al. 1997, Tornberg and Holmberg 1995). Roy et al. (2009) showed that despite restricted sequence diversity, there was some level of somatic hypermutation in the FACS-sorted peritoneal B1 cells; B1a IGHV segments containing 2.35 mutations/kb, and B1b IGHV containing 2.9-15.78 mutations/kb (Roy et al. 2009).

Fetal derived B1 cells also have decreased junctional diversity compared to B2 cells: in a study by Holodick et al. (2014), 57% of B1a cells derived from $Lin^{-}CD45R^{-/lo}CD19^{+}$ fetal liver progenitors had zero N-nucleotides, consistent with the absence of TdT expression at this stage of development in the mouse; in contrast, adult bone marrow derived $Lin^{-}CD45R^{-/lo}CD19^{+}$ B cell progenitors generate B1a cells with substantial N-nucleotide additions. The number of N-nucleotide additions increases in B1 cells as mice age: B1b cells have shown to use similar numbers of N-nucleotides as B2 cells in 8-week-old mice (Tornberg and Holmberg 1995), and B1a B cells have comparable frequencies of N-nucleotide additions as B2 cells by 6 to 10 months of age (Gu et al. 1990). The T15 idiotype specific for phosphorylcholine (PC) commonly generated by B1 cells does not contain N-nucleotides additions, and mice with forced expression of transgenic TdT during fetal life generate little T15 antibody, resulting in a failure of their serum to protect B1 deficient XID mice against pneumococcal challenge

(Benedict and Kearney 1999). Therefore, the limited immunoglobulin diversity of B1 cells, particularly B1a cells, can be beneficial for immune protection in some cases (Benedict and Kearney 1999).

B1 cells are important in host defense against infections with bacteria (e.g. *Streptococcus pneumonia* and *Borrelia hermsii*) (Alugupalli et al. 2004, Boes et al. 1998b, Haas et al. 2005), and viruses (e.g. influenza and vesicular stomatitis virus) (Baumgarth et al. 2000, Ochsenbein et al. 1999). Using chimeric mice with allotype-disparate congenic B1 and B2 cells, Baumgarth and colleagues have shown that B1-derived influenza-specific IgM is essential for maximum survival to influenza challenge, and B1a cells accumulate in the regional (mediastinal) lymph nodes, where they increase the local secretion of antibody (Baumgarth et al. 2000, Choi and Baumgarth 2008). The authors show that the B1a cells migrate to the local lymph node without evidence of proliferation or antigen specific clonal expansion, and recruited cells secrete both viral specific and non-specific antibody in an antigen-independent manner (Choi and Baumgarth 2008). B1 cell-derived natural antibodies promote immunity by a variety of different mechanisms: neutralization of viruses at the mucosa (Choi et al. 2012, Ochsenbein et al. 1999); complement fixation (Chen et al. 2009) and formation of immune complexes to facilitate phagocytosis (Jayasekera et al. 2007). Natural IgM has also been shown to be important in the generation of the adaptive humoral response: in mice deficient in B1 secretory (but not surface membrane) IgM, IgG response to T cell-dependent antigens is decreased (Baumgarth et al. 2000, Boes et al. 1998a) in part due to decreased formation of immune complexes trapped by follicular dendritic cells (Ehrenstein et al. 1998).

B1 cells also promote homeostasis and can modulate the immune response by secreting the anti-inflammatory cytokine IL-10. B1 cells secrete more IL-10 than B2 cells, and secretion is further increased upon mitogen stimulation (O'Garra and Howard 1992). Natural IgM antibodies that recognize PC and malondialdehyde (MDA) determinants exposed on the cell membranes of apoptotic cells promote complement deposition and phagocytosis of apoptotic corpses by immature DCs (Chen et al. 2009). B1a derived natural IgM has also been shown to limit atherosclerosis development in apolipoprotein E-deficient ($ApoE^{-/-}$) mice (Kyaw et al. 2011). B1 cells not only limit inflammation, but can also augment immune responses by efficiently stimulating T cells, in part due to expression of high basal levels of the co-stimulatory molecule CD86 (Holodick et al. 2009). Zhong et al. showed that B1 presentation of cognate OVA antigen to T cells induced greater proliferation measured by thymidine incorporation compared to that induced by B2 cells, and this effect was drastically reduced with anti-CD86 antibodies (Zhong et al. 2007). With these important functions influencing immune response, it is not surprising that B1 cells have also been implicated in some disease states. Natural IgM has been shown to be promote inflammation by initiating the classical complement cascade and causing tissue destruction by binding to epithelial antigens exposed upon ischemia-reperfusion injury (Zhang et al. 2004). B1 cells have also been implicated in various autoimmune diseases reviewed in (Berland and Wortis 2002).

Another functional distinction between B1 and B2 cells is their differential response to some types of BCR stimulation and downstream signaling events. B2 cells can be induced to proliferate upon IgM cross-linking with anti-IgM, while B1 cells fail to

respond (Morris and Rothstein 1993, Tumang et al. 2004). This is one mechanism proposed to limit expansion of potentially self-reactive B1 cells. Stimulation with phorbol esters such as PMA without an ionophore, however, induces peritoneal B1 but not splenic B1 or B2 cells to proliferate (Rothstein and Kolber 1988, Tanguay et al. 1999, Tumang et al. 2004). B1 and B2 cells have different developmental requirements for pre-BCR and BCR stimulation; accordingly, downstream signaling events also differ between these two subsets of cells. B1 cells fail to efficiently mobilize intracellular calcium upon BCR cross-linking measured with calcium sensitive immunofluorescent dyes. B1 cells also have basal tonic signaling downstream of the BCR, and activated molecules (Syk, PLCγ2, STAT3) can be measured with antibodies specific for the phosphorylated antigen in western blots and flow cytometry (Holodick et al. 2009, Karras et al. 1997). In contrast, these signaling molecules only become phosphorylated upon BCR stimulation in B2 cells (Holodick et al. 2009).

B1 cells in humans

Following the discovery of murine $CD5^+$ B cells with unique functions, CD5 expression (initially called Leu-1) was also observed in normal human B cells and chronic lymphocytic leukemia B cells (Casali et al. 1987, Hardy et al. 1987). Similar to mouse B1 cells, human $CD5^+$ B cells also secrete polyreactive natural antibodies, including against self-antigens (Casali et al. 1987, Hardy et al. 1987, Schutte et al. 1991) and reviewed in (Casali and Notkins 1989). $CD5^+$ B cells in humans are also more abundant early in ontogeny. In human cord blood and neonates, 70 to 90% of B cells are $CD5^+$ (Carsetti et al. 2004), and this percentage decreases to 10 to 20% in

peripheral blood with age (Carsetti et al. 2004, Gary-Gouy et al. 2002, Hardy et al. 1987). However, CD5 expression is more heterogeneous in human B cells than in mice. In mice, CD5 is expressed mainly in B1a cells but also in the small population of $CD1d^{hi}$ regulatory B cells that secrete high levels of IL-10 (Yanaba et al. 2009), and at very low levels in anergic B2 cells (Hippen et al. 2000). In humans, CD5 is expressed by pre-naïve, naïve transitional B cells, and can be detected at lower levels on other B cell subsets from the immature to memory B cell stages (Carsetti et al. 2004, Dalloul 2009, Lee et al. 2009). CD5 expression can be induced in response to certain stimuli (Appleyard and Wilkie 1998, Berland et al. 2010, Dalloul 2009, Yanaba et al. 2009), with simultaneous stimulation of both the BCR and CD40 being the most potent signal for CD5 upregulation (Gagro et al. 2000). It has been proposed that human B1 cells constitutively express CD5, while in B2 cells, the CD5 expression is inducible (Youinou et al. 1999).

Several investigators, therefore, worked to more narrowly describe the human equivalent of the mouse B1 cell reviewed in (Rothstein et al. 2013). Griffin et al. (2011a) identified a population of $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ cells in umbilical cord and adult peripheral blood leukocytes (PBL) that shared functional characteristics with murine B1 cells (Griffin et al. 2011a). These B1 cells represent 1 to 9% of B cells in adult PBL and decline with age (Descatoire et al. 2011, Griffin et al. 2011a, Griffin et al. 2011b, Griffin and Rothstein 2012a, Perez-Andres et al. 2011, Suchanek et al. 2012). The developmental origin of these cells is unknown. $CD19^{+}CD10^{+}CD27^{+}$ pre-B cells in the adult marrow bone marrow have high mRNA expression of the transcription factor LIN28B (McWilliams et al. 2013); the ectopic expression LIN28B has previously shown

to induce development of murine adult bone marrow hematopoietic precursors into innate lymphoid lineages, including the B1a, MZ B cells, and γδT cells (Yuan et al. 2012). It is also unknown whether human B1 cells are more concentrated in another tissue or body cavity such as murine B1 cells in the peritoneal cavity (Rothstein et al. 2013). Interestingly, 66% to 75% of $CD20^+CD27^+CD43^+$ cells have been shown to be $CD5^+$, and account for 35% of all $CD5^+$ B cells in the peripheral blood (Griffin et al. 2011a, Verbinnen et al. 2012). One study, however, reported that a smaller percentage (11.5%) of $CD20^+CD27^+CD43^+$ cells expressed CD5 (Suchanek et al. 2012). The $CD20^+CD27^+CD43^+$ B1 cells differed from activated human B cells by the lack of expression of activation markers CD69 and CD70 (Griffin et al. 2011a). In addition to sharing some protein markers with murine B1 cells (e.g. CD43 and CD5), meta-analysis of microarray data showed murine B1 and human $CD20^+CD27^+CD43^+CD70^-$ B1 cells shared 3 genes with greater than 2.0 fold higher expression compared to other B cell populations: CD5, CCR1, and SYT11 (Mabbott and Gray 2014).

The functional characteristics used to define $CD20^+CD27^+CD43^+CD70^-$ human B1 cells in the first publication by Griffin et al. (2011a) included spontaneous secretion of IgM; efficient stimulation of T cell proliferation in vitro; and tonic intracellular signaling via phosphorylated PLCγ2 and Syk upon phosphatase inhibition (Griffin et al. 2011a). These authors showed that B1 cells are enriched for specificities to PC and DWEYS tetramers similar to mouse B1 cells. Functionally and phenotypically distinct subsets of human B1 cells also exist. A minor fraction of $CD20^+CD27^+CD43^+$ cells (10-12.5%) has been shown to express CD11b in adult peripheral blood and cord blood (Griffin and Rothstein 2011). The $CD20^+CD27^+CD43^+CD11b^+$ cells also express CD11c and CD14,

unlike naïve, memory, or $CD20^{+}CD27^{+}CD43^{+}CD11b^{-}$ B1 cells, and have a distinct transcriptome (Griffin and Rothstein 2011). $CD11b^{-}$ B1 cells are almost all $CD5^{+}$ in contrast to $CD11b^{+}$ cells, which express none to low levels of CD5 (Griffin and Rothstein 2011). $CD11b^{-}$ B1 cells spontaneously secrete more IgM than $CD11b^{+}$ B1 cells (Griffin and Rothstein 2011). $CD5^{-}CD20^{+}CD27^{+}CD43^{+}$ cells isolated from people post pneumococcal vaccination (Pneumo23) secreted a greater amount of polysaccharide-specific IgM, IgG, and IgA antibodies, however, than the $CD5^{+}$ fraction (Verbinnen et al. 2012). In contrast, $CD11b^{+}$ B1 cells are more efficient than $CD11b^{-}$ B1 cells at both stimulating $CD4^{+}$ T cells to proliferate, in part due to higher CD86 expression, (Griffin and Rothstein 2011) and suppressing T cell activation by spontaneously secreting IL-10 (Griffin and Rothstein 2012b). Similar to murine B1 cells, human B1 cells have also been implicated in autoimmune disease, and $CD11b^{+}$ B1 cells were found to be more frequent in patients with systemic lupus erythematosus compared to healthy controls (Griffin and Rothstein 2011).

Other populations of human B cells have been shown to have innate B1-like functions reviewed in (Baumgarth 2011). In CVID patients, $CD21^{lo}CD19^{hi}CD23^{-}$ $CD24^{lo}CD27^{lo}CD38^{lo}CD86^{hi}IgM^{hi}$ IgD^{int} cells were found to be expanded and have B1 like characteristics, including immunoglobulin repertoires with few mutations in IGHV gene segments, reduced calcium mobilization and proliferation following BCR stimulation, and distinct gene expression profiles (Rakhmanov et al. 2009, Yamagata et al. 2006). The innate-like $CD21^{lo}$ cells also resemble $FCRL4^{+}$ B cells with elevated expression of SOX5 and FCRL4 (Rakhmanov et al. 2009). $FCRL4^{+}$ memory B cells are refractory to proliferation with BCR cross-linking similar to B1 cells, but with diverse

immunoglobulin repertoires and IgG isotype (Ehrhardt et al. 2005). $IgM^{hi}IgD^{lo}CD27^{+}$ memory cells have also been compared to murine B1 cells, as they are important in immunity to *Streptococcus pneumonia* (Haas et al. 2005, Kruetzmann et al. 2003). The $IgM^{hi}IgD^{lo}CD27^{+}$ immunoglobulin sequences contain mutations, albeit at a lower frequency than naïve B or isotype switched memory B cells (Weller et al. 2008).

B1 and $CD5^{+}$ B cells in other species

The presence of B1 cells has been examined in primates, rabbits, guinea pigs, cattle, and pigs. African green monkeys and cynomolgus macaques were shown to have peritoneal $CD19^{hi}CD21^{lo/-}CD11b^{+}$ with functional characteristics similar to those described for murine B1 cells (Yammani and Haas 2013). $CD19^{+}CD11b^{+}$ cells composed 25 to 40% of peritoneal B cells, and fractions of which also expressed CD5, CD27, and CD80 (Yammani and Haas 2013). The primate peritoneal $CD19^{+}CD11b^{+}$ cells displayed evidence of tonic signaling down stream of the BCR with constitutively active STAT3 (Yammani and Haas 2013). Peritoneal and splenic $CD19^{+}CD11b^{+}$ cells had BCR specificities enriched for PC, also similar to murine B1 cells (Yammani and Haas 2013). Following immunization with the TI-2 antigen trinitrophenyl (TNP)-Ficoll, antigen-specific $CD19^{+}CD11b^{+}$ increased in frequency similar to that observed with murine B1b B cells (Yammani and Haas 2013).

The rabbit, in which all B cells express CD5, has been proposed to exclusively have B1 cells (Raman and Knight 1992); supporting evidence includes the fact that rabbit B cells are primarily produced early in life, (Hayward et al. 1978) may be maintained by self-renewal (Adler and Adler 1984), and have restricted IGHV utilization

(Herzenberg and Herzenberg 1989, Lanning et al. 2000, Tunyaplin and Knight 1995). In contrast to murine B1 cells, rabbit B cells respond to protein vaccination (Zhang et al. 2014) and express extensively mutated immunoglobulins (Lanning et al. 2000). It is plausible that rabbit B cells are not distinctly B1 or B2, but perhaps a hybrid of the two. The single report describing $CD5^+$ B cells in the guinea pig is limited in the comparative and functional characterization of these cells (Dilwith and Wicher 1997).

Cattle $CD5^+$ B cells share some phenotypic and tissue distribution similarities with murine B1 cells (Naessens and Williams 1992, Naessens 1997). Upon infection with *Trypanosoma congolense*, $CD5^+$ B cells have been shown to undergo polyclonal activation and secretion of non-trypanosoma IgM antibodies (Buza et al. 1997) similar to that described for murine B1 cells in response to influenza (Choi and Baumgarth 2008). However, during *Trypanosoma congolense* infection more than 60% of peripheral blood B cells are $CD5^+$ (Naessens and Williams 1992), and such a response has not been reported for murine B1 cells.

In the pig, $CD5^+$ B cells share similarities with tissue distribution of B1 cells in mice, but they were not found to be more frequent early in ontogeny. Porcine $CD5^+$ B cells do not appear to share functional similarities with murine B1 cells in regards to immunoglobulin diversity or expression of IL-10 at the mRNA level (Appleyard and Wilkie 1998, Wilson and Wilkie 2007). Therefore based on the available evidence, CD5 expression does not appear to distinguish B1 cells in the pig. $CD5^+$ B cells had not been described in the horse until the studies presented in this thesis.

B lymphopoiesis in the horse

B lymphopoiesis can be readily detected at the molecular level in the equine fetus around 90 to 120 DG but potentially earlier in the yolk sac of the embryo (Tallmadge et al. 2009, Tallmadge et al. 2013, Tallmadge et al. 2014). The mRNA expression of B cell molecules CD20, CD21, CD22, CD27, CD40, CD45RA, CD79A, CD79B, and RAG-2 in the fetal liver, bone marrow, and spleen was measured at 90 to 120 DG (Tallmadge et al. 2009). The detection of CD27 in the fetal spleen and bone marrow was confirmed with quantitative RT-PCR (Tallmadge et al. 2009), and perhaps suggests, similarly to humans, that CD27 is not exclusively a memory marker but also found in fetal innate B cell lineages (Griffin et al. 2011a, McWilliams et al. 2013).

Limited information has been gathered about the generation of the immunoglobulin repertoire in the equine fetus, particularly with relevance to preparedness for fighting pathogens at birth, though there is growing knowledge about the immunoglobulin repertoire of the adult. The annotation of the equine heavy and light chain immunoglobulin genes by Sun et al. (2010) revealed the potential use of different heavy and light chain variable gene segments. The equine IGH locus contains at least 50 IGHV (14 of which are functional), 40 IGHD, and 8 IGHJ segments that can be used to construct the heavy chain (Sun et al. 2010). The adult horse, however, uses only a few IGHV genes to construct the majority of the heavy chain repertoire. Tallmadge et al. (2013) observed the use of 3 IGHV genes (IGHV2S2, IGHV2S3, and IGHV2S4) in more than 80% of expressed IGH sequences in the adult mesenteric lymph node. Similarly, Sun et al. (2010) found only 3 IGHV genes were used in the bone marrow of two adult horses, with IGHV2S2 and IGHV2S3 used in the majority of sequences. The use of only

a few IGHV genes to compose the majority of the immunoglobulin repertoire is also observed in pigs (Butler et al. 2011) and rabbits (Knight and Becker 1990). In contrast to IGHV gene segment use, a variety of different IGHD and IGHJ segments are used to construct equine heavy chains. Thirty-five out of 40 IGHD and all 8 IGHJ gene segments were observed in immunoglobulin sequences expressed in the adult bone marrow (Sun et al. 2010). Similarly 20 different IGHD and 5 different IGHJ gene segments were observed in 31 immunoglobulin sequences expressed in the adult mesenteric lymph node (Tallmadge et al. 2013).

For the light chain, the horse has at least 144 IGLV (27 functional), 7 IGLJ (4 functional), and 7 IGLC (4 functional) genes for the lambda light chain; and 60 IGKV (19 functional), 5 IGKJ (4 functional), and 1 IGKC for the kappa light chain (Sun et al. 2010). Reported allotypes of light chain gene segments that differ from the reference genomic sequences by up to 7.9% of nucleotides could potentially function as another layer of combinatorial diversity at the population level (Hara et al. 2012, Sun et al. 2010, Tallmadge et al. 2014). The horse uses the lambda light chain to construct more than 90% of circulating immunoglobulins (Ford et al. 1994). A variety of different IGLV and IGLJ gene segments are used, though IGLV genes in subgroup 8 (with a subgroup containing gene segments that share >75% identical base pairs with each other), particularly IGLV8-122, IGLV8-128, and IGLV8-24, are used in the majority of sequences from adult tissues (Hara et al. 2012, Sun et al. 2010, Tallmadge et al. 2014). Significant sequence and junctional diversity are also used in the adult immunoglobulin repertoire (Sun et al. 2010). Adult sequences show evidence of somatic hypermutation with IGH and IGL gene segments differing in nucleotide identity from germline sequence

by up to 26% and 16% of nucleotides, respectively (Tallmadge et al. 2013, Tallmadge et al. 2014). Junctional diversity includes zero to 37 N-nucleotide additions at IGHV-IGHD and IGHD-IGHJ junctions (Tallmadge et al. 2013), and 0 to 27 N-nucleotides at the IGLV-IGLJ junction (Tallmadge et al. 2014).

Studies in our lab concurrent with the preparation of this thesis described production of the immunoglobulin repertoire in the lymphoid tissues of the horse during phases of development (Tallmadge et al. 2009, Tallmadge et al. 2013, Tallmadge et al. 2014). These studies revealed that some aspects of immunoglobulin diversity were already established early in development, while others appeared to be developmentally programed. For example, bias in IGHV segments present in the equine adult repertoire were also present in the fetal spleen (Tallmadge et al. 2013), while IGLV segment utilization was less restricted in the fetal compared to adult immunoglobulin repertoire (Tallmadge et al. 2014). IGLV8-128 was used in the majority of sequences expressed in the foal and adult mesenteric lymph node, while a greater number of different IGLV gene segments were used in the fetal spleen, with IGLV8-12 the most frequently observed (Tallmadge et al. 2014). Fetal sequences also had evidence of mutations, albeit at a lower frequency than the foal and adult horse (Tallmadge et al. 2013, Tallmadge et al. 2014). TdT is expressed in fetal tissues between 90 to 120 DG, and low levels of N-nucleotide additions contribute to junctional diversity of the pre-immune repertoire (Tallmadge et al. 2009). Though the number of N-nucleotide additions increases with development, the median CDR3 lengths for both the heavy and lambda light chain are similar (Tallmadge et al. 2013, Tallmadge et al. 2014). The expression of TdT and the presence of N-nucleotide additions early in ontogeny is also observed in

many other species, including humans (Pascual et al. 1993), pigs (Sinkora et al. 2003), sheep (Tunyaplin and Knight 1995), but not mice (Feeney 1990). It remained unknown, however, if the degree of immunoglobulin diversity originated in the primary (e.g. liver and bone marrow) lymphoid tissues of the fetus or occurred with further diversification in secondary lymphoid tissues (e.g. spleen).

The expression of the seven heavy chain isotypes and lambda light chain also show a developmental progression at the mRNA and protein levels. The horse can express IGHM, IGHD, IGHA, IGHE, and multiple IGHG isotypes (Wagner et al. 2004). IGHM and IGL mRNA detected in the 90 to 120 DG fetal liver are consistent with the generation of immature B cells (Tallmadge et al. 2009). IGHD, IGHA, IGHG1, IGHG3/5, IGHG6, and IGHG7 were expressed in both the fetal spleen and bone marrow revealing that these tissues support maturation and immunoglobulin class switching during fetal life (Tallmadge et al. 2009). The distribution of these B cells was described with immunohistochemistry revealing that IgM^+ B cells were present in small clusters in the spleen of the fetus and organized into germinal centers in the pre-suckle neonate (Tallmadge et al. 2009). IgG and IgA expressing cells were not observed in the spleen until 1 to 3 months of life (Tallmadge et al. 2009). Endogenous antibodies at the protein level are detected midway (around 180 days) through gestation and, when challenged in utero, the equine fetus generates antigen-specific IgM and IgG antibody response by at least 200 days of gestation (DG) (Martin and Larson 1973, Morgan et al. 1975). In the pre-suckle foal, serum IgM concentrations are only 3 times lower than that of the adult horse, but IgG1 and IgG4/7 are 70 times and 120 times lower, respectively (Tallmadge et al. 2009).

Focus of the thesis

Common variable immunodeficiency is a late onset disease characterized by progressive B cell lymphopenia, hypogammaglobulinemia or agammaglobulinemia, failure in the humoral response to protein vaccination (e.g. tetanus toxoid), and recurrent infections that are eventually fatal (Flaminio et al. 2009). B cells in these patients are eventually nearly absent in the bone marrow, spleen and peripheral blood, with occasional cells or small clusters observed in the lymph nodes (Flaminio et al. 2009). Transcriptome and quantitative RT-PCR showed that CVID horses have decreased mRNA expression of PAX5 suggestive of a developmental block between the pre-pro B and pro-B cell stage (Tallmadge et al. 2012b). Similar to equines, the etiology of CVID in humans is largely unknown, with less than 15% of cases linked to a specific genetic mutation reviewed in (Kopecky and Lukesova 2007). In man, CVID is the most common clinically relevant primary immunodeficiency with a prevalence of 1:50,000 to 1:200,000 people in the general population (Kumar and Bhatia 2013). Differential methylation patterns of PAX5 are suggestive of an epigenetic etiology of disease in horses, a mechanism that has not yet been described to cause CVID in human patients (Tallmadge et al. 2015).

The B2 cell abundance and prominent function challenges the study of B1 cells; yet, the interest in the function B1 cells in humans has been growing. Our laboratory became interested in B1 cells when we gathered evidence that a population of B1-like cells are preferentially preserved in horses with CVID while B2-like cells are depleted, including the following observations: (1) lack of B cells in primary and secondary lymphoid tissue but presence of B cells in the peritoneal cavity, where B1 cells are

prominent in mice; (2) low serum IgM and IgG concentrations but (3) low-normal serum IgA concentrations, an isotype B1 cells preferentially switch to; (4) failure to respond to tetanus-toxoid vaccination, a T cell-dependent antigen that B2 cells respond to; and (5) specific response to pneumococcal polysaccharide vaccination, a T cell-independent antigen that B1 and MZ B cells respond to. CVID presents the only *natural* model in which B1-like cell function can be studied in the absence of B2 cells; however, some basic aspects of phenotype and function of the population of B1-like cells in the horse must first be described.

For many years, our laboratory has observed that the percentage of $CD5^+$ cells with forward- and side-scatter characteristics consistent with lymphocytes is greater than that of $CD3^+$ T cells, particularly in foals. Other cell types with similar lymphocyte light scatter characteristics include NK cells and B cells. Since NK cells are not reported to express CD5 in other species, the excess $CD5^+$ cells are assumed to be B cells that have not been previously described in the horse. Therefore, I became interested in learning about the frequency of $CD5^+$ B cells in the foal and adult horse, and how CD5 could be a marker of equine innate-like B1 cells, similar to murine B1a cells and a subset of human B1 cells.

B1 cells are predominant early in ontology in mice and humans; therefore, my studies of B cell development in the horse began in the primary lymphoid organs of the fetus. In the first part of my study, I tested how primary lymphoid tissues of the equine fetus were equipped to support B cell hematopoiesis and immunoglobulin diversity of the pre-immune repertoire. The horse is an ideal model to study the development of the humoral response during gestation, as the epitheliochorial placentation of the horse

does not allow transfer of maternal immunoglobulins to the fetus, eliminating this confounding element (Perryman et al. 1980). My original hypothesis was that B cell hematopoiesis in the equine fetus occurs in the liver and bone marrow with limited immunoglobulin diversity. The purpose of the second part of my study was to characterize $CD5^{+}$ B cells by describing ontogeny, tissue distribution, phenotype, and function. My hypothesis was that equine $CD5^{hi}$ B cells share a similar phenotype and function described for B1 cells in other species, with a distinct gene expression profile and limited immunoglobulin diversity when compared to $CD5^{lo}$ B cells.

My studies demonstrate that the liver and the bone marrow at approximately 100 DG are active sites of hematopoiesis and suggest that the B cells produced in these tissues generate a wide repertoire of pre-immune immunoglobulins for protection of the neonate at birth. I describe equine $CD5^{hi}$ B cells with phenotypic and functional characteristics attributed to B1 cells in other species, and the potential for targeted immunity in immunodeficient patients.

CHAPTER TWO

HEMATOPOIESIS IN THE EQUINE FETAL LIVER[a]

Abstract

My study investigated how the primary hematopoietic organs of the equine fetus are equipped to support B lymphopoiesis. The mRNA expression of molecules and transcription factors involved in early lineage-specific hematopoietic differentiation including c-KIT, CD34, IL7R, CXCL12, IRF8, PU.1, PAX5, NOTCH1, GATA1, and CEBPA were detected in the equine fetal liver using RT-PCR. Immunohistochemistry and flow cytometric analysis indicated that approximately 2% of the cells were positive for the hematopoietic stem cell (HSC) marker CD34, whereas 1-4% of cells stained positive for T lymphocyte markers (CD2, CD3, CD4, CD5, CD8) and B lymphocyte markers (CD19, IgM), and 3-15% of cells expressed myeloid markers (CD11b, CD172a). Taken together, the results of this study demonstrate active B lymphopoiesis in the equine fetal liver around 100 days of gestation.

Introduction

Understanding the development of the immune system is critical for the development of successful vaccines against infectious agents that continue to cause significant disease in neonates and in the young. During fetal life, the liver is a primary hematopoietic organ and supports differentiation and expansion of B cells until the bone marrow takes over this role (Butler et al. 2011, Timens and Kamps 1997, Yokota et al.

[a] Chapters 2 and 3 combined were published as one manuscript (Battista et al. 2014). The final publication is available at Springer via http://dx.doi.org/10.1007/s00251-014-0799-9.

2006). The purpose of this study was to learn how the liver and bone marrow of the equine fetus were equipped to support hematopoiesis, particularly B lymphoiesis.

Hematopoiesis and the generation of hematopoietic stem cells (HSCs) begins in the aorta-gonad-mesonephros (AGM) region and yolk sac (YS) of the mammalian embryo (Medvinsky and Dzierzak 1996, Moore and Metcalf 1970, Weissman et al. 1977). The HSCs then migrate to the fetal liver where they expand and differentiate into the erythroid, myeloid or lymphoid lineages (Houssaint 1981, Johnson and Moore 1975). Differentiation and commitment to the B lymphoid lineage involves gene regulatory networks, epigenetic modifications, and cytokine signaling (Nutt and Kee 2007, Schebesta et al. 2007). Hematopoiesis shifts to the bone marrow later in gestation (Sinkora et al. 2003), and this tissue becomes the primary hematopoietic organ in adult life; though in some mammalian species, such as rabbits (Vajdy et al. 1998) and sheep, (Reynolds and Morris 1983) gut-associated lymphoid tissues (GALT) play a major role in post-natal B cell development.

B lymphopoiesis can be readily detected at the molecular level in the equine fetus around 90 to 120 DG (about 1/3rd of gestation), and possibly earlier in the yolk sac of the embryo (Tallmadge et al. 2009, Tallmadge et al. 2013, Tallmadge et al. 2014). Previous studies in our laboratory have focused on the generation of B cells and production of the immunoglobulin repertoire in the secondary lymphoid tissues of the horse during phases of development. In my study, I tested how primary lymphoid tissues of the equine fetus were equipped to support B lymphopoiesis during gestation. My hypothesis was that B lymphopoiesis in the equine fetus occurs in the liver and bone

marrow. My study shows active B lymphopoiesis in the equine fetal liver and bone marrow around 100 DG.

Materials and methods

Equine tissue samples

These experiments were approved by the Cornell University Center for Animal Resources and Education and Institutional Animal Care and Use Committee for the use of vertebrates in research. Three mares (2 Thoroughbreds, 1 Warmblood) at the Cornell Equine Park were bred and abortions were chemically induced with prostaglandin injections (2.5mg intramuscularly every 12 hours until abortion, Lutalyse, Pfizer, New York, NY) at approximately 100 DG (101-105 DG) (Douglas and Ginther 1976). The fetal livers (n=3) were dissected out within an hour of abortion using sterile technique. Part of the tissues were snap frozen in liquid nitrogen for RNA isolation, or preserved in Tissue-Tek O.C.T. Compound (optimal tissue culture medium, SakurFineteck U.S.A., Inc., Torrance, CA) for immunohistochemistry; these tissues were stored at -80 °C until analysis. Single cell suspension was made from the remaining fetal liver by pressing the tissue through first a metal mesh (size 80 mesh, Sigma-Aldrich, St. Louis, MO) and then a nylon mesh (70μm, BD Falcon, Franklin Lakes, NJ). Mononuclear leukocytes were isolated by Ficoll gradient centrifugation (density 1.077, GE Healthcare, Piscataway, NJ) as previously described (Flaminio et al. 2000). Cells were immediately used for flow cytometric analysis or frozen in media containing dimethyl sulfoxide and stored in liquid nitrogen until additional flow cytometric analysis (CD5, CD34) or RNA isolation. Upon thawing for flow cytometric analysis, the cells were again subjected to Ficoll gradient

centrifugation to enrich for viable cells. Fetal bone marrow was harvested from the same fetuses and snap frozen in liquid nitrogen for RNA isolation, and stored at -80 ^{o}C until use. The adult horse bone marrow sample was collected immediately post-mortem from a healthy research adult horse belonging to another investigation at Cornell University College of Veterinary Medicine. The tissue was harvested within 1 hour of euthanasia, snap frozen in liquid nitrogen for RNA isolation, and stored at -80 ^{o}C until use.

RT-PCR to detect expression of genes associated with hematopoiesis

Snap frozen equine fetal whole liver, fetal whole bone marrow, adult whole bone marrow tissue, and frozen fetal liver isolated leukocytes were homogenized with the QIAshredder columns (Qiagen, Valencia, CA). Total RNA was isolated with the RNeasy® Kit (Qiagen) and genomic DNA was degraded with the RNase-Free DNase Set® (Qiagen) or DNAse I (Life Technologies, Grand Island, NY) following the manufacturer's instructions. cDNA was made from 1μg RNA with the RevertAID™ first strand cDNA synthesis kit using Oligo(dT)$_{18}$ (Thermo Fisher Scientific Inc., Waltham, MA) according to manufacturer's instructions. PCR reactions were performed by adding 50ng cDNA template to 0.2mM dNTP, 0.6μM each forward and reverse primers (Table 2.1), and 1.25u DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc.) in 1X DreamTaq™ buffer. Thermal cycling conditions were as follows: initial denaturation 95°C x 1min, [denaturation 95°C x 30s, annealing 58°C x 30s, extension 72°C x 1min] repeated 40 times, and final extension 72°C x 5min. Amplicons were visualized using electrophoresis on a 1% agarose gel stained with GelGreen nucleic acid stain (Phenix

Table 2.1 RT-PCR primer sequences for the hematopoietic genes studied

Gene	GenBank ID	Primer Sequence (5'-3')		Product (bp)
CD34	XM_001491596	Forward	CTAGGGTGTGCTCCTTGCTC	209
		Reverse	GACCAGTGCAATCAGGGTCT	
CEBPA	NA[a]	Forward	TGGACAAGAACAGCAACGAG	167
		Reverse	GCGGCTCAGTTGTTCCAC	
CXCL12	XM_001489644	Forward	CAGCCTGAGCTACAGATGTCC	280
		Reverse	CCTTTTCTGAGCAGCCTTTC	
GATA1	XM_005614118	Forward	TGTTTCCAGCAGTGCCTATG	247
		Reverse	CCTGTTCTGCCCATTCATCT	
IL7	CX592622	Forward	AAAGACGGCAAAGAATATCGA	142
		Reverse	ACAGAGGTTCCTTATTATCATCAC	
IL7R	CD469725	Forward	ACATGCCTGCTCTGGTCTCT	580
		Reverse	TCACGTGCATCCAATCATTT	
IGHM	NW_001876796	Forward	CTTCACTACGGAAGAGGTGC	295
		Reverse	ACTCAGGCTGTCATAGGTGC	
IRF8	XM_001502568	Forward	ACGTGGTGGTCAAGGTCTTC	207
		Reverse	AGCTCTTCCCAGCTTCTTCC	
KIT	XM_001492243	Forward	AGACCTGGAAGACCTGCTCA	376
		Reverse	CATTCGGAAACCTTCCTTGA	
NOTCH1	XM_001498582	Forward	TCCTTCCTGACCTGGATGAC	393
		Reverse	CCATGTTGTCCTGGATGTTG	
PAX5	XM_001504306	Forward	CATCAAGCCTGGGGTAATTG	157
		Reverse	CACGGTGTCATTGTCACACA	
PU.1	JN979560	Forward	GAGACCATCCAGCTCCAGAC	315
		Reverse	CAGCTCGGTGAAGTGGTTCT	
β-Actin	NM_001081838.1	Forward	TCCCTGGAGAAGAGCTACGA	350
		Reverse	GTGGACAATGAGGCCACAAT	

[a]The forward primer was designed based on the consensus sequence for the alignment of human (NM_004364), cow (NM_176784), mouse (NM_007678), and pig (XM_003127015) sequences, while the reverse primer was designed from the horse sequence NW_001867363.

Research Products, Candler, NC) and the Gel Doc™ EZ Imager (Bio-Rad Laboratories Inc., Hercules, CA).

A panel of 12 genes known to participate in hematopoiesis were tested, including transcription factors essential for commitment and differentiation of the hematopoietic lineages: (1) GATA1 for erythropoiesis (Tsiftsoglou et al. 2009); (2) CEBPA for myelopoiesis (Koschmieder et al. 2009); (3) PAX5 for B cell lymphopoiesis (Cobaleda et al. 2007, Pridans et al. 2008); (4) IFR8 and (5) PU.1 for both myelopoiesis and B lymphopoiesis (Kastner and Chan 2008, Wang and Morse 2009); (6) NOTCH1 receptor essential for T cell commitment (Rothenberg 2011); HSC markers (7) CD34, a sialomucin important in HSC migration and homing (Nielsen and McNagny 2009), and (8) KIT, a transmembrane receptor essential for HSC signaling (Kent et al. 2008), also expressed during B cell ontogeny from the lymphoid-primed multipotent progenitor through common lymphoid progenitor (CLP) and pro-B cell stages, and acts synergistically with IL-7 to promote proliferation and differentiation of pro-B cells (McNiece et al. 1991); (9) IL7R expressed by the CLP through the early pre-B cell stage and signaling promotes survival, proliferation, and differentiation (Fry and Mackall 2002); (10) IgM expressed only by immature and mature B cells; the chemokine (11) CXCL12 and (12) IL-7 expressed by microenvironment stromal cells to support hematopoiesis (Nagasawa 2006), for the homing of HSCs to the fetal liver and bone marrow, and the generation of pre-pro and pro-B cells (Nagasawa et al. 1996). The house-keeping gene β-ACTIN was used as a positive control. Primers (Table 2.1) were designed using the Primer3 program accessible at http://frodo.wi.mit.edu/ (Rozen and Skaletsky 2000). To prevent amplification of genomic DNA, the primers spanned introns

when possible. Destruction of genomic DNA from RNA samples was confirmed by performing the cDNA synthesis reaction without the addition of the RevertAID™ Reverse Transcriptase (Thermo Fisher Scientific Inc.) followed by the PCR reaction with the β-ACTIN primers to show no genomic DNA product was amplified. The amplicons were confirmed by sequencing.

Immunohistochemistry of leukocytes in the equine fetal liver

Whole fetal liver harvested from two different fetuses and frozen in Tissue-Tek O.C.T. were cut into 7μm thick sections on a cryotome and fixed with acetone for 10 minutes. The sections were stained as previously reported (Tallmadge et al. 2009) using the following primary antibodies (Kydd et al. 1994, Lunn et al. 1998, Parrish et al. 1982): CD2 (clone HB88A, Washington State University (WSU) Monoclonal Antibody Center, Pullman, WA), CD3 (F6G.3(G12), J. Stott, University of California, Davis, CA), CD4 (HB61A, WSU Monoclonal Antibody Center), CD5 (HT23A, WSU Monoclonal Antibody Center), CD8 (HT14A, WSU Monoclonal Antibody Center), CD19-like (cz2.1, D.F. Antczak, Cornell University, Ithaca, NY), IgM (CM7, AbDSerotec, Raleigh, NC), CD172A (IGHD59B, WSU Monoclonal Antibody Center), major histocompatibility complex (MHC) class I (cz3, D.F. Antczak), MHC class II (cz11, D.F. Antczak), anti-canine parvovirus (negative control, CPV12, C. Parrish, Ithaca, NY). Images of the slides were taken with an Olympus BX-50 microscope, visualized with Metamorph software (Molecular Devices**,** Sunnyvale, California**),** and representative images were selected from both fetuses**.**

Flow cytometric immunophenotyping of the equine fetal liver isolated leukocytes

One million cells per sample were blocked with 10% normal goat serum and washed with PBS, then labeled with the same antibodies as above for the immunohistochemistry including CD2, CD3, CD4, CD5, CD8, CD19-like, IgM, CD172A, MHC class I, MHC class II, in addition to CD11b-FITC (M1/70.15.11.5, Miltenyi Biotec, Auburn, CA) and CD34 (1H6, R&D Systems, Minneapolis, MN). Cells stained with unconjugated primary antibodies were stained with goat anti-mouse IgG(H+L)-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA)**.** Finally the cells were fixed with 2% paraformaldehyde in phosphate buffered solution, and fluorescence was measured with the BD FACScalibur flow cytometer using an argon laser (Bio-Rad Laboratories Inc.). The negative control were cells stained only with the goat anti-mouse IgG(H+L)-FITC secondary antibody. One hundred thousand ungated events were collected in all but one case (CD5 staining of fetal liver #1, 68,000 events counted).

Cytology of the equine fetal liver isolated leukocytes

Cytospins of approximately $5x10^4$ isolated cells per slide were made using centrifugation at 500xg for 3 minutes, and stained with Wright's stain in an automated stainer (Modified Wright's stain, Hema-tek 1000, Siemens Healthcare Diagnostics Inc., Tarrytown, NJ) at the Clinical Pathology Laboratory, Cornell University College of Veterinary Medicine. A 200 cell differential count was performed by a single blinded observer (Dr. Tracy Stokol).

Results

Molecular evidence of hematopoiesis

In order to characterize the fetal liver and bone marrow as active hematopoietic sites early in gestation, the expression of relevant hematopoietic developmental genes was confirmed. Twelve selected genes important in hematopoiesis KIT, CD34, IL7R, IGHM, CXCL12, IL7, PU.1, IRF8, PAX5, NOTCH1, CEPBA, and GATA1 were detected at the mRNA level in the adult horse bone marrow, and 100-day equine fetal liver and bone marrow whole tissues (Figure 2.1). The same hematopoiesis-related genes were expressed in the isolated mononuclear cells from these tissues. The fetal bone marrow PCR products consistently ran slower than those from the other tissues, and direct sequencing of a subset of PCR reactions confirmed the bands were the amplicons of interest.

Organization of leukocytes in the fetal liver

I next investigated how leukocytes were organized in the fetal liver, and in what proportion they were present in order to understand the relative extent of B lymphopoiesis. Immunohistochemistry staining was used for qualitative analysis and showed the presence of leukocytes in the equine fetal liver at 100 DG (Figure 2.2). T cell ($CD3^+$, $CD4^+$, $CD8^+$) and B cell ($CD19^+$ or IgM^+) markers stained cells with large nuclear to cytoplasmic ratios, consistent with the morphology of lymphocytes. These cells were randomly distributed throughout the tissue sections as isolated cells or in small clusters. CD2 and CD5, which are expressed by subsets of both T and B cell lymphocytes, stained cells in a similar distribution. The T cell marker CD8 stained

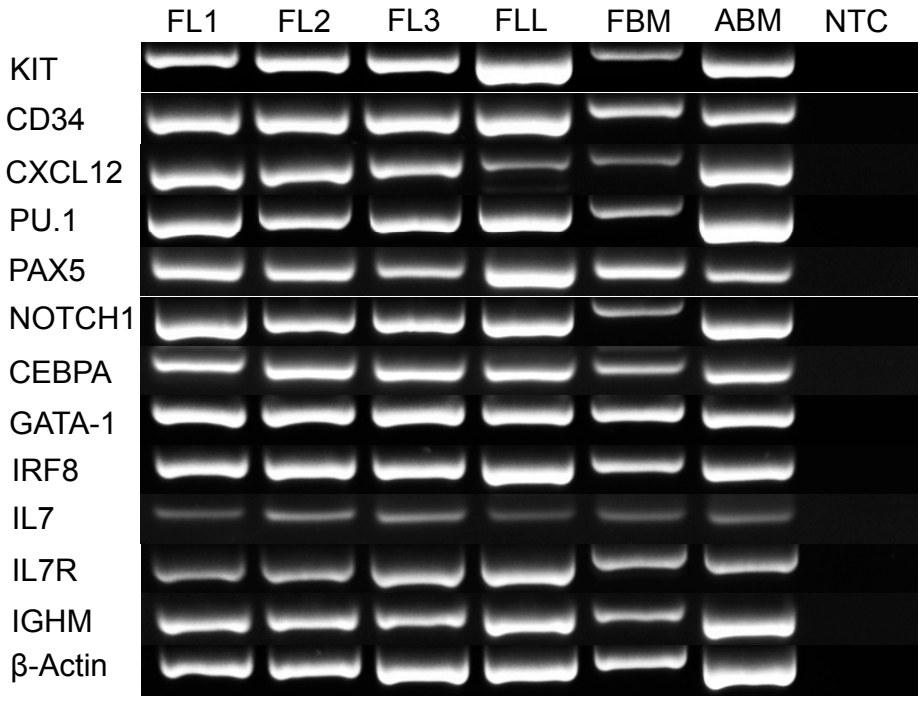


Figure 2.1 Expression of hematopoietic genes in the equine fetal liver.
RNA was isolated from equine fetal liver and bone marrow collected at 100 days of gestation, and from adult horse bone marrow for expression analysis of the early genes in hematopoiesis using RT-PCR. FL1 = fetal liver 1, FL2 = fetal liver 2, FL3 = fetal liver 3, FLL = fetal liver leukocytes, FBM = fetal bone marrow, ABM = adult horse bone marrow, NTC = no template control.

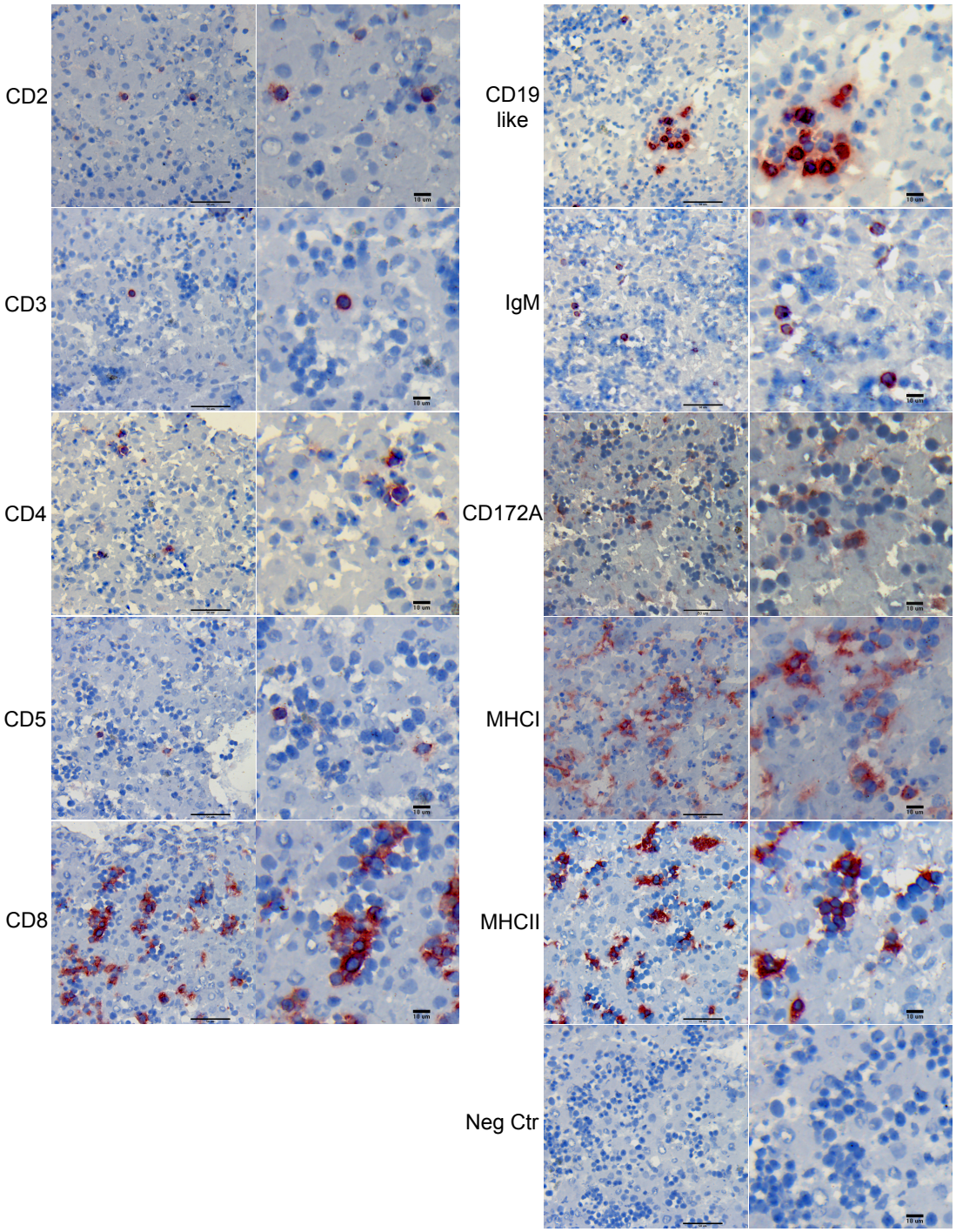


Figure 2.2 Leukocyte distribution in the equine fetal liver.
Whole fresh-frozen equine fetal liver was stained with monoclonal antibodies against equine leukocyte markers, and representative images were selected from fetuses #2 and #3. The figures on the right of each panel show positive cells at higher magnification of the corresponding image on the left. Scale bars measure 50μm on the left column, and 10μm on the right column for each panel.

a greater number of cells compared to the other T cell markers, and positive cells resembled lymphocytes and cells characteristic of the myeloid lineage, with small nuclear to cytoplasmic ratios. The CD172A marker, expressed by monocytes, macrophages, and neutrophils, stained large and small cells with small nuclear to cytoplasmic ratios, and were distributed in small clusters. Cells staining positive for MHC class I and MHC class II were randomly distributed throughout the tissue. MHC class I positive cells were most often found in large groups (>10 cells), while MHC class II positive cells were identified generally in small groups or isolated. Similar to CD8, MHC class I and MHC class II positive cells resembled both the lymphocyte and myeloid lineages.

Distribution of leukocytes in the fetal liver

Isolated leukocytes from fetal liver were quantitated with flow cytometric analysis (Table 2.2). Approximately 2% of the total mononuclear cells isolated from the fetal liver expressed the HSC marker CD34, between 0.4-4.1% T or B cell markers, and 11-15% expressed the CD172A myeloid marker. The technique applied for leukocyte isolation (Ficoll gradient centrifugation) favors mononuclear cell enrichment, but not purification from neutrophils. In contrast, only 3% of cells stained positive with CD11b, which is expressed in monocytes, neutrophils, and B1 cells (Montecino-Rodriguez and Dorshkind 2006). Consistent with the immunohistochemistry staining, 24-30% cells expressed MHC class I, and 13-24% cells expressed MHC class II, the latter was similar to the percentage of B cells and monocytes together. Since only 12-19% of the cells isolated from the fetal liver could be accounted for as lymphoid or myeloid using

Table 2.2. Surface molecule expression of equine fetal liver isolated leukocytes

	Percent Positive Cells Measured with Flow Cytometry			
Markers	Fetus 1	Fetus 2	Fetus 3	Cells that express marker
CD2	1.9	2.1	2.0	T cells, subset of B cells
CD3	0.8	0.9	1.3	T cells
CD4	1.9	1.4	2.7	T cells, subset of macrophages or dendritic cells
CD5	3.5	1.5	2.4	T cells, subset of B cells
CD8	4.1	1.0	3.0	T cells, subset of macrophages or dendritic cells
CD19-like	1.0	0.4	1.6	B cells
IgM	0.9	0.5	1.4	B cells
CD11b	2.6	NM	3.2	monocytes, macrophages, neutrophils, subset of B cells
CD172A	15.5	11.8	14.4	macrophages, neutrophils
CD34	2.3	NM	1.4	hematopoietic stem cells
MHCI	29.8	24.5	24.7	mature nucleated cells
MHCII	20.8	13.7	23.8	monocytes, macrophages, dendritic cells, B cells

'NM' indicates that the surface expression of the marker was not measured

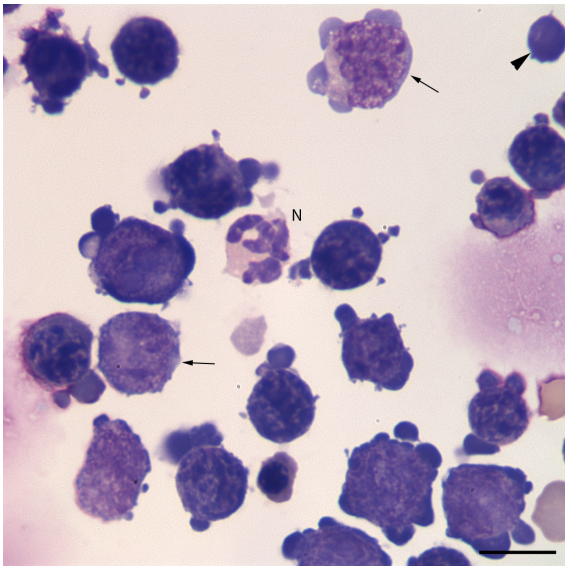
the antibodies available for the horse, which do not include an erythrocyte lineage marker, the cells were also classified based on morphology using cytospin preparations. The differential cell count (Figure 2.3) showed that the majority of cells (80-86%) were of the erythroid lineage, followed by cells with morphologic features of small lymphocytes (8-9%), monocytes (4-7%), and neutrophils (1-5%). The predominance of erythroid cells is consistent with the fetal liver's function at this phase of life.

Discussion

The study herein reports hematopoietic activity in the liver and bone marrow of the equine fetus as early as 100 DG. The mRNA expression of genes that represent hematopoiesis of lymphoid, myeloid, and erythroid lineages (Figure 2.1) was observed in whole tissues and isolated cells from the fetal liver. Immunohistochemical (Figure 2.2), flow cytometric (Table 2.2), and cytologic (Figure 2.3) results of fetal liver tissues or isolated cells corroborate with active hematopoiesis, with predominant erythropoiesis.

T and B cells were observed at this developmental stage. T cells were mostly seen as isolated or small groups of cells, suggesting that they were likely circulating through the liver, as their primary development happens in the fetal thymus. B cells were seen isolated but more often in small clusters, suggesting B cell developmental niches. Although CD2 is considered typically a T cell marker, this molecule is also expressed in B cells, and tissues from one fetus displayed $CD2^+$ cells in small clusters, similarly to the distribution of B cells. Sinkora et al. (1998) showed that, in the pig, CD2 is expressed in B cells during early development, and the number of $CD2^+$ B cells

a.



b.

Cell Type	Fetus 1	Fetus 2	Fetus 3
Erythrocytes	86%	79.5%	85%
Lymphocytes	9%	9%	8%
Neutrophils	1%	4.5%	3%
Monocytes	4%	6.8%	4%

Figure 2.3 Leukocyte differential cell count in the equine fetal liver.
(a) Cytospin slides of isolated equine fetal liver leukocytes were made and stained with Wright's stain. A sample image of fetus #3 is shown. Most of the nucleated cells were erythroid progenitors, with fewer differentiating monocytes (arrows), neutrophils (N) and lymphocytes (arrowhead). The scale bar measures 10μm. (b) Differential cell counts from each fetus sample show that the majority of cells were erythrocytes, followed by lymphocytes, monocytes, and neutrophils.

decreases with age and exposure to microflora. It is possible that, like the pig, equine B cells express CD2 in fetal life, and further characterization of this B cell population is pursued in Chapter 4. Also similar to previous studies, a relatively large population of $CD8^+$ cells was detected in the fetal liver with IHC. Many of the $CD8^+$ cells observed in this study had morphologic characteristics of myeloid cells, which are known to express the CD8 molecule in other species, including humans (monocytes) (Gibbings et al. 2007) and rats (monocytes and macrophages), (Hirji et al. 1997, Lin et al. 2000) and further investigation is warranted.

The presence of $CD34^+$ cells supports the potential for hematopoiesis, and reveals the possibility for isolation and application of liver-derived HSC in regenerative and developmental studies. The percentage of $CD34^+$ HSCs was assessed on frozen stored and thawed cells when an antibody with cross-reactivity to the equine antigen became available. Accordingly, the percentage of HSCs in the fetal liver may actually be greater than measured in this study if the equine HSCs are more sensitive to mortality than other leukocytes upon freezing.

In summary, my study demonstrates active B lymphopoiesis in the primary lymphoid tissues of the equine fetus at 100 DG, both at molecular and cellular levels. No difference was observed in the presence of expression of a subset of genes known to be important in hematopoiesis between the fetal liver and bone marrow. Previous molecular studies in our laboratory demonstrated the presence of only IGHM mRNA transcripts in the fetal liver while IGHD, IGHA, and some IGHG transcripts could be detected in the fetal bone marrow and spleen (Tallmadge et al. 2009). These results suggest that differences in B cell diversification and immunoglobulin repertoire may

exist between the fetal liver and other hematopoietic tissues during fetal life, and this possibility is explored in the following chapter.

CHAPTER THREE

DEVELOPMENT OF THE PRE-IMMUNE IMMUNOGLOBULIN REPERTOIRE IN THE PRIMARY LYMPHOID TISSUES OF THE EQUINE FETUS[b]

Abstract

My study investigated how the equine fetus prepares its pre-immune humoral repertoire for an imminent exposure to pathogens in the neonatal period, particularly how the primary hematopoietic organs are equipped to support development of immunoglobulin diversity. To assess immunoglobulin diversity achieved during the production of B cells, V(D)J segments were sequenced in primary lymphoid organs of the equine fetus and adult horse, revealing that similar heavy chain VDJ segments and CDR3 lengths were most frequently used independent of life stage. In contrast, different lambda light chain segments were predominant in the fetal compared to adult stage and, surprisingly, the fetus had less restricted use of variable gene segments to construct the lambda chain. Fetal immunoglobulins also contained elements of sequence diversity, albeit to a smaller degree than that of the adult horse. My data suggest that the B cells produced in the liver and bone marrow of the equine fetus generate a wide repertoire of pre-immune immunoglobulins for protection; and the more diverse use of different lambda variable gene segments in fetal life may provide the neonate an opportunity to respond to a wider range of antigens at birth.

[b] Chapters 2 and 3 combined were published as one manuscript (Battista et al. 2014). The final publication is available at Springer via http://dx.doi.org/10.1007/s00251-014-0799-9.

Introduction

In the equine fetus, endogenous antibodies are detected midway through gestation (around 180 days) and, when challenged in utero, the fetus generates an antigen-specific IgM and IgG antibody response by at least 200 days of gestation (DG) (Martin and Larson 1973, Morgan et al. 1975). Limited information is known about the generation of the immunoglobulin repertoire in the equine fetus, particularly with relevance to preparedness for fighting pathogens. The purpose of my study was to learn how primary lymphoid tissues of the equine fetus were equipped to support immunoglobulin diversity.

The generation of a functional immunoglobulin molecule requires somatic recombination of the V(D)J loci for both the heavy and light chain genes. The pre-immune immunoglobulin receptor repertoire develops in the absence of exogenous antigens in the primary lymphoid tissues during fetal life, and diversity is generated primarily by combinatorial and junctional diversities. Combinatorial diversity is produced by joining different heavy chain and light chain gene segments. The horse has available 14 IGHV, 40 IGHD, and 8 IGHJ functional gene segments to construct the heavy chain (Sun et al. 2010). The number of gene segments used to construct immunoglobulin molecules varies by species, and the use of 14 IGHV genes by the horse is intermediate between mice, which use more than 90 IGHV segments, and the chicken, which uses only one (Das et al. 2008). Light chains are constructed using either the lambda or kappa loci. The horse uses lambda chains in more than 90% of circulating antibodies (Ford et al. 1994), constructed from 27 IGLV, 7 IGLJ, and 7 IGLC potentially functional genes (Sun et al. 2010). In some species, combinatorial diversity differs

during phases of development, and certain immunoglobulin (Ig) segments are preferentially utilized in fetal or adult life (Butler et al. 2000, Gontier et al. 2005, Jiang et al. 2011, Koti et al. 2010, Yancopoulos et al. 1984, Zemlin et al. 2001).

The Ig segments have been divided into subgroups, and each subgroup is composed of gene segments sharing >75% nucleotide identity (Sun et al. 2010). For the heavy chain, the 14 IGHV genes are grouped into 7 subgroups; the 40 IGHD genes into 28 subgroups; and the 8 IGHJ genes into 2 subgroups. The 27 IGLV genes were grouped into 11 subgroups. Recently, there was a change in nomenclature for the heavy chain Ig genes in accordance with the International ImMunoGeneTics information system based on these subgroups, and "VH5" was renamed "IGHV2S3", indicating gene 3 of subgroup 2 (Tallmadge et al. 2013). Unlike the heavy chain nomenclature, for the lambda light chain the subgroup number is not followed by an "S" but a "-", and the gene number based on chromosomal location from 3` to 5` in the locus (as annotated by Sun et al. 2010). Accordingly, "Vλ5" was renamed IGLV4-66, indicating subgroup 4 and the 66th IGLV gene from the 3` end of the locus (Tallmadge et al. 2014). IGLJ genes names do not include a subgroup designation, and are named based on chromosomal location from 5` to 3` in the locus (Lefranc 2001).

Junctional diversity is created by the deletion and addition of base pairs at the junctions of the Ig gene segments during recombination events. The complement determining regions (CDRs) are the sequences that come in contact with antigen, and are the most diverse regions of the immunoglobulin molecule. CDR3 contains the greatest degree of sequence variability and spans the junction of the IGHV, IGHD, and IGHJ gene segments in the heavy chain, and IGLV and IGLJ gene segments in the light

chain. Diversity of the CDR3 region is influenced by both combinatorial and junctional diversity. CDR3 length differs in fetal compared to adult life in certain species (Bangs et al. 1991, Gontier et al. 2005, Zemlin et al. 2001).

Previous studies in our lab have described the generation of B cells and production of the immunoglobulin repertoire in the secondary lymphoid tissues of the horse during phases of development, revealing some aspects of immunoglobulin diversity were already established early in development, while others appeared to be developmentally programed (Tallmadge et al. 2009, Tallmadge et al. 2013, Tallmadge et al. 2014). For example, bias in IGHV segments present in the equine adult repertoire were also present in the fetal spleen (Tallmadge et al. 2013), while IGLV segment utilization was less restricted in the fetal compared to adult immunoglobulin repertoire (Tallmadge et al. 2014). However, it was unknown if the degree of immunoglobulin diversity originated in the primary (liver and bone marrow) lymphoid tissues of the fetus, or if further diversification occurred in secondary lymphoid tissues (e.g. spleen). In this study, I tested how primary lymphoid tissues of the equine fetus were equipped to support immunoglobulin diversity of the pre-immune repertoire during gestation. My original hypothesis was that limited immunoglobulin diversity originated in the fetal liver and bone marrow. This study shows the generation of a surprisingly diverse pre-immune immunoglobulin repertoire that sets up humoral protection after birth.

Materials and methods

Equine tissue samples

These experiments were approved by the Cornell University Center for Animal Resources and Education and Institutional Animal Care and Use Committee for the use of vertebrates in research. Three mares (2 Thoroughbreds, 1 Warmblood) at the Cornell Equine Park were bred and abortions were chemically induced with prostaglandin injections (2.5mg intramuscularly every 12 hours until abortion, Lutalyse, Pfizer, New York, NY) at approximately 100 DG (101-105 DG) (Douglas and Ginther 1976). The fetal livers (n=3) were dissected out within an hour of abortion using sterile technique, snap frozen in liquid, and stored at -80 °C until analysis. The adult horse bone marrow samples were collected immediately post-mortem from three healthy research adult horses (1 Thoroughbred, 2 breed unknown) belonging to another investigation at Cornell University College of Veterinary Medicine. The tissue was harvested within 1 hour of euthanasia, snap frozen in liquid nitrogen and stored at -80 ^{o}C until use.

Immunoglobulin heavy and light chain V(D)J sequencing

Snap frozen equine fetal liver, fetal bone marrow, and adult bone marrow were homogenized with the QIAshredder columns (Qiagen, Valencia, CA). Total RNA was isolated with the RNeasy® Kit (Qiagen) and genomic DNA was degraded with the RNase-Free DNase Set® (Qiagen) or DNAse I (Life Technologies, Grand Island, NY) following the manufacturer's instructions. 5'-rapid amplification of cDNA ends (RACE) library was constructed with the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA) following manufacturer's instructions. The 5'-RACE PCRs were

performed with a 5` RACE primer for the heavy chain VDJ sequence that spans the last 24 nucleotides conserved among IGHJ segments, and a 5` RACE primer for the lambda light chain sequence that spans a conserved region in the lambda constant segments, as previously described (Tallmadge et al. 2013, Tallmadge et al. 2014). The lambda chain was chosen as the horse uses primarily this light chain in circulating antibodies (Ford et al. 1994). The PCR products were purified, ligated into the pJET1.2 vector (CloneJET™ PCR Cloning Kit, Thermo Fisher Scientific Inc.), transformed into either JM107 (TransformAID™ Bacterial Transformation Kit, Thermo Fisher Scientific Inc.) or NEB-5 alpha (New England BioLabs, Ipswich, MA) competent *E.coli*, expanded, and sequenced at the Cornell University Institute of Biotechnology (Ithaca, NY), as previously described (Tallmadge et al. 2013, Tallmadge et al. 2014). Heavy and lambda light chain sequences were obtained from fetal liver, fetal bone marrow, and adult bone marrow (n = 3). To recover a minimum of 30 unique and productive Ig sequences per tissue (10 per individual), 31 fetal liver, 49 fetal bone marrow, 32 adult horse bone marrow heavy chain clones and 33 fetal liver, 49 fetal bone marrow, and 33 adult horse bone marrow lambda light chain clones were sequenced. Minimal lambda light chain sequence diversity was obtained from donor fetal liver #2, despite sequencing 11 clones, resulting in only 2 unique clones from this donor.

Immunoglobulin heavy and lambda light chain sequence analysis

Ig sequences determined in this study are available through GenBank with accession numbers KF748612 - KF748792. Ig sequences were analyzed and nucleotide identity plots generated using Geneious Pro R6-1 (Drummond et al. 2011),

(Biomatters Ltd, Auckland, New Zealand). Ig gene segments were identified by comparing the cloned sequences against the EquCab2.0 equine reference genome annotated by Sun et al. (2010) using the NCBI *Equus caballus* BLAST tools as previously described (Tallmadge et al. 2013, Tallmadge et al. 2014). All BLAST hits were evaluated for identity, alignment length, and orientation. With one exception (IGVDJ66), all annotated IGHD segments were at least 7bp long and shared greater than 65% nucleotide identity with the genomic sequence. In total, IGHD segments could be annotated in 86% of sequences. For the remainder of sequences with IGHD segments of insufficient length or nucleotide identity, IGHD segments were designated as “not determined”.

Ig gene sequence identities between expressed sequences and the genome reference sequences were calculated with the Geneious Pro R6-1 software. The length of the heavy chain (CDR3H) and lambda light chain (CDR3L) were determined as previously described (Ford et al. 1994, Sun et al. 2010). Variability plots were made as described by Wu and Kabat (1970) with the variability index calculated as the number of different amino acids at a given position divided by the frequency of the most common amino acid at that position.

Statistical analysis

The Shapiro-Wilk normality test performed with GraphPad Prism version 6.0c (GraphPad Software, San Diego, California) revealed that most of the data was not normally distributed, and the appropriate non-parametric test was performed. Pairwise nucleotide identity, nucleotide identity to genome, number of N-nucleotide additions,

nucleotide deletions at segment junctions, IGHD segment length, and CDR3 lengths were evaluated with the Kruskal-Wallis Rank Sum test for three way comparisons between fetal liver, fetal bone marrow, and adult horse bone marrow, and the Wilcoxon-Mann-Whitney Rank Sum test for two-way comparisons between the different life stages or tissue with KaleidaGraph (Synergy Software, Reading, PA). IGHV, IGHD, and IGHJ segment usage was assessed by Chi^2 analysis (Graphpad Prism version 6.0c). The Chi^2 test was not valid for pairwise comparisons between tissues for IGLV segment use due to the use of many different gene segments resulting in a small frequency for any individual gene; therefore, the Fisher exact test was performed using Graphpad Prism for IGL segment usage. All data was treated as unpaired. A p-value $\leq$ 0.05 was considered significant.

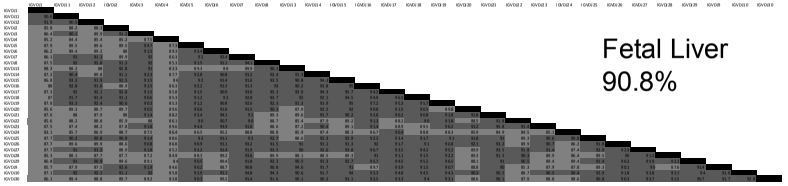
Results

Sequence diversity in the pre-immune immunoglobulin repertoire

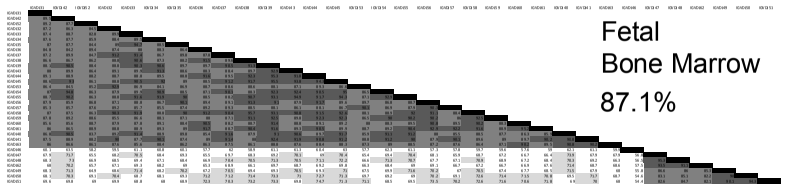
Previous studies showed that a small degree of sequence diversity was present in immunoglobulins derived from equine fetal spleen (Tallmadge et al. 2013, Tallmadge et al. 2014). In this part of my experiments, I asked if sequence diversity originated already in the primary lymphoid tissues of the fetus, and how that compared to the sequence diversity observed in the primary lymphoid tissue of the adult horse. Heavy and lambda light chain sequence variation was determined for equine fetal liver, fetal bone marrow, and adult horse bone marrow. The median percent nucleotide identity of heavy chain VDJ segments was compared between all tissues. Significant differences (p-values < 0.0001) were found between the fetal and adult horse tissues (Figure 3.1).

Figure 3.1 Pairwise nucleotide identity matrices for the equine heavy and lambda light chain variable regions. Pairwise comparisons of nucleotide identity were made for the heavy chain (left) and lambda light chain (right) sequences from fetal liver (top), fetal bone marrow (middle), and adult horse bone marrow (bottom). The levels of identity are depicted with shading: dark gray 90-100%, medium gray 80-89%, light gray 70-79%, and white <70%. The median nucleotide identities noted. The median nucleotide identities were compared between all tissues: fetal liver and fetal bone marrow, fetal liver and adult horse bone marrow, fetal bone marrow and adult horse bone marrow, all fetal and adult horse bone marrow, and the three-way comparison between fetal liver, fetal bone marrow, and adult horse bone marrow. Significant differences (p-values <0.0001) of median nucleotide identity between tissues were observed for the heavy chain variable region for all comparisons. No significant differences (p-values >0.16) were found between median nucleotide identities for the lambda light chain variable region between tissues for all comparisons.

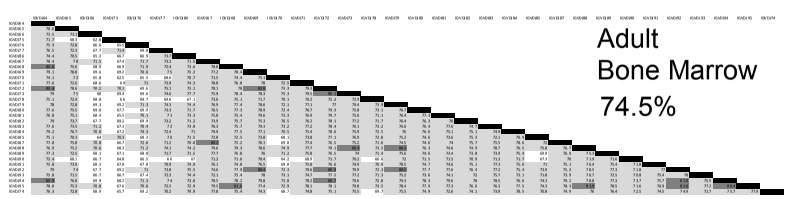
Heavy Chain



Fetal Liver
90.8%

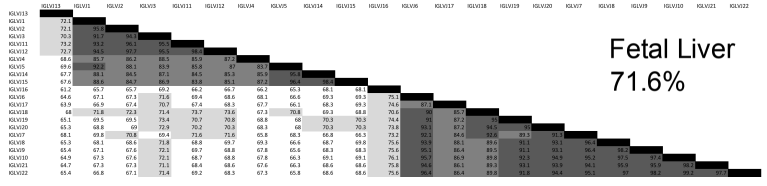


Fetal
Bone Marrow
87.1%

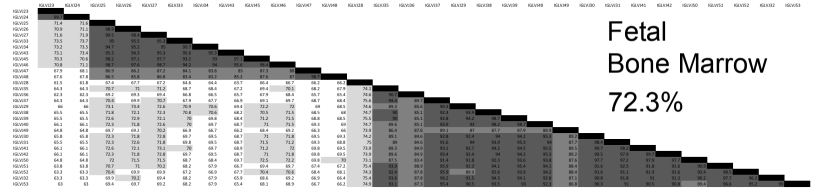


Adult
Bone Marrow
74.5%

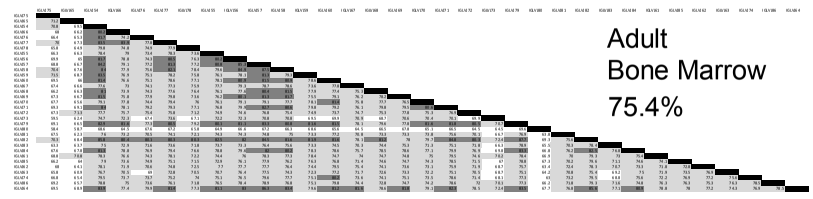
Lambda Light Chain



Fetal Liver
71.6%



Fetal
Bone Marrow
72.3%



Adult
Bone Marrow
75.4%

Sequences

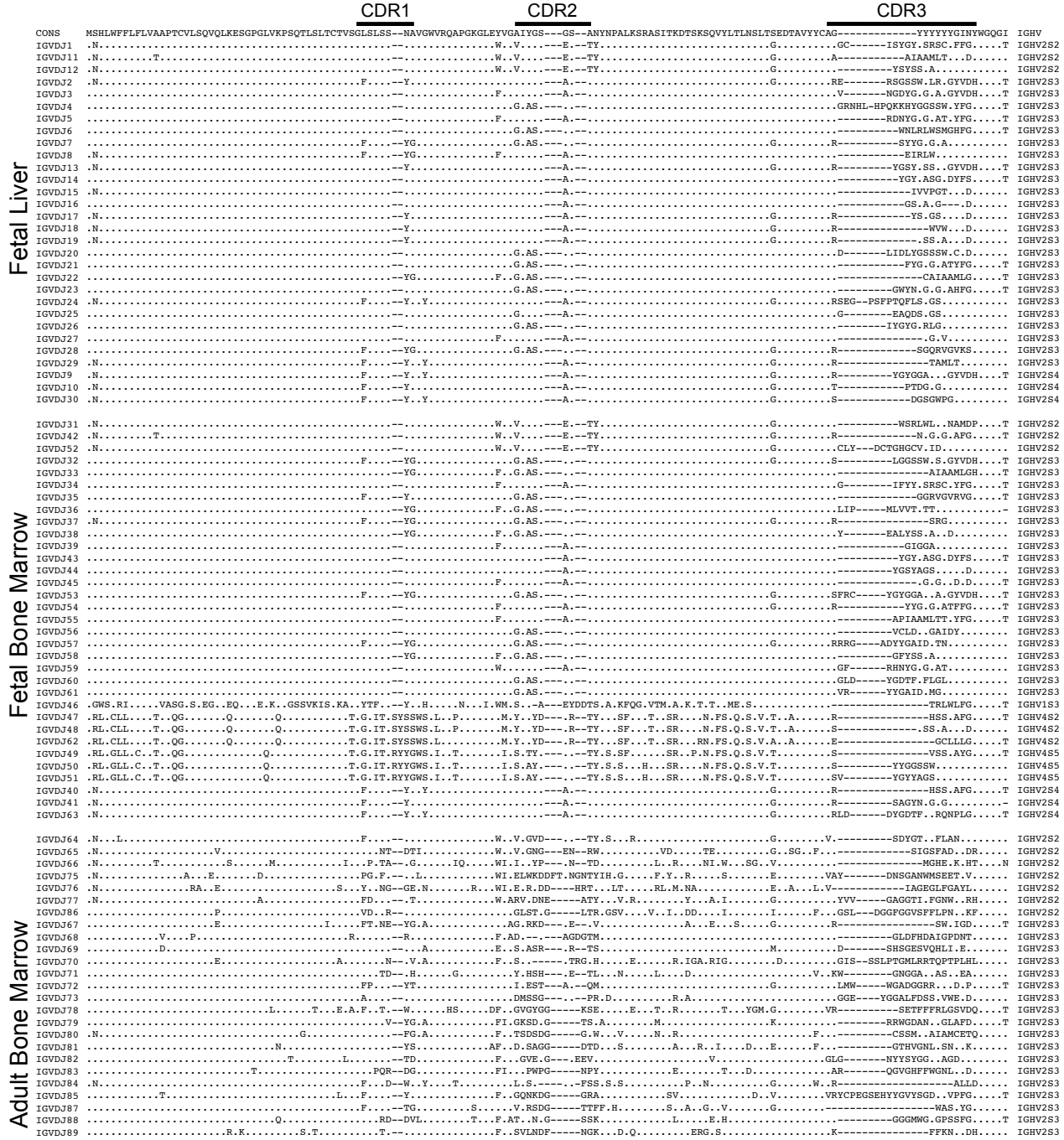
Sequences

In contrast to the heavy chain, the lambda light chain VJ segments had similar pairwise nucleotide identities between fetal and adult horse tissues (p-values > 0.16) (Figure 3.1).

In the fetal tissues, these nucleotide differences were predominately in the CDR3H region (Figure 3.2) largely reflecting the use of different IGHD segments resulting in the greatest variability of amino acid identity in the positions spanning the CDR3H (Figure 3.3). In the adult horse, the mutations and amino acid variability were concentrated in the CDR3H but also CDR2H, and to a lesser degree, CDR1H region (Figures 3.2 and 3.3). The amino acid differences in the lambda fetal sequences were more evenly distributed throughout the variable region with a smaller degree of clustering around the CDRs (Figures 3.3 and Figure 3.4) than the fetal heavy chain sequences. In the adult horse bone marrow, however, the highest degree of amino acid variability was concentrated in the CDRs (Figure 3.3).

Fetal heavy chain sequences also had a higher median percentage (> 98%) of identical nucleotides with the reference genome sequence than sequences from horse bone marrow (89.4% for IGHV, 88.7% for IGHD, and 89.8% for IGHJ), and these germline identity values were significantly different (p-values < 0.03); germline identity values did not differ (p-values > 0.4) between fetal liver and fetal bone marrow (Table 3.1). Similar to the heavy chain, lambda chain segments sequenced from equine fetal tissues, trended towards higher nucleotide identity to the reference genome sequences than the adult (91-100% for fetal, 71.7-100% for adult) (Table 3.2). Consistent with somatic hypermutation, adult horse sequences had higher mutation rates at adenosine nucleotides, and greater percentage of transition mutations for IGHV2S3 (35% in fetal

Figure 3.2 Amino acid sequence alignment of immunoglobulin heavy chain variable region in equine fetal and adult horse primary lymphoid tissues. Immunoglobulin heavy chain variable region nucleotide sequences and their respective translated amino acid sequences were obtained from equine fetal (liver and bone marrow) and adult horse (bone marrow) whole tissues. Alignment of the heavy chain variable region with amino acids identical to the consensus sequence are represented as dots, and residues different from the consensus noted. Gaps in the alignment are represented by dashes. The three CDR regions are indicated above the consensus sequence (Sun et al. 2010; Tallmadge et al. 2013). Sequences from each tissue are ordered based on IGHV gene indicated to the right of each amino acid sequence.


CDR1
CDR2
CDR3
Fetal Liver
Fetal Bone Marrow
Adult Bone Marrow

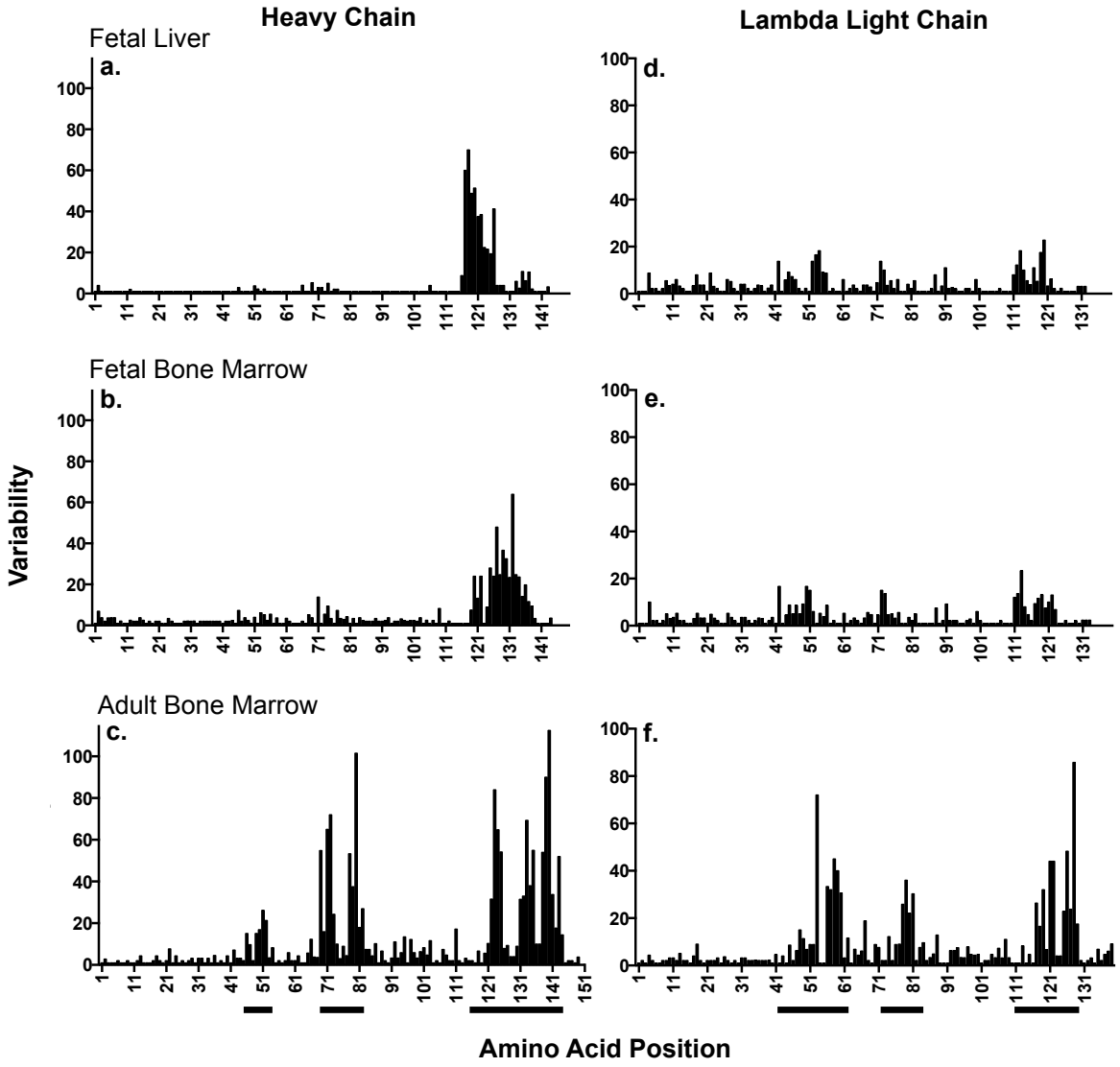


Figure 3.3 Variability in immunoglobulin amino acid sequence in equine fetus and adult horse primary lymphoid tissues. Heavy and light chain immunoglobulin variable region nucleotide sequences, and their respective translated amino acid sequences were obtained from equine fetal (liver and bone marrow) and adult horse (bone marrow) whole tissues. Amino acid variability at each residue of the immunoglobulin variable region was calculated as describe by Wu and Kabat (1970) for (a) fetal liver heavy chain, (b) fetal bone marrow heavy chain, (c) adult horse bone marrow heavy chain, (d) fetal liver lambda light chain, (e) fetal bone marrow lambda light chain, and (f) adult horse bone marrow lambda light chain. The three CDR regions are underlined bellow the X-axis.

Figure 3.4 Amino acid sequence alignment of immunoglobulin lambda light chain variable region in equine fetal and adult horse primary lymphoid tissues. Immunoglobulin lambda light chain nucleotide sequences and their respective translated amino acid sequences were obtained from equine fetal (liver and bone marrow) and adult horse (bone marrow) whole tissues. Alignment of the lambda chain variable region with amino acids identical to the consensus sequence are represented as dots, and residues different from the consensus noted. Gaps in the alignment are represented by dashes. The three CDR regions are indicated above the consensus sequence (Shimanuki et al. 2013; Sun et al. 2010). Sequences from each tissue are ordered based on IGLV gene indicated to the right of each amino acid sequence.

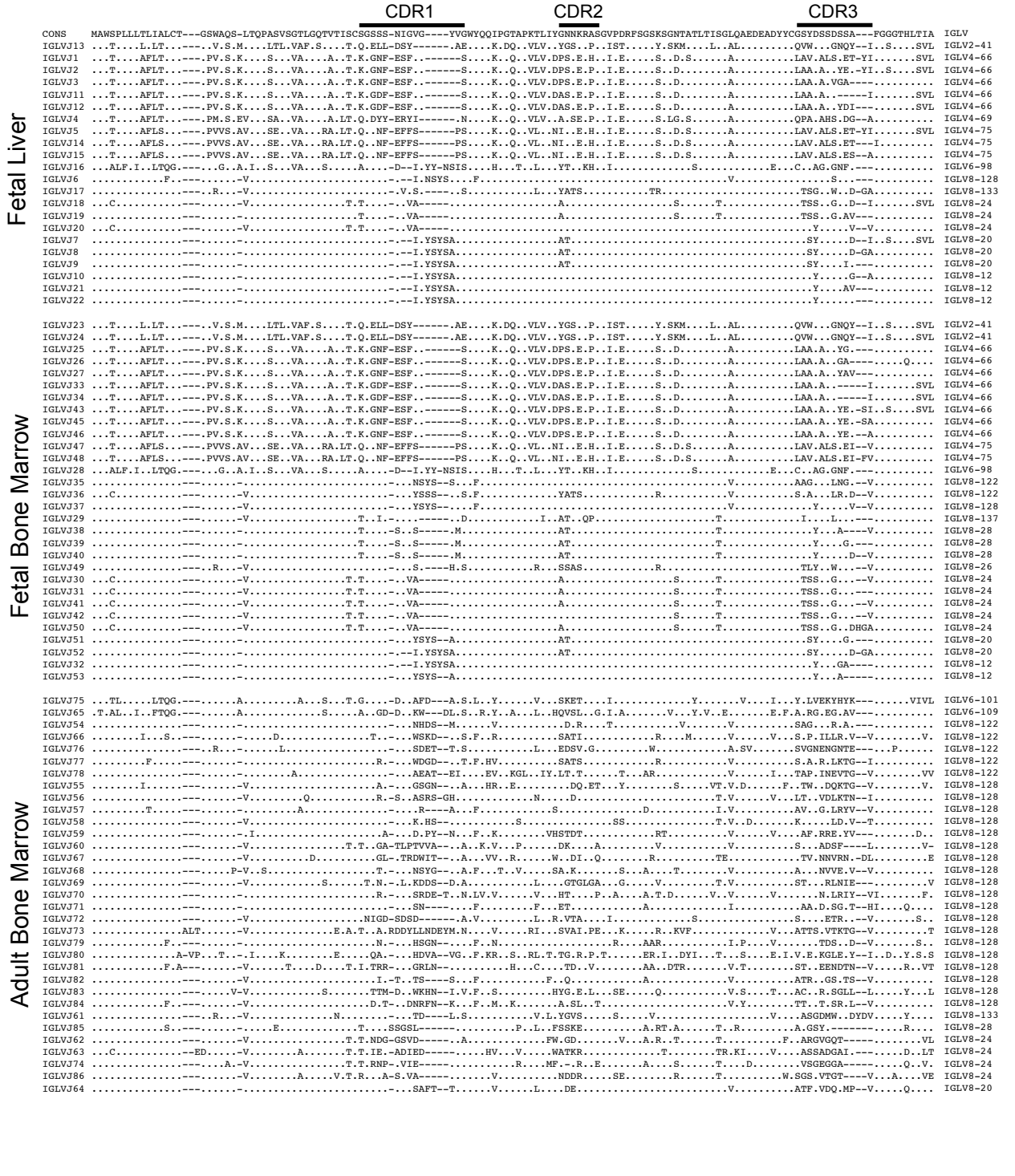

CDR1
CDR2
CDR3
Fetal Liver
Fetal Bone Marrow
Adult Bone Marrow

Table 3.1 Immunoglobulin heavy chain germline segment use, nucleotide identity with reference genome sequence, and junctional nucleotides.

	Sequence	IGHV		VD	IGHD		DJ	IGHJ	
		Gene	NI[a]	J[b]	Gene	NI[a]	J[b]	Gene	NI[a]
Fetal Liver (n = 30)									
Fetus 1	IGVDJ1	IGHV2S2	100.0	13	IGHD7S1	100	2	IGHJ1S3	100
	IGVDJ2	IGHV2S3	99.1	7	IGHD15S2	100	3[c]	IGHJ1S2	100
	IGVDJ3	IGHV2S3	98.8	14	IGHD18S1	84.2	0	IGHJ1S2	100
	IGVDJ4	IGHV2S3	97.4	35	IGHD15S2	100	0	IGHJ1S3	100
	IGVDJ5	IGHV2S3	98.8	19	IGHD10S1	84.6	3[c]	IGHJ1S3	100
	IGVDJ6	IGHV2S3	97.4	10	IGHD18S1	92.3	9	IGHJ1S3	100
	IGVDJ7	IGHV2S3	97.7	4[c]	IGHD18S1	84.2	0	IGHJ1S5	100
	IGVDJ8	IGHV2S3	98.0	7	IGHD20S2	84.6	0	IGHJ1S5	100
	IGVDJ9	IGHV2S4	98.8	2	IGHD18S1	100	0	IGHJ1S2	100
	IGVDJ10	IGHV2S4	99.4	9	IGHD20S2	80	0	IGHJ1S5	100
Fetus 2	IGVDJ11	IGHV2S2	99.7	1	IGHD17S1/S2	100	0	IGHJ1S5	94.3
	IGVDJ12	IGHV2S2	100.0	3	IGHD17S1/S2	100	0	IGHJ1S5	100
	IGVDJ13	IGHV2S3	99.4	0	IGHD13S1	100	0	IGHJ1S2	100
	IGVDJ14	IGHV2S3	98.8	5	IGHD9S1	100	2	IGHJ1S3	96.6
	IGVDJ15	IGHV2S3	98.5	7	IGHD15S2	100	0	IGHJ1S5	96.8
	IGVDJ16	IGHV2S3	98.8	3	IGHD17S1/S2	91.7	0	IGHJ1S5	96.8
	IGVDJ17	IGHV2S3	99.4	0	IGHD10S1	100	0	IGHJ1S5	94.1
	IGVDJ18	IGHV2S3	99.4	ND[c]	ND	ND	ND	IGHJ1S5	96.7
	IGVDJ19	IGHV2S3	99.4	0	IGHD17S1/S2	100	0	IGHJ1S5	96.8
	IGVDJ20	IGHV2S3	97.7	16	IGHD15S2	92.3	0	IGHJ1S5	96.2
Fetus 3	IGVDJ21	IGHV2S3	97.4	6	IGHD18S1	84.2	2	IGHJ1S3	100
	IGVDJ22	IGHV2S3	96.8	6	IGHD17S1/S2	100	0	IGHJ1S3	93.1
	IGVDJ23	IGHV2S3	97.7	7	IGHD9S1	72	3	IGHJ1S3	100
	IGVDJ24	IGHV2S3	98.6	33[c]	IGHD10S1	100	0	IGHJ1S5	97.1
	IGVDJ25	IGHV2S3	98.5	17	IGHD10S1	100	0	IGHJ1S5	97.1
	IGVDJ26	IGHV2S3	97.7	13	IGHD18S1	100	8	IGHJ1S5	100
	IGVDJ27	IGHV2S3	98.8	4	IGHD18S1	90	1	IGHJ1S5	100
	IGVDJ28	IGHV2S3	97.7	8[c]	IGHD24S1	100	5	IGHJ1S5	100
	IGVDJ29	IGHV2S3	98.6	1	IGHD17S1/S2	100	1	IGHJ1S5	100
	IGVDJ30	IGHV2S4	98.8	3	IGHD20S1	100	5	IGHJ1S5	100
Fetal bone Marrow (n = 33)									
Fetus 1	IGVDJ31	IGHV2S2	100.0	0	IGHD18S1	66.7	0	IGHJ1S6	97.4
	IGVDJ32	IGHV2S3	97.7	2	IGHD15S2	91.3	0	IGHJ1S2	100
	IGVDJ33	IGHV2S3	97.1	4	IGHD17S1/S2	100	0	IGHJ1S3	95.7
	IGVDJ34	IGHV2S3	98.8	15	IGHD7S1	100	0	IGHJ1S3	100
	IGVDJ35	IGHV2S3	97.4	9	IGHD24S1	100	3	IGHJ1S3	100
	IGVDJ36	IGHV2S3	96.8	8	IGHD15S1	100	1[c]	IGHJ1S5	100
	IGVDJ37	IGHV2S3	97.4	ND[c]	ND	ND	ND	IGHJ1S5	100
	IGVDJ38	IGHV2S3	96.8	16	IGHD17S1/S2	100	1	IGHJ1S5	100
	IGVDJ39	IGHV2S3	98.8	9	IGHD18S1	100	0	IGHJ1S5	100
	IGVDJ40	IGHV2S4	98.8	1	IGHD17S1/S2	93.8	0	IGHJ1S3	100
	IGVDJ41	IGHV2S4	99.4	15	IGHD18S1	92.3	0	IGHJ1S5	100
Fetus 2	IGVDJ42	IGHV2S2	99.4	5	IGHD18S1	84.2	0	IGHJ1S3	100
	IGVDJ43	IGHV2S3	98.8	5	IGHD9S1	100	0	IGHJ1S3	96.6
	IGVDJ44	IGHV2S3	98.8	5	IGHD7S2	95.7	0	IGHJ1S5	97
	IGVDJ45	IGHV2S3	98.8	3	IGHD20S2	83.3	0	IGHJ1S7	100
	IGVDJ46	IGHV1S3	99.7	1	IGHD18S1	100	0	IGHJ1S3	96.3
	IGVDJ47	IGHV4S2	99.7	1	IGHD17S1/S2	100	0	IGHJ1S3	100
	IGVDJ48	IGHV4S2	99.7	0	IGHD17S1/S2	100	0	IGHJ1S5	96.8
	IGVDJ49	IGHV4S5	99.7	1	IGHD17S1/S2	100	0	IGHJ1S3	96

Table 3.1 Continued

	Sequence	IGHV		VD	IGHD		DJ	IGHJ	
		Gene	NI[a]	J[b]	Gene	NI[a]	J[b]	Gene	NI[a]
	IGVDJ50	IGHV4S5	98.6	0	IGHD15S2	100	0	IGHJ1S5	100
	IGVDJ51	IGHV4S5	98.6	3	IGHD7S2	100	0	IGHJ1S5	100
Fetus 3	IGVDJ52	IGHV2S2	100.0	3[c]	IGHD11S1	100	2	IGHJ1S5	100
	IGVDJ53	IGHV2S3	97.7	10	IGHD18S1	100	4	IGHJ1S2	100
	IGVDJ54	IGHV2S3	99.1	1[c]	IGHD18S1	85	5	IGHJ1S3	96.4
	IGVDJ55	IGHV2S3	98.8	10	IGHD17S1/S2	100	2[c]	IGHJ1S3	100
	IGVDJ56	IGHV2S3	97.4	13	IGHD22S1	100	0	IGHJ1S5	100
	IGVDJ57	IGHV2S3	97.7	12	IGHD22S1	100	3	IGHJ1S5	100
	IGVDJ58	IGHV2S3	96.8	6	IGHD17S1/S2	95	0	IGHJ1S5	100
	IGVDJ59	IGHV2S3	98.5	17	IGHD18S1	85	2	IGHJ1S5	100
	IGVDJ60	IGHV2S3	97.4	9	IGHD4S1	100	8	IGHJ1S5	97.1
	IGVDJ61	IGHV2S3	97.4	9	IGHD22S1	100	4	IGHJ1S5	100
	IGVDJ62	IGHV4S2	98.6	5	IGHD18S1	100	0	IGHJ1S3	93.1
	IGVDJ63	IGHV2S4	98.8	4	IGHD4S1	100	14	IGHJ1S4	91.7
Adult Horse Bone Marrow (n = 32)									
Adult A	IGVDJ64	IGHV2S2	94.8	19	IGHD4S1	100	6	IGHJ1S5	96
	IGVDJ65	IGHV2S2	89.4	ND	ND	ND	ND	IGHJ1S5	82.8
	IGVDJ66	IGHV2S2	82.9	0	IGHD5S6[d]	100	0	IGHJ1S7	77.8
	IGVDJ67	IGHV2S3	88.5	0	IGHD15S2	100	7	IGHJ1S4	95.2
	IGVDJ68	IGHV2S3	90.7	33	IGHD26S1	87.5	2	IGHJ1S5	94.4
	IGVDJ69	IGHV2S3	92.4	14	IGHD1S1	81.8	7	IGHJ1S5	86.1
	IGVDJ70	IGHV2S3	88.7	5	IGHD5S5	100	50	IGHJ1S5	90
	IGVDJ71	IGHV2S3	89.0	5	IGHD18S1	95	19	IGHJ1S5	94.4
	IGVDJ72	IGHV2S3	95.4	16[c]	IGHD15S2	90.9	9	IGHJ1S7	90.9
	IGVDJ73	IGHV2S3	93.3	ND	ND	ND	ND	IGHJ1S7	88.9
	IGVDJ74	IGHV2S4	86.3	ND	ND	ND	ND	IGHJ1S5	81.5
Adult B	IGVDJ75	IGHV2S2	79.7	0	IGHD26S1	88.9	34	IGHJ1S5	100
	IGVDJ76	IGHV2S2	82.4	24	IGHD15S1	70	2	IGHJ1S5	100
	IGVDJ77	IGHV2S2	89.4	7	IGHD18S1	73.9	6	IGHJ1S5	78.1
	IGVDJ78	IGHV2S3	83.6	ND	ND	ND	ND	IGHJ1S2	88.2
	IGVDJ79	IGHV2S3	92.2	23	IGHD16S1	92.9	4	IGHJ1S3	95.8
	IGVDJ80	IGHV2S3	90.5	11	IGHD5S2	70	12	IGHJ1S5	86.7
	IGVDJ81	IGHV2S3	89.0	23	IGHD8S3	87.5	4	IGHJ1S5	88.9
	IGVDJ82	IGHV2S3	89.7	12	IGHD10S1	88.5	5	IGHJ1S5	100
	IGVDJ83	IGHV2S3	89.5	ND	ND	ND	ND	IGHJ1S5	92.9
	IGVDJ84	IGHV2S3	91.1	ND	ND	ND	ND	IGHJ1S5	100
	IGVDJ85	IGHV2S3	89.1	23[c]	IGHD13S1	82.4	6	IGHJ1S6	76.3
Adult C	IGVDJ86	IGHV2S2	87.1	10	IGHD18S1	83.3	18	IGHJ1S5	93.1
	IGVDJ87	IGHV2S3	88.9	0	IGHD14S1	100	8	IGHJ1S3	90.5
	IGVDJ88	IGHV2S3	91.0	19	IGHD18S1	100	8	IGHJ1S3	100
	IGVDJ89	IGHV2S3	87.8	ND	ND	ND	ND	IGHJ1S5	89.7
	IGVDJ90	IGHV2S3	80.5	18	IGHD26S1	85.7	4	IGHJ1S5	95
	IGVDJ91	IGHV2S3	87.4	ND	ND	ND	ND	IGHJ1S5	85.7
	IGVDJ92	IGHV2S3	92.4	10	IGHD18S1	76.2	6	IGHJ1S5	78.1
	IGVDJ93	IGHV2S3	89.8	ND	ND	ND	ND	IGHJ1S5	73.5
	IGVDJ94	IGHV2S3	91.4	14	IGHD9S1	100	2	IGHJ1S7	82.8
	IGVDJ95	IGHV2S3	91.3	ND	ND	ND	ND	IGHJ1S7	87.1

a. Identity represents the percent identical nucleotides shared with the germline sequence
b. Junction indicates the number of N + P-nucleotides present at segment junction
c. Junctional nucleotides may include P-nucleotides
d. The annotated IGHD segment of IGVDJ66 was only 4 nucleotides long, however there were only 4 nucleotides between the annotated IGHV and IGHJ segments that were identical to the genomic IGHD5S6.
'ND' indicates that the germline gene segment could not be determined

Table 3.2. Immunoglobulin lambda light chain germline segment use, nucleotide identity with reference genome sequence, and junctional nucleotides

	Sequence	IGLV		VJ	IGLJ	
		Gene	Identity[a]	Junction[b]	Gene	Identity[a]
Fetal Liver (n = 22)						
Fetus 1	IGLVJ1	IGLV4-66	95.7	2[c]	IGLJ1	97.4
	IGLVJ2	IGLV4-66	99.4	0	IGLJ1	100
	IGLVJ3	IGLV4-66	99.4	1	IGLJ5	100
	IGLVJ4	IGLV4-69	99.7	0	IGLJ5	100
	IGLVJ5	IGLV4-75	100.0	0	IGLJ1	97.4
	IGLVJ6	IGLV8-128	99.2	0	IGLJ7	100
	IGLVJ7	IGLV8-20	98.6	0	IGLJ1	100
	IGLVJ8	IGLV8-20	98.6	0	IGLJ4	92.3
	IGLVJ9	IGLV8-20	98.6	1	IGLJ5	100
	IGLVJ10	IGLV8-12	100.0	0	IGLJ5	100
Fetus 2	IGLVJ11	IGLV4-66	100.0	0	IGLJ1	97.1
	IGLVJ12	IGLV4-66	100.0	0	IGLJ1	97.1
Fetus 3	IGLVJ13	IGLV2-41	100.0	0	IGLJ1	100
	IGLVJ14	IGLV4-75	100.0	1[c]	IGLJ5	100
	IGLVJ15	IGLV4-75	100.0	0	IGLJ7	100
	IGLVJ16	IGLV6-98	99.4	0	IGLJ4	94.4
	IGLVJ17	IGLV8-133	94.9	0	IGLJ5	100
	IGLVJ18	IGLV8-24	99.7	0	IGLJ1	97.1
	IGLVJ19	IGLV8-24	98.8	2	IGLJ5	97.1
	IGLVJ20	IGLV8-24	97.1	1	IGLJ5	97.1
	IGLVJ21	IGLV8-12	100.0	2	IGLJ5	97.1
	IGLVJ22	IGLV8-12	100.0	0	IGLJ5	100
Fetal Bone Marrow (n = 30)						
Fetus 1	IGLVJ23	IGLV2-41	99.7	0	IGLJ1	100
	IGLVJ24	IGLV2-41	100.0	0	IGLJ1	100
	IGLVJ25	IGLV4-66	99.4	1	IGLJ4	94.4
	IGLVJ26	IGLV4-66	99.4	2	IGLJ4	91.7
	IGLVJ27	IGLV4-66	99.4	1	IGLJ5	97.1
	IGLVJ28	IGLV6-98	99.4	0	IGLJ5	100
	IGLVJ29	IGLV8-137	100.0	0	IGLJ5	100
	IGLVJ30	IGLV8-24	100.0	1	IGLJ5	97.1
	IGLVJ31	IGLV8-24	100.0	0	IGLJ5	100
	IGLVJ32	IGLV8-12	99.7	0	IGLJ5	100
Fetus 2	IGLVJ33	IGLV4-66	100.0	0	IGLJ1	97.1
	IGLVJ34	IGLV4-66	99.7	0	IGLJ1	97.1
	IGLVJ35	IGLV8-122	96.6	0	IGLJ4	97.3
	IGLVJ36	IGLV8-122	91.0	0	IGLJ4	97.2
	IGLVJ37	IGLV8-128	98.9	1	IGLJ5	97.1
	IGLVJ38	IGLV8-28	99.4	0	IGLJ4	97.4
	IGLVJ39	IGLV8-28	99.1	1	IGLJ4	97
	IGLVJ40	IGLV8-28	99.4	0	IGLJ4	97.2
	IGLVJ41	IGLV8-24	99.7	0	IGLJ4	97.3
	IGLVJ42	IGLV8-24	99.4	1	IGLJ7	97.1
Fetus 3	IGLVJ43	IGLV4-66	99.4	2[c]	IGLJ1	100
	IGLVJ45	IGLV4-66	99.4	1[c]	IGLJ5	100
	IGLVJ46	IGLV4-66	99.4	0	IGLJ5	100
	IGLVJ47	IGLV4-75	100.0	1	IGLJ5	97.1
	IGLVJ48	IGLV4-75	100.0	4	IGLJ5	97.1
	IGLVJ49	IGLV8-26	98.9	1	IGLJ5	97.1
	IGLVJ50	IGLV8-24	99.7	3[c]	IGLJ7	100

Table 3.2 Continued

	Sequence	IGLV		VJ	IGLJ	
		Gene	NI[a]	J[b]	Gene	NI[a]
	IGLVJ51	IGLV8-20	98.3	1	IGLJ4	97
	IGLVJ52	IGLV8-20	98.6	0	IGLJ7	100
	IGLVJ53	IGLV8-12	100.0	0	IGLJ7	100
Adult Horse Bone Marrow (n = 33)						
Adult A	IGLVJ54	IGLV8-122	93.2	0	IGLJ4	94.7
	IGLVJ55	IGLV8-128	86.5	14	IGLJ4	91.7
	IGLVJ56	IGLV8-128	90.9	20	IGLJ5	96.9
	IGLVJ57	IGLV8-128	90.8	0	IGLJ5	100
	IGLVJ58	IGLV8-128	92.5	2	IGLJ5	100
	IGLVJ59	IGLV8-128	88.0	2	IGLJ7	91.4
	IGLVJ60	IGLV8-128	89.7	5	IGLJ7	96.6
	IGLVJ61	IGLV8-133	88.6	3[c]	IGLJ5	89.5
	IGLVJ62	IGLV8-24	88.6	17	IGLJ1	93.8
	IGLVJ63	IGLV8-24	86.6	19	IGLJ5	85.3
	IGLVJ64	IGLV8-20	90.1	8	IGLJ5	90.3
Adult B	IGLVJ65	IGLV6-109	85.6	30	IGLJ7	90.3
	IGLVJ66	IGLV8-122	84.5	1	IGLJ7	94.3
	IGLVJ67	IGLV8-128	84.8	20	IGLJ7	93.5
	IGLVJ68	IGLV8-128	88.8	19	IGLJ7	100
	IGLVJ69	IGLV8-128	85.7	1	IGLJ7	96.8
	IGLVJ70	IGLV8-128	88.5	17	IGLJ7	86.7
	IGLVJ71	IGLV8-128	90.9	10	IGLJ7	93.3
	IGLVJ72	IGLV8-128	85.1	5	IGLJ7	93.5
	IGLVJ73	IGLV8-128	79.2	25	IGLJ7	81.6
	IGLVJ74	IGLV8-24	89.4	15	IGLJ7	83.8
Adult C	IGLVJ75	IGLV6-101	93.2	18	IGLJ7	78.1
	IGLVJ76	IGLV8-122	86.1	4	IGLJ4	78.8
	IGLVJ77	IGLV8-122	87.8	16	IGLJ5	93.5
	IGLVJ78	IGLV8-122	83.8	7	IGLJ7	89.5
	IGLVJ79	IGLV8-128	93.3	0	IGLJ5	91.4
	IGLVJ80	IGLV8-128	71.7	20	IGLJ5	77.4
	IGLVJ81	IGLV8-128	82.3	15	IGLJ7	87.1
	IGLVJ82	IGLV8-128	92.0	4	IGLJ7	94.3
	IGLVJ83	IGLV8-128	83.3	11	IGLJ7	87.5
	IGLVJ84	IGLV8-128	88.1	5	IGLJ7	100
	IGLVJ85	IGLV8-28	88.1	15	IGLJ7	93.9
	IGLVJ86	IGLV8-24	89.3	14	IGLJ7	85.7

a. Identity represents the percent identical nucleotides shared with the germline sequence
b. Junction indicates the number of N + P-nucleotides present at segment junction
c. Junctional nucleotides may include P-nucleotides

verses 47% in adult horse), and IGLV8-128, IGLV8-24, IGLV8-12 (40% in fetal verses 45% in adult horse) (Di Noia and Neuberger 2007, Tonegawa 1983)

Combinatorial diversity in the pre-immune repertoire

The next question related to the extent of combinatorial diversity generated in the primary lymphoid tissues, and how it compared to secondary lymphoid tissues of the equine fetus or the adult. The immunoglobulin repertoire of the equine fetal liver and bone marrow showed similarities to that of the adult horse bone marrow, particularly in regards to combinatorial diversity of the heavy chain. IGHV2S2, IGHV2S3, and IGHV2S4 were observed in all tissues tested, all of which belong to IGHV Subgroup 2 as indicated by “2S” in the segment names, meaning that these IGHV germline genes share at least 75% identity at the nucleotide level to each other (Figure 3.5a; Sun et al. 2010, Tallmadge et al. 2013). Additionally IGHV segments from subgroups 1 and 4 were identified in the fetal bone marrow. IGHJ1S2, IGHJ1S3, and IGHJ1S5 were observed at all life stages with IGHJ1S4, IGHJ1S6, and IGHJ1S7 found in the fetal and adult horse bone marrow (Figure 3.5b). Twenty-three of the forty IGHD genes were annotated including 10 different IGHD segments in the fetal liver, 12 in the fetal bone marrow, and 15 in the adult horse bone marrow (Figure 3.5c). IGHD17S1 and IGHD17S2 share identical coding sequences and could not be distinguished from each other. There was one potential IGHD-IGHD rearrangement observed in IGVDJ75 in which there was a second IGHD segment that aligned between the first IGHD and IGHJ with junctional diversity on both the 5` and 3` junctions. However, only short 8-9 nucleotide sequences of both IGHD segments could be aligned and, therefore, the

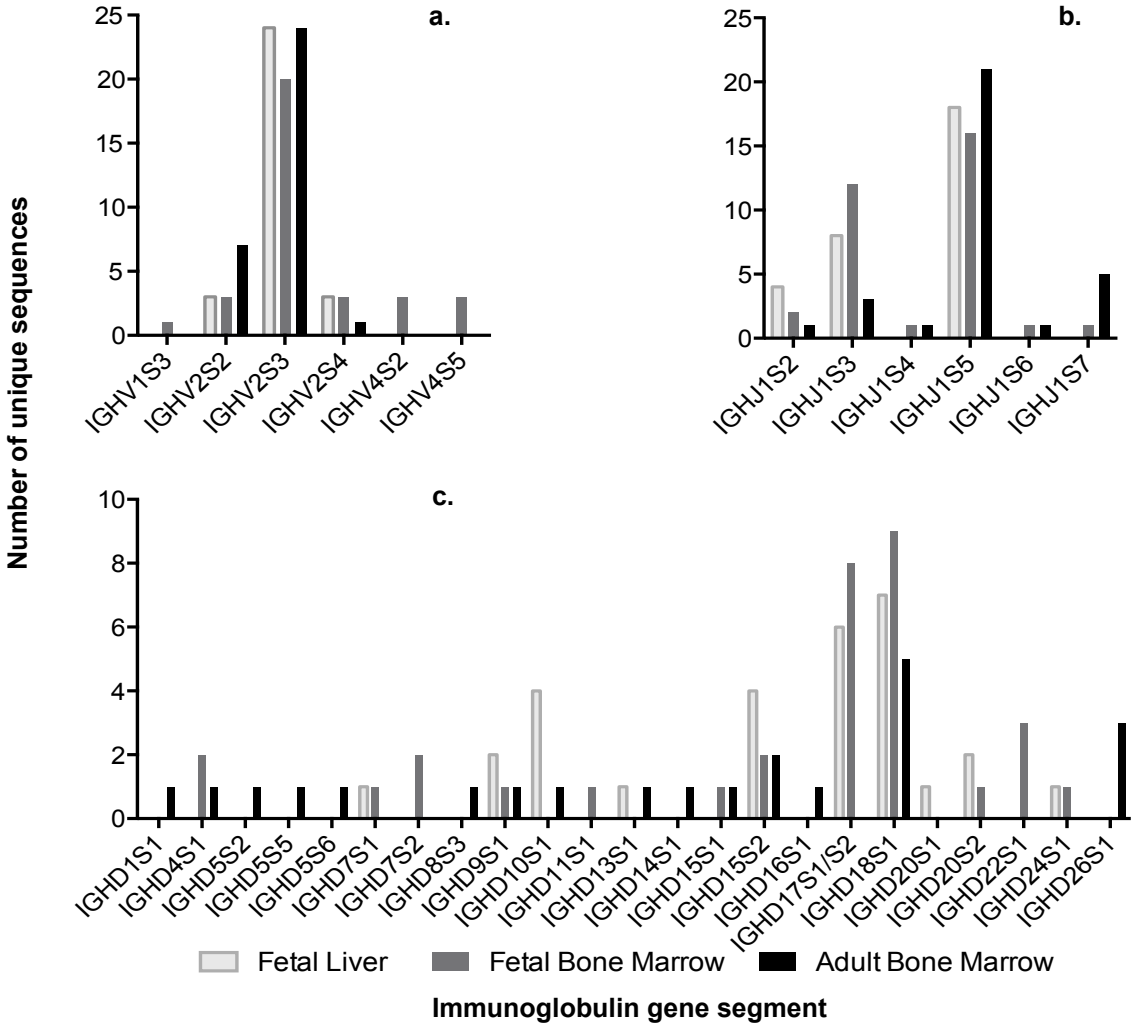


Figure 3.5 Immunoglobulin heavy chain gene segment usage in equine fetus and adult horse primary lymphoid tissues. (a) IGHV, (b) IGHJ, and (c) IGHD gene segments were studied in the fetal liver (light gray), fetal bone marrow (dark gray), and adult horse bone marrow (black). The quantity of unique sequences utilizing each gene segment is shown. No statistically significant difference (p-value > 0.1) was found by Chi^2 analysis in usage of the collection of most common IGHV (IGHV2S2, IGHV2S3, IGHV2S4) and IGHD (IGHD17S1/S2, IGHD18S1, IGHD15S2) sequences when compared between fetal liver, fetal bone marrow and adult horse bone marrow, or pairwise comparisons between fetal and adult horse, fetal liver and fetal bone marrow, and fetal liver and adult horse bone marrow; the difference in the utilization of the most common IGHJ segments (IGHJ1S2, IGHJ1S3, IGHJ1S5) was significantly different (p-value = 0.05) between fetal bone marrow and adult horse bone marrow, and not (p-values > 0.06) between fetal bone marrow and fetal liver or any other comparison between fetal and adult horse sequences.

second shorter IGHD segment could not be confidently annotated. IGHV2S3, IGHJ1S5, IGHD18S1 were the most frequently utilized gene segments in fetal and adult horse tissues as previously reported (Sun et al. 2010, Tallmadge et al. 2013). When the composition of the most commonly used VDJ segments were compared, there was no statistically significant difference (p-value > 0.1) in usage of IGHV and IGHD segments between fetal liver and fetal bone marrow or between fetal and adult horse sequences; the difference in IGHJ utilization was significant only between fetal bone marrow and adult horse bone marrow but not between fetal bone marrow and fetal liver or any other comparison between fetal and adult horse sequences, making the biologic importance of this finding likely unimportant.

Sixteen different IGLV segments were observed (Figure 3.6a) belonging to 4 different subgroups (Table 3.2). Segments belonging to subgroups IGLV2 and IGLV4 were only isolated from fetal tissues, while those in IGLV6 and IGLV8 were found in all tissues. Subgroup IGLV2 consists of only one gene segment (IGLV2-41), while subgroups IGLV4, IGLV6, and IGLV8 have multiple gene segments (Sun et al. 2010). The four IGLJ gene segments identified were observed in all tissues tested (Figure 3.6b). Fetus #2 had limited diversity with only two unique immunoglobulin lambda sequences collected, despite sequencing 11 clones. To make sure that the 5` RACE library for this tissue was not biased, a second library was constructed, the lambda 5`RACE PCR repeated, and the resulting amplicons directly sequenced. The sequencing trace obtained was consistent with the clones already in hand so additional sequences were not pursued. IGLV4-66 and IGLJ5 were the most common segments used in fetal tissues, while IGLV8-128 and IGLJ7 were more frequently found in the

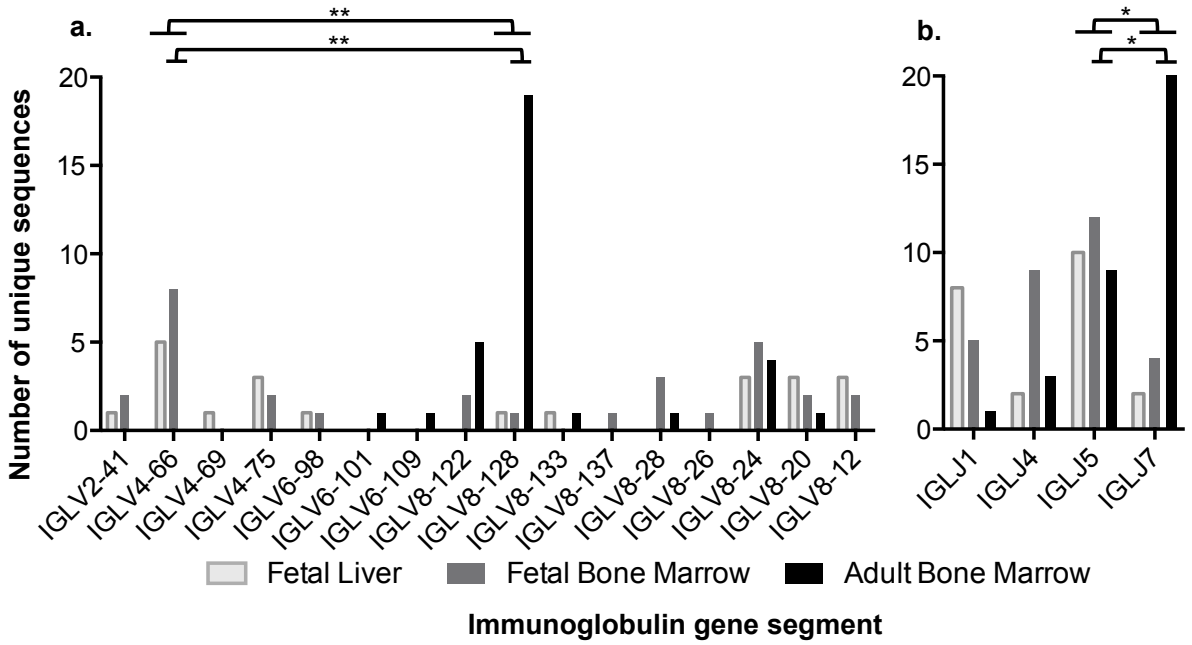


Figure 3.6 Immunoglobulin lambda light chain segment usage in equine fetal and adult horse primary lymphoid tissues. (a) IGLV and (b) IGLJ gene segments were studied in the fetal liver (light gray), fetal bone marrow (dark gray), and adult horse bone marrow (black). The quantity of unique sequences utilizing each gene segment is shown. Significant differences (p-values ≤ 0.0001**) between the two most common IGLV segments (IGLV4-66, IGLV8-128) were observed with the Fisher's Exact Test between fetal liver and adult horse bone marrow, fetal bone marrow and adult horse bone marrow, and all fetal and adult horse bone marrow; fetal liver and fetal bone marrow were not significantly different (p-value = 1.0). Significant differences (p-values ≤ 0.01*) were also observed for the two most common IGLJ (IGLJ5, IGLJ7) segments between fetal liver and adult horse bone marrow, fetal bone marrow and adult horse bone marrow, and all fetal and adult horse bone marrow; fetal liver and fetal bone marrow gene segment usages were not significantly different (p-value = 0.7).

adult horse bone marrow; this bias in IGLV and IGLJ segment use between fetal and adult horse sequences was statistically significant (p-values ≤ 0.0001 IGLV and ≤ 0.01 IGLJ, respectively) (Figure 3.6).

A range of 14-28 unique heavy and lambda light chain Ig gene combinations were observed in fetal and adult horse tissues (Figure 3.7). The most frequently used heavy chain and light chain segments were not necessarily the most frequent combinations; for example, the most frequently used IGH segments were IGHV2S3, IGHJ1S5, IGHD18S1 but the most frequent combination in the fetal bone marrow was IGHV2S3-IGHD22S1-IGHJ1S5 (Table 3.1). Similarly the most frequently observed IGL segments were IGLV4-66 and IGLJ5 in the fetus while the most frequent combination was IGLV4-66 -IGLJ1 in the fetal liver (Table 3.2). Altogether, these data indicate that there is bias in variable region segment use in the lambda light chain but not the heavy chain between life stages as previously reported in secondary lymphoid tissue (Tallmadge et al 2013, Tallmadge et al. 2014), and suggests that the heavy and lambda light chain rearrangements are regulated differently in primary fetal lymphoid tissue.

Junctional diversity in the pre-immune immunoglobulin repertoire

The 100 DG equine fetus also had evidence of junctional diversity in the immunoglobulin heavy chain sequences, created by the deletion and addition of nucleotides at the V(D)J gene segment junctions. Non-template (N) nucleotides were present in fetal heavy chain sequences with medians of 7 (liver, range 0-35) and 5 (bone marrow, range 0-17) nucleotides at the IGHV-IGHD junction, and 0 nucleotides

Figure 3.7 Immunoglobulin heavy and lambda light chain gene segment combinations in equine fetal and adult horse primary lymphoid tissues. Unique (a) heavy and (b) lambda light chain V(D)J combinations observed in the fetal liver (closed squares), fetal bone marrow (closed diamonds), and adult horse bone marrow (open circles). The data indicates that both the fetal and adult horse tissues used a variety of unique V(D)J combinations

a.
IGHJ1S2
IGHJ1S3
IGHJ1S4
IGHJ1S5
IGHJ1S6
IGHJ1S7
ND
IGHD20S2
IGHD26S1
IGHD24S1
IGHD22S1
IGHD20S2
IGHD5S6
IGHD15S2
IGHD5S5
IGHD18S1
IGHD17S1/ S2
IGHD16S1
IGHD15S1
IGHD8S3
IGHD14S1
IGHD7S2
IGHD13S1
IGHD11S1
IGHD10S1
IGHD5S2
IGHD9S1
IGHD7S1
IGHD4S1
IGHD1S1
IGHV2S2
IGHV2S3
IGHV1S3
IGHV4S2
IGHV4S5
IGHV2S4
Fetal liver ■
Fetal bone marrow ◆
Adult bone marrow O
b.
IGLV8-12
IGLV8-20
IGLV8-24
IGLV8-26
IGLV8-28
IGLV8-137
IGLV8-133
IGLV8-128
IGLV8-122
IGLV6-109
IGLV6-101
IGLV6-98
IGLV4-75
IGLV4-69
IGLV4-66
IGLV2-41
IGLJ1
IGLJ4
IGLJ5
IGLJ7

(liver range 0-9, bone marrow range 0-14) at the IGHD-IGHJ junction, similarly to secondary lymphoid tissue at this stage of development (Tallmadge et al. 2013). A significantly (p-values < 0.03) greater number of N-nucleotides were observed in adult horse heavy chain sequences with a median of 13 nucleotides (range 0-33) at the IGHV-IGHD, and 6 (range 0-50) at the IGHD-IGHJ junction when compared to both fetal liver and fetal bone marrow. There was no significantly statistical difference (p-values > 0.4) in the number of N-nucleotides in the fetal liver compared to fetal bone marrow at any junction. Similar trends were observed for the lambda light chain when the expressed sequences were compared to the reference genome. The junctional nucleotides contained many homopolymers ranging from dimers to hexamers, and trended towards higher guanosine content, consistent with TdT activity.

Although there was a difference in number of N-nucleotides, the median CDR3H length, which span the VDJ junctions was similar (p-values > 0.3) in fetal (liver 15 codons, bone marrow 16 codons) and adult horse (bone marrow 16 codons) tissues (Figure 3.8a). Similar results were found in the light chain: the median CDR3L length was 11 codons in all tissues (p-values > 0.7) (Figure 3.8b). The CDR3H and CDR3L lengths determined for the equine fetal liver and adult horse bone marrow were similar to those previously reported in the adult horse (Almagro et al. 2006, Sun et al. 2010, Tallmadge et al. 2013).

In order to further understand the similar CDR3 lengths despite a greater number of N-nucleotide additions in adult horse tissues, the lengths of IGHD segments and deletions off the 3` end of the IGHV and 5` end of the IGHJ gene segments were

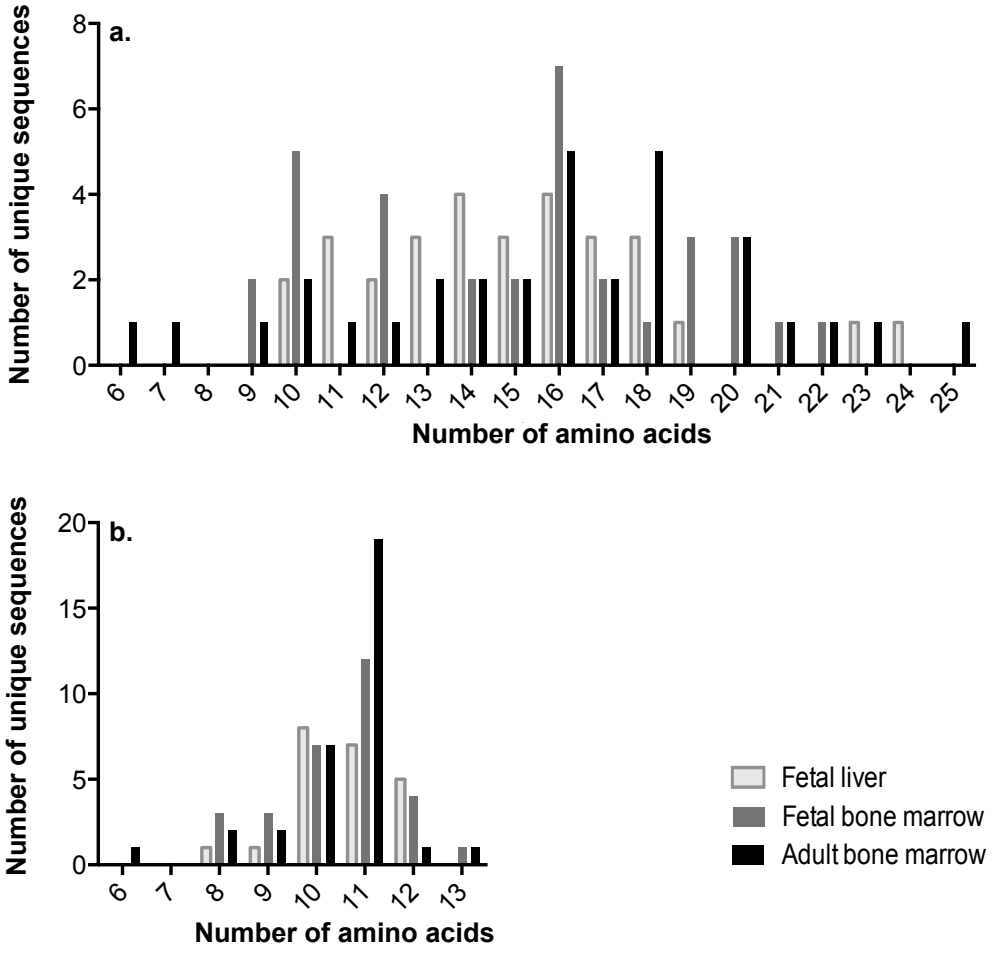


Figure 3.8 CDR3 lengths of equine fetal and adult horse immunoglobulins in primary lymphoid tissues. Distribution of CDR3 amino acid lengths for the (a) heavy chain and (b) lambda light chain in the fetal liver (light gray), fetal bone marrow (dark gray), and adult horse bone marrow (black). No statistical differences were measured between the median CDR3 nucleotide lengths for fetal liver, fetal bone marrow, and adult horse bone marrow, all fetal and adult horse, or any two way comparisons for the immunoglobulin heavy (p-values > 0.3) or lambda light (p-values > 0.7) chains.

examined. The median IGHD segment nucleotide lengths annotated in expressed sequences, subjected to exonuclease activity were significantly longer ($p < 0.005$) in fetal liver (14 nucleotides, range 9-26) and fetal bone marrow (20, range 7-33) than those in the adult horse bone marrow (10, range 4-26). There was also significantly less ($p < 0.001$) nucleotides removed from the 5` end of the IGHJ gene segments of fetal sequences compared to the adult horse bone marrow, with no difference, however in the number of nucleotides deleted from the 3` end of the IGHV segments (Table 3.3).

Discussion

The diversity of the pre-immune immunoglobulin repertoire in the primary lymphoid tissues of the equine fetus was not as limited as expected. No differential bias in IGHV segment use or CDR3 length between fetal and adult horse sequences were detected. Previously, our lab described no differences in heavy chain variable gene segments in secondary lymphoid tissue of the fetus and horse (Tallmadge et al. 2013). My study shows no differences in the primary lymphoid tissue IGH repertoire; the preference for IGHV2S3, IGHD18S1, and IGHJ1S5 to construct the heavy chain originates during fetal development, and is maintained in adulthood. In contrast, in this study I observed a bias for IGLV4-66 and IGLJ5 in fetal life but IGLV8-128 and IGLJ7 in the adult horse for the lambda light chain. Biases in V(D)J segment utilization are reported in fetal life in many species (Butler et al. 2006, Gontier et al. 2005, Jiang et al. 2011, Koti et al. 2010, Pascual et al. 1993), and have been proposed to be due to a variety of factors such as genomic location and accessibility (Souto-Carneiro et al. 2005, Yancopoulos et al. 1984, Zemlin et al. 2001), segment length and resulting CDR3

Table 3.3 Nucleotides removed from the 3` ends of IGHV segments and 5` ends of IGHJ segments

	IGHV Median (range)	IGHJ Median (range)
Fetal liver	4 (0 – 5)	2 (0 – 17)
Fetal bone marrow	4 (0 – 6)	2 (0 – 11)
Adult horse bone marrow	4 (0 – 7)	7 (0 – 20)

size (Shiokawa et al. 1999), and response to environmental conditions (Butler et al. 2000). However, the equine fetus uses broader range of IGLV segments, with the dominant IGLV segment present in 25% of fetal sequences but in 57% of adult horse lambda chain sequences, suggesting that none of the above mechanisms regulate immunoglobulin variable gene segment use in the horse. There were also a greater number of different IGLV-IGLJ combinations observed in the fetus (16 liver, 18 bone marrow) than the adult (14). Decreased restriction during fetal life also occurs in sheep, in which there is no bias for IGHJ selection in fetal or neonatal stages but IGHJ1 is most frequently used in the adult (Gontier et al. 2005). In contrast, humans (Lee et al. 2000) and swine (Butler et al. 2013, Wertz et al. 2013) have restricted IGLV utilization in their pre-immune immunoglobulin repertoires. It remains unknown what governs the shift to a predominance of IGLV8-128 and IGLJ7 in the adult horse. The same biases have been observed in horses of different breeds and geographic location (Hara et al. 2012, Sun et al. 2010, Tallmadge et al. 2014). In the fetus, the bias for IGLV4-66 and IGLJ5 may reflect the predominance of B1-like cells in this life stage, as B1 cells in other species preferentially use different V(D)J segments than B2 cells (Berland and Wortis 2002). IGLJ5, IGLJ6, and IGLJ7 have identical nucleotide sequences and can only be distinguished by the associated IGLC segment (Sun et al. 2010); therefore, the bias of IGLJ7 by the adult horse observed in this study is likely biologically insignificant from that of IGLJ5 in the fetus. The diversity of the pre-immune antibody repertoire in the primary lymphoid tissues of the equine fetus was assessed by analyzing 30-33 unique and productive Ig sequences donated by 3 different fetuses and adults (except for fetal liver, where IGL n=22 as discussed previously). Albeit relatively limited, the sequences

were sufficient to demonstrate immunoglobulin combinatorial diversity in B cells produced during the fetal developmental period. The use of 3 different fetuses of distinct breeding parents accounts for individual variations; however, expansion of data with sequencing and bioinformatic technologies are being pursued to confirm and reveal further similarities and differences in the equine fetal and adult antibody repertoire.

CDR3 length influences the folding pattern, shape, and positioning of key amino acids in the CDR3 loop and, consequently, antigen specificity of the antibody (Barrios et al. 2004, Miqueu et al. 2007). The length of the CDR3 region is the result of both combinatorial and junctional diversity. The lengths of the V(D)J segments, bases removed from these segments by exonucleases, and N- and P-nucleotides added at the junction all contribute to CDR3 length (Desiderio et al. 1984, Lafaille et al. 1989, Sanz 1991). By 100 DG, there were significantly less N-nucleotides at the IGHV-IGHD and IGHD-IGHJ junctions. P-nucleotides could not be confidently identified, as there were few full-length exons annotated; however, it was suspected that 17% fetal and 6% adult horse heavy chain sequences contained P-nucleotides (Table 3.1). Despite the difference in N-nucleotide additions, equine fetal liver CDR3 lengths are similar to that of the adult horse as previously shown in secondary lymphoid tissue (Tallmadge et al. 2013). The length of the genomic V(D)J segments used could not fully explain this discrepancy; alternatively, the similar CDR3 lengths could be due to greater exonuclease activity in the adult horse or a greater number of mutations preventing the annotation of the actual 5` and 3` ends of the segments. Swine also have similar CDR3H lengths in fetal and adult life with early expression of TdT, and the longer IGHD segments used in fetal life are trimmed to a greater extent by exonucleases (Butler et al.

2000, Sinkora et al. 2002). In contrast to horses (Tallmadge et al. 2013, Tallmadge et al. 2014) and pigs (Butler et al. 2000), CDR3H lengths in humans (Delassus et al. 1998, Shiokawa et al. 1999) and mice (Bangs et al. 1991) increase with development. Longer CDR3s increase the potential nucleotide diversity, and restricting this length is hypothesized to be one of the mechanisms controlling the range of antigen binding sites during ontogeny (Shiokawa et al. 1999, Zemlin et al. 2001).

Pairwise nucleotide identity was higher in fetal life for the heavy chain, while no difference was observed at the lambda light chain locus, likely due to the wider variety of IGLV segments belonging to different subfamilies, composing the majority (~350bp) of the variable region (~400bp). Heavy and lambda light chain sequences expressed in fetal tissues have a higher percentage of identical nucleotides with the reference genome sequence than adult sequences; this difference is expected, as adult immunoglobulin molecules undergo somatic hypermutation and affinity maturation in response to antigenic stimulation in the periphery. Fetal tissues contain essentially naïve B cells, while those of the adult are colonized by newly generated naïve B cells in addition to antigenic experienced plasma cells (Nagasawa 2006).

Though at a lesser degree than that in the adult horse, fetal sequences did contain a small number of mutations identified by comparing the expressed sequences to that of the reference genome. Different allotypes have been described at the equine lambda locus, and nucleotide differences identified herein from the reference genomic sequence could be the result of allelic variation, sequence variation between different gene segments, and mutations (Hara et al. 2012, Sun et al. 2010, Tallmadge et al. 2014). Expressed IGLV sequences in the fetus and the reference genomic sequences

differed in nucleotide identity by as much as 9% (Table 3.2); this difference is greater than the up to 4% of nucleotide differences between IGLV gene alleles observed (Tallmadge et al. 2014) likely reflecting sequence variation between IGL gene segments and mutations**.** Cattle fetus immunoglobulin genes also present mutations and undergo a small degree of somatic hypermutation in the absence of external antigen (Koti et al. 2010). Somatic hypermutation greatly contribute to antibody diversity, as *HC1*$^{+/0}$ *IgH*$^{-/-}$ *Igκ*$^{-/-}$ mice express only one IGHV gene, and can respond to a number of different antigens with high affinity using junctional diversity and somatic hypermutation alone (Xu and Davis 2000). Therefore, even the minimal mutations in the V(D)J sequences generated in the presumed sterile environment of the womb may significantly contribute to the diversity of the equine immunoglobulin repertoire during fetal life.

My study suggests that the B cells produced in the liver and bone marrow of the equine fetus generate a wide repertoire of pre-immune immunoglobulins. The B cells that develop in the primary lymphoid tissues during gestation have already elements of combinatorial, junctional, and sequence diversity in the immunoglobulin repertoire observed in the adult horse, with similar heavy chain VDJ segments and CDR3 lengths. By 100 DG, I observed minor combinatorial diversity differences between the fetal liver and fetal bone marrow tissues, which suggests common regulatory mechanisms occurring at the two hematopoietic sites at this stage of development. A smaller degree of sequence diversity was also detected in the fetal primary lymphoid tissues, presumably reflecting the assumed sterile environment of the womb. The use of a greater number of different lambda variable gene segments in fetal life may provide the neonate an opportunity to respond to a wider range of antigens at birth.

CHAPTER FOUR

EQUINE $CD5^+$ B LYMPHOCYTES

Abstract

B lymphocytes expressing CD5 have been described in many species with distinct developmental, phenotypic, and functional characteristics. In mice, CD5 distinguishes the innate B1a B cells from follicular B2 B cells, the conventional players in adaptive humoral immunity. My study tested for the first time aspects of ontogeny, phenotype, and function of equine $CD5^{hi}$ B cells. I learned that $CD5^{hi}$ B cells represented a greater proportion of B cells early in development and in the peritoneal cavity. Peripheral blood $CD5^{hi}$ B cells more frequently co-expressed CD2, CD21, and CD11b than $CD5^{lo}$ B cells when measured with flow cytometry, but had similar mRNA expression of B1 and B2 signature genes including DGKA, FGL2, PAX5, and IGHM when measured with qRT-PCR. Sequencing of lambda light chain segments revealed that $CD5^{hi}$ and $CD5^{lo}$ B cells generate similar levels of immunoglobulin diversity; however, $CD5^{hi}$ B cells more frequently bound to fluorescence-labeled phosphorylcholine and secreted IL-10 in vitro. $CD5^{hi}$ B cells also represented a greater proportion of remaining B cells in horses with common variable immunodeficiency. Taken together, my study shows that equine $CD5^{hi}$ B cells share some of the same characteristics attributed to B1 cells in other species, and may be a target for immune protection in patients with humoral immunodeficiency.

Introduction

The development of the humoral immune system begins during gestation with the production of two major types of B cells: B1 and B2 (Montecino-Rodriguez and

Dorshkind 2012). B2 cells are the classic players in adaptive humoral immunity, continuously produced by the bone marrow in late gestation and following birth. B1 cells are considered components of innate immunity and have important developmental, phenotypic, and functional differences from B2 cells (Baumgarth 2011, Berland and Wortis 2002, Montecino-Rodriguez and Dorshkind 2012). In mice, CD5 distinguishes the innate B1a B cells from follicular B2 B cells (Kantor and Herzenberg 1993). CD5 expression on human B cells is more heterogeneous (Carsetti et al. 2004, Dalloul 2009, Lee et al. 2009); however, the majority of the human equivalent to mouse B1 cells described by Griffin et al. (2011a) are also $CD5^+$.

In mice, B1 cells are primarily produced during fetal life (Godin et al. 1993, Montecino-Rodriguez and Dorshkind 2012), and human B1 cells have been described in umbilical cord blood (Griffin et al. 2011a). B1 cells play important roles in immunity to pathogens by secreting natural antibodies (Baumgarth et al. 2000, Boes et al. 1998b, Haas et al. 2005, Ochsenbein et al. 1999) and by acting as effective antigen presenting cells (Zhong et al. 2007). B1 cells help maintain homeostasis by promoting efferocytosis (Chen et al. 2009) and by secreting the anti-inflammatory cytokine IL-10 (Griffin and Rothstein 2012b, O'Garra and Howard 1992). B1 cells have also been implicated in autoimmune disease (Berland and Wortis 2002). B1 cells are further distinguished from B2 cells by their response to BCR cross-linking and mitogen stimulation (Morris and Rothstein 1993, Rothstein and Kolber 1988, Tumang et al. 2004), tonic BCR signal transduction events (Holodick et al. 2009, Karras et al. 1997), gene expression profiles (Mabbott and Gray 2014, Rothstein et al. 2013, Yamagata et al. 2006), and limited immunoglobulin repertoires (Kantor et al. 1997, Roy et al. 2009, Tornberg and

Holmberg 1995). $CD5^+$ B cells have been observed in different veterinary species, including in pigs (Appleyard and Wilkie 1998), cattle (Naessens and Williams 1992), rabbits (Raman and Knight 1992), and guinea pigs (Dilwith and Wicher 1997). In the horse, CD5 is considered a T cell marker and its expression on B cells has not yet been characterized.

CD5 is a transmembrane glycoprotein, belonging to the scavenger receptor cysteine-rich superfamily, expressed by all T and a subset of B cells (Berland and Wortis 2002, Dalloul 2009). CD5 physically associates with the antigen receptor complexes of T and B cells and modulates signaling through these receptors (Lankester et al. 1994). In B cells, CD5 association with the BCR has been shown by co-immunoprecipitation with CD79a (Lankester et al. 1994). CD5 functions as a negative regulator of antigen receptor signaling by raising the threshold for B or T cell activation following cross linking of the BCR or TCR (Berland and Wortis 2002). This was demonstrated in B cells with CD5 knockout mice; $CD5^+$ B1 cells from wild type mice do not proliferate in response to anti-IgM stimulation, however, peritoneal B1 cells from CD5 knock out mice proliferate in response to this stimulus, similar to B2 cells (Bikah et al. 1996). CD5 expression in the Daudi human B cell line has been shown to result in increased IL-10 production (Gary-Gouy et al. 2002) a cytokine shown to promote B cell survival (Go et al. 1990). Therefore, Gary-Gouy et al. (2002) propose that CD5 may promote B cell survival after BCR engagement by both down-regulating BCR-mediated signaling that may lead to overstimulation and activation-induced cell death, and promoting IL-10 production (Gary-Gouy et al. 2002).

In horses, our research laboratory has observed that the percentage of peripheral blood $CD5^+$ lymphocytes is greater than that of $CD3^+$ lymphocytes, although both are traditionally T cell markers in the horse. During ontogeny, CD5 is expressed on early thymic progenitor cells and subsequently lost on NK committed progenitors (Dalloul 2009). Therefore, this unaccounted for population of $CD5^+$ lymphocytes is likely a subset of B cells. The purpose of this study was to test how aspects of ontogeny, tissue distribution, phenotype, and function differed between $CD5^+$ and $CD5^-$ B cells. My hypothesis was that equine $CD5^+$ B cells share a similar phenotype and function described for B1 cells in other species, with a distinct gene expression profile and limited immunoglobulin diversity when compared to $CD5^-$ B cells. I found that CD5 expression on B cells was not distinctly present or absent similar to that described in pigs (Appleyard and Wilkie 1998), and therefore herein CD5 expression on equine B cells was more specifically referred to as either $CD5^{hi}$ or $CD5^{lo}$. My studies show, for the first time, the presence of $CD5^{hi}$ B cells in the equine and reveal that these cells share some characteristics that have been described for B1 cells in other species. In addition, I investigated the presence of $CD5^{hi}$ B cells in horses affected with common variable immunodeficiency (CVID), a late-onset condition with impairment in the production of B2 cells and, consequently, B2 cell depletion and hypogammaglobulinemia (Flaminio et al. 2009). In these patients, serum IgA concentrations may be within the low normal range when IgG and IgM are low or undetectable, and an IgA-isotype (but not IgG) response to pneumococcal polysaccharide vaccine can be measured (Flaminio et al. 2009). I learned that a greater proportion of remaining B cells were $CD5^{hi}$ in horses with CVID

when compared to healthy adult horses; this finding has implications to a better understanding of immunity in patients with humoral deficiency.

Materials and methods

Equine tissue samples

These experiments were approved by the Cornell University Center for Animal Resources and Education and Institutional Animal Care and Use Committee for the use of vertebrates in research. Fetal liver leukocytes were obtained as previously described (Chapter 2). In brief, 2 mares (1 Thoroughbred and 1 Warmblood) at the Cornell Equine Park were bred and abortions were chemically induced with prostaglandin injections (Lutalyse, Pfizer, New York, NY) at approximately 100 days of gestation (102-105DG) (Douglas and Ginther 1976), and the fetal livers dissected and processed (see below) within an hour of abortion. Peripheral blood was obtained from 4 healthy foals (Warmbloods) also belonging to the Cornell Equine Park at days 3 (range 2-4), 21 (range 20-24), 28 (range 27-28), 35 (range 31-39), and 42 (range 42-45) of life. Peripheral blood was also collected from 8 healthy adult horses (6 Warmbloods, 1 Quarter Horse, 1 Thoroughbred cross, ages 11-19yrs), and 7 horses diagnosed with common variable immunodeficiency (1 Warmblood, 1 Quarter Horse, 5 Thoroughbreds, ages 8-23yrs). Adult horse spleen (n=6), bone marrow (n=6), and peritoneal samples (n=5) were collected immediately post-mortem from healthy research adult horses belonging to other investigations at Cornell University College of Veterinary Medicine (10 Thoroughbreds, 2 Quarter Horses, 1 Arabian, 1 breed unknown, ages 1-25yrs). Single cell suspensions were made from fetal liver and adult horse spleen by pressing

the tissue through a metal mesh (size 80 mesh, Sigma-Aldrich, St. Louis, MO) followed by a nylon mesh (70μm, BD Falcon, Franklin Lakes, NJ). Mononuclear leukocytes were isolated from blood, bone marrow, peritoneal cells, and single cell suspensions derived from fetal liver and adult horse spleen using Ficoll gradient centrifugation (density 1.077, GE Healthcare, Piscataway, NJ) as previously described (Flaminio et al. 2000). If analysis was not done immediately, cells were frozen in either CryoStor CS10 (Stem Cell Technologies, Vancouver, BC, Canada) or Recovery (Thermo Fisher Scientific, Wilmington, DE) cell culture freezing medium and stored in liquid nitrogen.

Flow cytometric cell immunophenotyping

One million cells per sample were blocked with 10% normal goat serum and then labeled with a B cell monoclonal antibody CD19-like (clone cz2.1, D.F. Antczak, Cornell University, Ithaca, NY) followed by goat anti-mouse IgG(Fab)-PE (Jackson ImmunoResearch Laboratories, West Grove, PA), and then a cocktail of CD3-FITC or CD3-Alexa Fluor 488 (F6G.3(G12), J. Stott, University of California, Davis, CA), CD5-PerCP-CY5.5 (HT23A, Washington State University (WSU) Monoclonal Antibody Center Pullman, WA) and either CD2-APC (HB88A, WSU Monoclonal Antibody Center), CD21-APC (B-ly4, BD Biosciences, San Jose, CA), IgM-APC (CM7, AbDSerotec, Raleigh, NC), major histocompatibility complex (MHC) class II-Alexa Fluor 647 (cz11, D.F. Antczak), or CD11b-APC (M1/70.15.11.5, Miltenyi Biotec, Auburn, CA). CD5, CD2, and IgM antibodies were conjugated to PerCP-CY5.5 or APC with LYNX Rapid Conjugation Kits (AbDSerotec) and MHC class II to Alexa Fluor 647 with the Molecular Probe Alexa Fluor 647 Antibody Labeling Kit (Thermo Fisher Scientific) according to

manufacturers' instructions. Negative isotype controls included mouse IgG1-FITC, IgG1-PE, IgG1-PerCP-CY5.5, IgG1-APC (MOPC-21, BD Biosciences) and rat IgG2b-APC (ES26-5E12.4, Miltenyi Biotec). Cells were fixed with 2% paraformaldehyde in phosphate buffered solution, and fluorescence was measured with a BD FACScalibur flow cytometer using argon and red diode lasers (BD Biosciences). A minimum of 10,000 events (or until the solution in the tube exhausted) were collected in a gate determined based on lymphocyte light scattering characteristics, and analyzed with BD CellQuest Pro Software (BD Biosciences). Isotype controls confirmed that non-specific staining was at least less than 2% of lymphocyte-gated cells.

FACS sorting $CD19^{+}CD3^{-}CD5^{hi}$ and $CD19^{+}CD3^{-}CD5^{lo}$ B cells

A custom mouse anti-equine CD19 antibody against epitopes designed by our laboratory was made by Abmart (Shanghai, China). The monoclonal antibody production service included design and generation of multi-epitope protein fragments to be used as antigens and immunization of BALB/c mice. Our laboratory screened several ascites fluids, and the clone 13706-1-5/C347 (raised against the epitope NRSSLNQNHSQD) was determined to be the most sensitive and specific based on flow cytometry and Western blot. For antibody production, the hybridoma cell line was grown in RPMI-1640 medium supplemented with 15% fetal bovine serum, penicillin and streptomycin (Thermo Fisher Scientific) until medium exhaustion. Supernatants were collected and centrifuged at 300 x *g* for 5 min to remove cells and debris, and aliquots stored at 4^{o}C. Isolated equine peripheral blood leukocytes (PBL) were stained with the hybridoma supernatant followed by a goat anti-mouse IgG(H+L)-FITC secondary

antibody (Jackson ImmunoResearch Laboratories) and fluorescence was analyzed with flow cytometry to determine the optimal reagent dilutions compared with the equine anti-CD19-like (clone cz2.1) monoclonal antibody.

Between 20 to 30x10^6 isolated PBL were labeled as described above with the custom monoclonal antibody anti-equine CD19 followed by secondary antibody goat anti-mouse IgG(H+L)-Alexa Fluor 647 secondary antibody (Jackson ImmunoResearch Laboratories), Alexa Fluor 488-conjugated CD3, and PerCP-CY5.5-conjugated CD5 antibodies in RPMI 1640 and 5% fetal calf serum (FCS, Thermo Fisher Scientific). Leukocytes were resuspended in RPMI with 5% FCS, and $CD19^+CD3^-CD5^{hi}$ and $CD19^+CD3^-CD5^{lo}$ B cells were sorted with the BD FACSAria III Cell Sorter at the Cornell Biomedical Sciences Flow Cytometry Core Lab (Cornell University, Ithaca, NY). For molecular experiments, cells were sorted directly into RNA lysis buffer (Zymo Research, Irvine, CA). For cell culture experiments, cells were sorted into RPMI with 10% FCS and 1x antibiotics and antimycotics (Thermo Fisher Scientific), and reanalysis of the $CD19^+CD3^-CD5^{lo}$ cells performed for every sample confirmed enrichment with less than 0.3% of cells contaminating the $CD5^{hi}$ gate.

qRT-PCR measurement of gene expression profiles

Peripheral blood $CD19^+CD3^-CD5^{hi}$ or $CD19^+CD3^-CD5^{lo}$ leukocytes from 5 equine healthy donors (4 adult horses, 1 21-day-old foal) were sorted as described above directly into RNA lysis buffer, and RNA was isolated with the Quick-RNA™ MicroPrep kit with DNAse I treatment to destroy genomic DNA (Zymo Research) according to

manufacturer's instructions. The concentration of RNA was quantified using a NanoDrop (Thermo Scientific), and 4ng of RNA were used per qRT-PCR reaction.

A panel of signature genes differentially expressed by murine B1 and human B1-like cells was assembled, including: CD5, diacylglycerol kinase alpha (DGKA), fibrinogen-like protein 2 (FGL2), paired box 5 (PAX5), interleukin-10 (IL-10), and immunoglobulin mu heavy chain (IGHM). Two differential genes are part of the B1 and B2 cell signature described by Yamagata et al. (2006): DGKA attenuates BCR signaling and is highly expressed in B2 cells (Wheeler et al. 2013), and FGL2 with roles of immunosuppression (Wang et al. 2014), also highly expressed in B1 cells. PAX5, the transcription factor responsible for commitment and maintenance of the B cell identity has been shown to have lower or comparable expression in B1 compared with B2 cells in 2 different studies (Fuxa and Busslinger 2007, Tumang et al. 2005). IL-10 and IGHM have greater expression in B1 cells (O'Garra and Howard 1992, Rothstein et al. 2013). Finally, IGK and IGL mRNA expression was measured in peripheral blood $CD19^{+}CD3^{-}CD5^{hi}$ and $CD19^{+}CD3^{-}CD5^{lo}$ B cells from 3 adult horse donors.

Ten-microliter qRT-PCR reactions were performed in triplicate with 500nM of primer and iScript™ One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) in a CFX96 Real-Time PCR Detection System (Bio-Rad). Cycling parameters were: 50 °C x 10 min, 95 °C x 5 min, [95 °C x 10 sec, 60 °C x 30 sec] repeated 40 cycles, followed by melt curve analysis. Exceptions to the protocol included: (1) 300nM of primer were used for IGK, (2) annealing temperatures was 63 °C for IGK, 61 °C for CD5, 58 °C for IL-10, and 57 °C for FGL2. SYBR primers (Table 4.1) were designed with Beacon Designer 7.91 software (PREMIER Biosoft International, Palo Alto, CA)

Table 4.1 Primer sequences for B1 and B2 signature genes

Gene	GenBank ID	Primer (5'-3')		Product (bp)
SYBR Primers				
CD5	XM_005598215	Forward	GGTGAAGAGATTCCGCCAG	85
		Reverse	GTGGTTGCGATGGAAAGACA	
DGKA	XM_001504786	Forward	AGATGACTCTGAAGGACAATGG	86
		Reverse	GCTTGACTCGCACAGGTT	
FGL2	XM_001488486	Forward	ATGGCAAATGTTCCTCTAAG	132
		Reverse	GTGTAACTCTGTAGGTCTCA	
PAX5[a]	XM_001504306	Forward	GCTGAGTATAAACGCCAAA	94
		Reverse	CGGATGATCCTGTTGATG	
IGHM[a]	NW_001876796	Forward	CATCCCTCCCTCCTTTGC	81
		Reverse	TCATAGGTGCCCAGGTTTG	
IGL[b]	NC_009151.2	Forward	TCCAGGCTGAGGACGAGGC	135
		Reverse	GGAAGAGAGAGACCGAGGGT	
IGK[b]	XM_003362951	Forward	TTCAGTGGCAGTGGATCTGG	184
		Reverse	CAGAAGACGGTGGGAAGATG	
IL-10[c]	NM_001082490	Forward	GTCATCGATTTCTGCCCTGT	181
		Reverse	GCTTCGTTCCCTAGGATGC	
B-ACTIN[a]	NM_001081838.1	Forward	GATGCAGAAGGAGATCACAGC	96
		Reverse	GAGCCGCCGATCCATACG	
Clone primers for standard curve				
IGL[b]	NC_009151.2	Forward	TCCTCACCCTCATCGCTCTC	423
		Reverse	CTCAGAGGAGGGCGGGAA	
IGK[b]	XM_003362951	Forward	RACE UPM[d]	737
		Reverse	AATGTCTATGCCTGCGAGGTCTCCC	

a. (Tallmadge et al. 2012b)
b. (Badial et al. under submission)
c. (Sanchez-Matamoros et al. 2013)
d. SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA)

and synthesized by Eurofins MWG operon (Eurofins Genomics Company, Huntsville, AL). All primers spanned intron boundaries except for IGHM, and no RT reactions revealed that genomic DNA contributed to less than 0.13% of amplification. Reaction efficiency was between 90 and 110%, and no primer dimers were observed on the melt curve analysis. Gene expression was normalized with an endogenous control (B-ACTIN), and relative expression levels were plotted as the inverse of the normalized Ct averaged for the triplicates with GraphPad Prism 6.0f (GraphPad Software, Inc., La Jolla, CA).

Absolute quantification was performed for IGL and IGK, and RNA standard curves were prepared for each of these genes as previously described (Badial et al. under submission). A region that included the SYBR primers was amplified from whole peripheral blood cDNA (Table 4.1) with iProof polymerase (Bio-Rad) for IGL or with 5'-rapid amplification of cDNA ends (RACE) for IGK (as described in Chapter 3, Badial et al. under submission), cloned into pJET1.2 vector (CloneJET™ PCR Cloning Kit, Thermo Scientific), and sequenced at the Cornell University Institute of Biotechnology (Ithaca, NY). The plasmid DNA was linearized, and in vitro transcription was performed from the T7 vector site (Thermo Scientific). RNA transcripts were purified with the RNA Clean & Concentrator™ kit (Zymo Research) and quantified using the NanoDrop (Thermo Scientific). Ten-fold serial dilutions were made and absolute quantification of mRNA transcripts was determined from the RNA standard curve with the CFX Manger software (Bio-Rad).

Immunoglobulin lambda light chain sequencing

RNA was isolated from peripheral blood $CD19^{+}CD3^{-}CD5^{hi}$ or $CD19^{+}CD3^{-}CD5^{lo}$ B cells from 2 healthy equine donors (1 adult horse, 1 21-day-old foal) as described above. cDNA was prepared from 45ng RNA with the Sensiscript Reverse Transcription Kit (Qiagen, Valencia, CA) with 0.5mM dNTP, 1μM Oligo-dT primer, Rnase inhibitor (10 units, Thermo Scientific), and 1μl Sensiscript Reverse Transcriptase in 1x Buffer RT in a 20μl reaction incubated for 60 min at 37 °C. IGL sequences were amplified with forward (5` AGATCTCCACCATGGCCTG 3`) and reverse (5` GGGGACAGTTTCTTCTCCAC 3`) primers to conserved regions of the lambda variable and constant gene segment, respectively, designed based on RACE immunoglobulin sequences previously described (Tallmadge et al. 2014). The RT-PCR reaction was performed with the iProof™ High-Fidelity PCR kit in a 50μl reaction including 10ng cDNA, and 1x iProof HF buffer, 1.5mM MgCl, 0.2mM dNTPs, 0.5μM each primer, and 0.02 U/μl iProof DNA polymerase (a high fidelity polymerase with an error rate of 4.4×10^{-7}). Thermal cycling conditions were as follows: initial denaturing 98 °C x 3 min, [denaturing 98 °C x 10 sec, annealing 60 °C x 10 sec, extension 72 °C x 10 sec] repeated 40 times, and final extension 72 °C x 10 min. Up to 40μl of the PCR product was visualized with electrophoresis on a 1% agarose gel and treated with GelGreen nucleic acid stain (Phenix Research Products, Candler, NC) using the Gel Doc™ EZ Imager (Bio-Rad). PCR products at expected size (~700bp) were excised, purified with the GeneJET™ Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific), ligated into the pJET1.2 vector (CloneJET™ PCR Cloning Kit, Thermo Fisher Scientific), and transformed into NEB-5 alpha (New England BioLabs, Ipswich, MA) competent *E.coli*.

Plasmids were purified with the GeneJET Plasmid Miniprep kit (Thermo Fischer Scientific) from expanded clones, and sequenced at the Cornell University Institute of Biotechnology, as previously described (Tallmadge et al. 2014).

Germline alleles were sequenced for two of the most frequently observed IGLV genes, IGLV8-122 and IGLV8-128, in unsorted leukocytes from the same healthy adult horse and foal as above. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. In order to obtain germline IGLV8-128 alleles, CD19 depleted PBL from each horse was used as a source for genomic DNA to reduce the number of mutated genes presented by mature B cells. CD19 depletion was done by labeling 7-10 million PBL with the anti-equine CD19 (clone 13706-1-5/C347) followed by goat anti-mouse IgG(H+L)-Alexa Fluor 647 (Jackson ImmunoResearch Laboratories) monoclonal antibody, and collecting $CD19^-$ leukocytes with the BD FACSAria III Cell Sorter as described above. PCR reactions were performed as described above with the iProof™ High-Fidelity PCR kit with 50ng genomic DNA and 1x iProof GC buffer (Bio-Rad). Primers designed to amplify genomic IGLV8-122 and IGLV8-128 were previously described (Tallmadge et al. 2014). IGLV8-122 primers (5' CAAAGGAAGCAGCTGACACG 3' and 5' GGGGCTGTGATTTGCATGTG 3') amplified a 705bp product, and IGLV8-128 primers (5'CAAAGGAAGCAGCTGACGTG 3' and 5' CTCAGCTTTCCGTGAGGGTT 3', amplified an 855bp product (Tallmadge et al. 2014). Thermal cycling parameters included initial denaturing at 98 °C for 3 min; 35 cycles of 98 °C for 10 sec, 60 °C for 10 sec, 72 °C for 15 sec, with a final extension at 72 °C for 10 min. PCR products were visualized, cloned, and sequenced as described above. Multiple identical clones were

obtained to confirm each allele, and sequence of a novel allele (IGLV8-128*04) is available through GenBank with accession number KR190601.

Immunoglobulin lambda light chain analysis

Sixty-four $CD19^{+}CD3^{-}CD5^{hi}$ (32 from each donor) and 67 $CD19^{+}CD3^{-}CD5^{lo}$ (34 from the foal and 33 form the adult horse) unique and productive immunoglobulin (Ig) sequences were collected and are available through GenBank with accession numbers KR190470 - KR190600. The IGLV gene segments were identified by comparing the cloned cDNA sequences against the EquCab2.0 equine reference genome using the NCBI *Equus caballus* BLAST tools with the IGL annotations by Sun et al. (2010) as previously described (Tallmadge et al. 2014). The top BLAST hits were evaluated for identity, alignment length, and orientation. IGLV8-122 and IGLV8-128 nucleotide identities between expressed sequences and the germline sequences were calculated with the Geneious Pro R6-1 software; the first 8 nucleotides that overlapped with the forward primer were not included in the analysis to avoid potential bias. The lambda light chain CDR3 was measured between the YCC motif in the IGLV and FGG motif in the IGLJ gene segments as previously described (Ford et al. 1994, Sun et al. 2010).

BCR specificity for phosphorylcholine

Phosphorylcholine-specific B cells were identified by incubating $1x10^{6}$ cells with 40μg/ml phosphorylcholine-BSA-fluorescein (PC-FITC, Biosearch Technologies, Petaluma, CA) at 37 °C in RPMI 1640 media with 5% FCS as previously described (Griffin et al. 2011a). Cells were then stained with anti-equine CD19 (clone 13706-1-

5/C347) followed by goat anti-mouse IgG(H+L)-Alexa Fluor 647 secondary antibody, and PerCP-CY5.5-conjugated CD5 as above, though RPE-conjugated (AbDSerotec) CD4 (HB61A, WSU Monoclonal Antibody Center) and RPE-conjugated CD8 (HT14A, WSU Monoclonal Antibody Center) were used instead of Alexa Fluor 488-CD3. Fluorescence was measured as described above using the BD FACScalibur (BD Biosciences), collecting at least 30,000 events in a gate determined based on lymphocyte light scattering characteristics, or until the solution in the tube was exhausted, and analyzed with BD CellQuest Pro Software (BD Biosciences). Positive gates were set based on staining with isotype negative controls, and a fluorescence-minus-one control (antibody cocktail without PC-FITC).

IL-10 measurement in the supernatant of cultured B cells

Fifty thousand FACS sorted $CD19^{+}CD3^{-}CD5^{hi}$ or $CD19^{+}CD3^{-}CD5^{lo}$ B cells from 3 adult horses were cultured in RPMI 1640 (Thermo Fisher Scientific) with 10% FCS (Thermo Fisher Scientific) and 1x antibiotics and antimycotics (Thermo Fisher Scientific) for 3 days in medium alone or supplemented with 2.5mg/ml pokeweed mitogen (Sigma-Aldrich) in a 96 well plate (200µl total volume per well). IL-10 concentrations in the supernatants were measured with the Luminex fluorescent bead-based assay (Luminex Corp., http://www.luminexcorp.com/) using anti-equine IL-10 antibodies (clone 492-2, 165-2 (Wagner et al. 2008), and analyzed with a Luminex IS 100 instrument (Luminex Corp., http://www.luminexcorp.com/) as previously described (Wagner and Freer 2009) at the Cornell University Animal Health Diagnostic Center. The lower limit of sensitivity

of the standard curve was 3.84pg/ml, and values below this concentration were indicated as zero.

Statistical Analysis

The Shapiro-Wilk normality test performed with Graphpad Prism version 6.0f (GraphPad Software) showed that most of the data was not normally distributed, and therefore the appropriate non-parametric tests were performed. The Wilcoxon-Mann-Whitney Rank Sum test was used for two-way comparisons and Kruskal-Wallis Rank Sum test was used for three-way comparisons with KaleidaGraph (Synergy Software, Reading, PA). Data comparing $CD5^{hi}$ and $CD5^{lo}$ B cells from the same horse were treated as paired, and data between horses as unpaired, except for IGL nucleotide percent identity and CDR3 length, which were treated as unpaired. Three-way comparisons were treated as unpaired. IGLV segment usage was assessed by Chi^2 analysis (Graphpad Prism version 6.0f). IGLV4-66 versus IGLV8-24 utilization for the foal, and IGLV8-122 versus IGLV8-128 for the adult horse were analyzed with Fishers Exact Test. A p-value ≤ 0.10 was considered significant.

Results

Distribution of $CD5^{hi}$ B cells during development

I first investigated the distribution of $CD5^{hi}$ B cells in different phases of development to learn if these cells were more abundant early in ontogeny as described for murine and human B1 cells. Multicolor flow cytometric analysis was used to measure the percentage of $CD5^{hi}$ B cells in 100 DG-fetal liver isolated leukocytes, and PBL of 3-

to 42-day-old foals and adult horses (Figure 4.1). The median percentage of $CD5^{hi}$ B cells was the greatest in the fetal liver and foal leukocytes at days 21-28 of life (20-21%), and the lowest in the adult horse leukocytes (14%), with statistically significant differences, between the fetal liver and adult horse (p-value = 0.09), and the 21-day-old foal and adult horse (p-value = 0.02) (Figure 4.1). Overall, CD5 expression in $CD19^{+}CD3^{-}$ B cells was dimmer than in $CD3^{+}$ T cells, in agreement with a study in mice (Manohar et al. 1982). In the adult, 93.4-98.1% of $CD5^{+}$ lymphocytes were $CD3^{+}CD19^{-}$ T cells with $CD5^{hi}$ B cells accounting for 0.2-3.2% of $CD5^{+}$ lymphocytes; the remaining were negative for CD19 and CD3 or excluded as potential T:B cell doublets.

Distribution of $CD5^{hi}$ B cells in different lymphoid tissues

The frequency of murine B1 cells varies between different lymphoid tissues. To test for variation of the distribution of $CD5^{hi}$ B cells in different lymphoid tissues in the horse, multicolor flow cytometric analysis was used to measure the percentage of $CD5^{hi}$ B cells in the peritoneal cavity, spleen, and bone marrow samples. A statistically significant greater (p-value = 0.03) median percentage of B cells were $CD5^{hi}$ in the peritoneal cavity (26%) when compared to PBL, similar in the bone marrow (10%, p-value = 0.11), and less in spleen (7%, p-value = 0.008) (Figure 4.2). Statistically significant different median percentages of $CD5^{hi}$ B cells were also observed for pairwise comparisons between the peritoneal cavity and either bone marrow or spleen (p-values = 0.004), and between bone marrow and spleen (p-value = 0.06) samples (Figure 4.2).

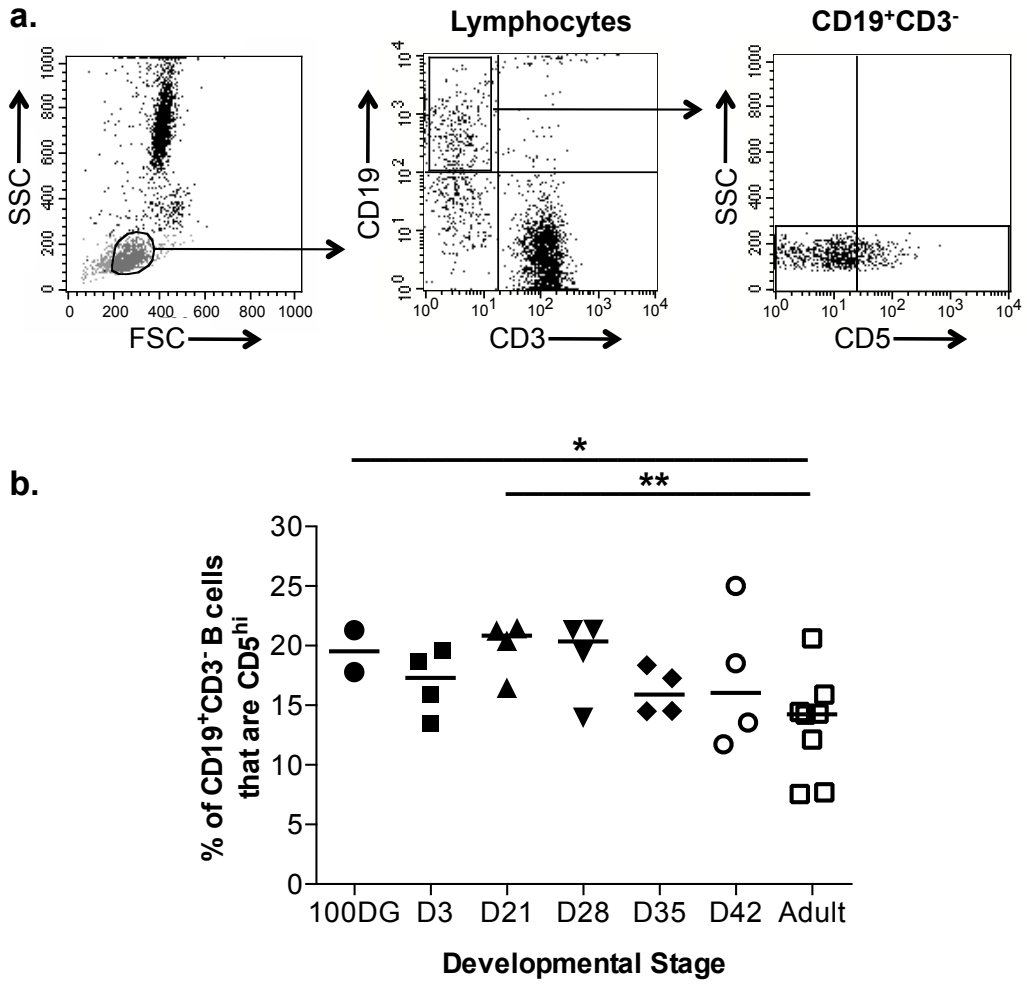


Figure 4.1 Frequency of $CD5^{hi}$ B cells during development.
(a) Representative dot plots showing the gating strategy to measure the frequency of $CD5^{hi}$ B cells. Cells were first gated on light scattering characteristics for lymphocytes, then B cells ($CD19^{+}CD3^{-}$). (b) The frequency of $CD5^{hi}$ B cells was measured in isolated fetal liver leukocytes (FLL) at 100 DG, peripheral blood leukocytes (PBL) in D3 to D42 foals, and PBL of adult horses, showing that these cells represent a greater proportion of B cells early in life. *p-value ≤ 0.10, **p-value ≤ 0.05

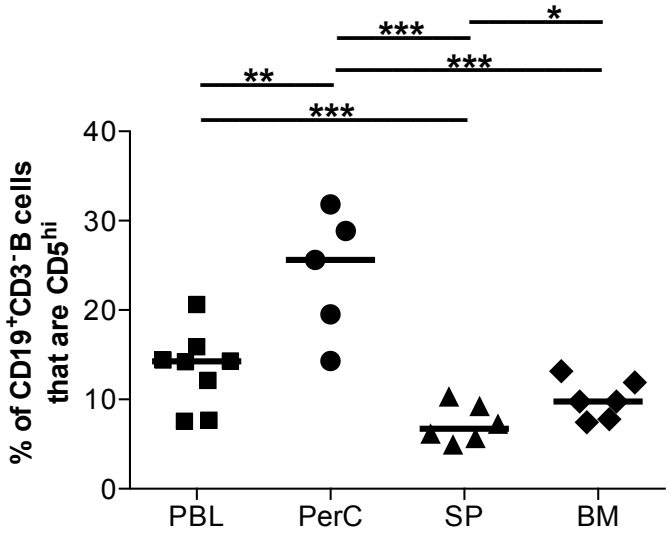


Figure 4.2 Frequency of $CD5^{hi}$ B cells in different lymphoid tissues.
The frequency of $CD5^{hi}$ B cells was measured in the peritoneal cavity (PerC), spleen (SP), and bone marrow (BM) of adult horses. The frequency of $CD5^{hi}$ B lymphocytes measured in adult horse PBL in figure 4.1 was included to facilitate comparisons between tissues. $CD5^{hi}$ B cells represented the greatest proportion of B cells in the peritoneal cavity, followed by PBL, bone marrow, and spleen.
*p-value ≤ 0.10, **p-value ≤ 0.05, ***p-value ≤ 0.01

Expression of developmental and B cell surface molecules in CD5^{hi} B cells

The co-expression of CD21, CD2, IgM, and MHC class II in CD5^{hi} or CD5^{lo} B cells was measured using multicolor flow cytometric analysis in 3- to 42-day-old foal and adult horse PBL (Figure 4.3). CD21 is a complement receptor (CR2) and part of the B cell co-receptor that promotes B cell activation, proliferation, and survival (Chen et al. 2000, Molnar et al. 2008). Greater (p-values ≤ 0.02) percentages of foal CD5^{hi} (median range 79-89%) and CD5^{lo} B cells (median range 64-88%) were CD21^{+} when compared to adult horse CD5^{hi} (median 48%) and CD5^{lo} B cells (median 41%), respectively, for all time points measured. Within the adult horse PBL samples, a statistically significant greater (p-value = 0.02) percentage of CD5^{hi} B cells were CD21^{+}; the geometric mean fluorescence intensity (MFI) for CD21 expression, however, was similar (p-value = 0.5) between adult horse CD5^{hi} and CD5^{lo} B cells (Figure 4.3b and 4.3c).

CD2 functions in cell adhesion and activation (Moingeon et al. 1989) and has traditionally been considered a T cell marker in the horse. CD2 expression on B cells increased with development, as greater (p-values ≤ 0.05) percentage of adult horse CD5^{hi} (median 94%) and CD5^{lo} B cells (median 84%) expressed CD2 compared to 3-day-old foal CD5^{hi} (median 81%) and CD5^{lo} B cells (median 74%). Within the adult horse PBL samples, the percentage of CD5^{hi} B cells that co-expressed CD2 and the MFI of CD2 expression was greater (p-values ≤ 0.02) than for CD5^{lo} B cells (Figure 4.3b and 4.3c).

The percentage of CD5^{hi} and CD5^{lo} B cells that co-expressed IgM was similar (p-value ≥ 0.2) between the 3-day-old foal (medians for CD5^{hi} 49%, CD5^{lo} 60%) and the

Figure 4.3 Co-expression of developmental and B cell markers by $CD5^{hi}$ and $CD5^{lo}$ B cells in the peripheral blood. (a) Representative dot plots for the expression of CD21, CD2, IgM, and MHC class II in $CD5^{hi}$ and $CD5^{lo}$ B cells. (b) The frequency of $CD5^{hi}$ (filled squares) and $CD5^{lo}$ B cells (open squares) co-expressing CD21, CD2, and IgM was measured in PBL of D3 to D42 foals, and adult horses. The frequency of $CD5^{hi}$ and $CD5^{lo}$ B cells and $CD3^{+}$ T cells (open triangles) co-expressing MHC class II was measured in 100 DG fetal liver leukocytes, and PBL of foal and adult horses. (c) The geometric mean fluorescent intensity (MFI) of CD21, CD2, IgM, and MHC class II in $CD5^{hi}$ and $CD5^{lo}$ in adult horse PBL. *p-value ≤ 0.10, **p-value ≤ 0.05, ***p-value ≤ 0.01

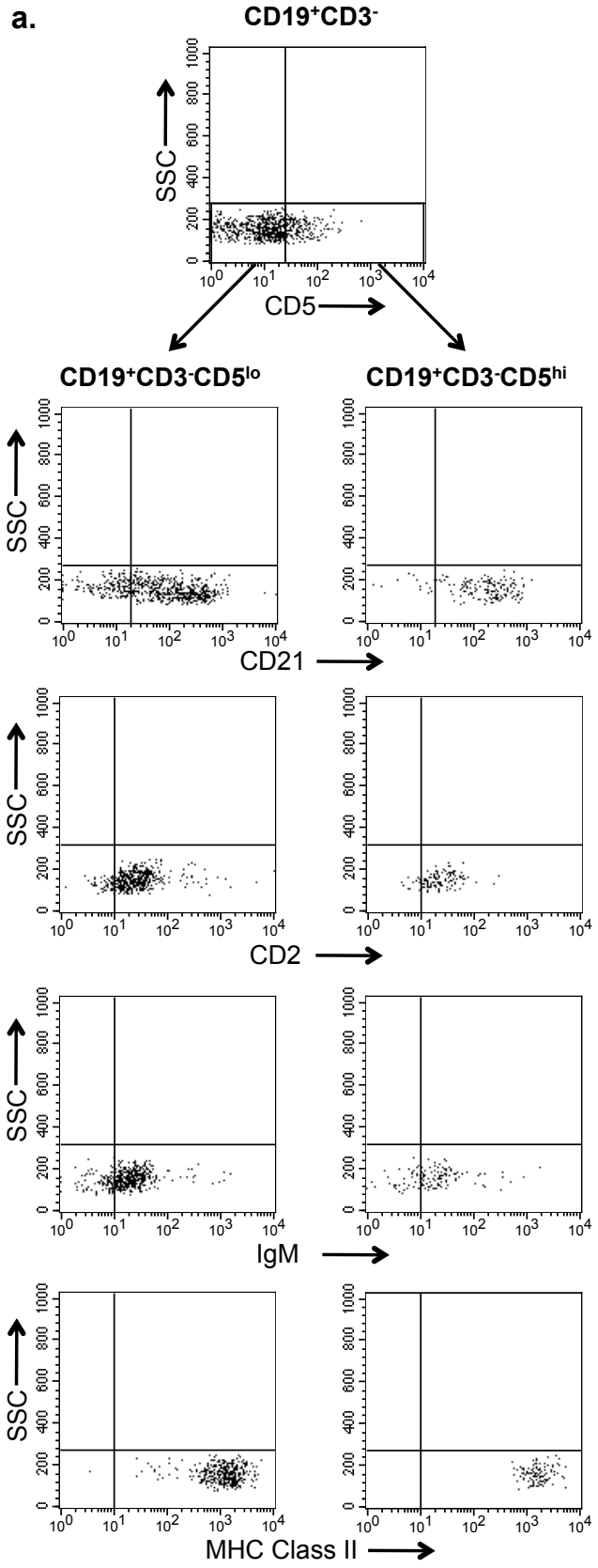
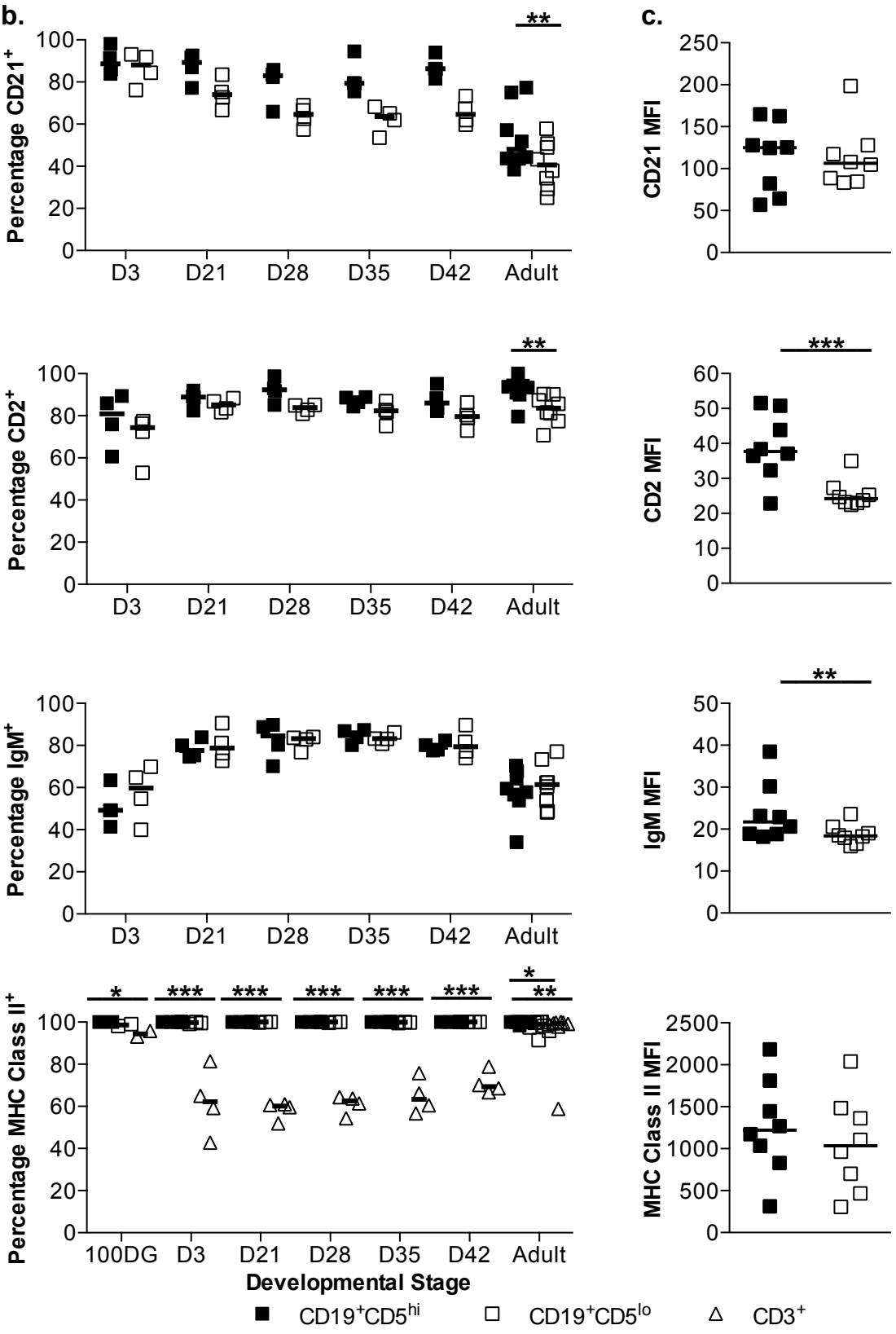

a.
CD19+CD3-
SSC
CD5
CD19+CD3-CD5lo
CD19+CD3-CD5hi
CD21
CD2
IgM
MHC Class II

Figure 4.3 Continued



adult horse (medians for $CD5^{hi}$ 59%, $CD5^{lo}$ 61%); in contrast, a greater (p-values ≤ 0.03) percentage of $CD5^{hi}$ (median range 78-85%) and $CD5^{lo}$ B cells (median range 79-83%) in the 21-, 28-, 35-, and 42-day-old foals expressed IgM compared to adult horse $CD5^{hi}$ and $CD5^{lo}$ B cells, respectively. Similar percentage of $CD5^{hi}$ and $CD5^{lo}$ B cells co-expressed IgM at each developmental stage (p-values ≥ 0.3) (Figure 4.3b). However, the MFI of IgM expression in adult horse $CD5^{hi}$ was greater (p-value = 0.05) than that of $CD5^{lo}$ B cells (Figure 4.3c).

Greater than 98% (medians ≥ 99) of $CD5^{hi}$ and $CD5^{lo}$ B cells in the fetal liver and foal, and 92% (medians ≥ 99) in the adult horse expressed MHC class II. In contrast, median percentage of $CD3^{+}$ T cells that expressed MHC class II in the 3 to 42-day-old foals ranged from 60-69%, while in the adult horse a median of 99% of $CD3^{+}$ T cells expressed MHC class II (Figure 4.3b). Surprisingly, 94% of the $CD3^{+}$ T cells from the two equine fetal livers expressed MHC class II. Statistically significant differences were observed for three way comparisons with $CD5^{hi}$ B cells, $CD5^{lo}$ B cells, and $CD3^{+}$ T cells in the fetus (p-value = 0.10), foal (p-values ≤ 0.01), and adult horse (p-value = 0.04) (Figure 4.3b). In the adult horse, the median percentage of $CD5^{hi}$ (100%) expressing MHC class II molecule was comparable to $CD5^{lo}$ B cells (99%), though this difference was statistically significant (p-value = 0.06) (Figure 4.3b); no difference (p-values ≥ 0.4) in the percentage of $CD5^{hi}$ and $CD5^{lo}$ B cells expressing MHC class II was observed at other developmental stages. MFI for MHC class II expression was similar (p-value = 0.15) in adult horse $CD5^{hi}$ and $CD5^{lo}$ B cells (Figure 4.3c).

Expression of B cell molecules in CD5^{hi} B lymphocytes of different lymphoid tissues

The co-expression of CD21, CD2, or IgM in CD5^{hi} and CD5^{lo} B cells of spleen and bone marrow samples was also measured with multicolor flow cytometric analysis (Figure 4.4). The co-expression of the B cell markers was not measured in peritoneal leukocytes because insufficient numbers of CD5^{hi} B cells were available for analysis using my gating strategy. Comparisons for the percentage of cells co-expressing and the MFI of expression for each marker were made between CD5^{hi} B cells between tissues, CD5^{lo} B cells between tissues, and CD5^{hi} and CD5^{lo} B cells within each tissue. The greatest percentage of CD5^{hi} and CD5^{lo} B cells that co-expressed CD21 (medians 94 and 92%, respectively) were found in the spleen, followed by the bone marrow (medians 71 and 77%, respectively), and PBL (medians 48 and 41%, respectively) (p-values $\leq$ 0.05 for all pairwise comparisons). Similarly, the MFI of CD21 expression in CD5^{hi} and CD5^{lo} B cells in the spleen was greater (p-values $\leq$ 0.009) than in CD5^{hi} and CD5^{lo} B cells, respectively, in both the PBL and bone marrow (Table 4.2). No statistical difference was observed in the percentage of CD5^{hi} and CD5^{lo} B cells co-expressing CD21 or the MFI of CD21 expression within the bone marrow (p-values $\geq$ 0.2) or spleen (p-values $\geq$ 0.4) (Figure 4.4, Table 4.2).

A similar (p-value $\geq$ 0.2) percentage of CD5^{hi} and CD5^{lo} B cells in the spleen co-expressed CD2 (medians 86 and 85%, respectively) with similar (p-values $\geq$ 0.4) MFI of CD2 expression, when compared with CD5^{hi} and CD5^{lo} B cells in PBL (Table 4.2). Fewer (p-values $\leq$ 0.05) CD5^{hi} and CD5^{lo} B cells in the bone marrow co-expressed CD2 (medians 79 and 64%, respectively) when compared to CD5^{hi} and CD5^{lo} B cells in the PBL and spleen. While the MFI of CD2 expression in bone marrow CD5^{hi} B cells was

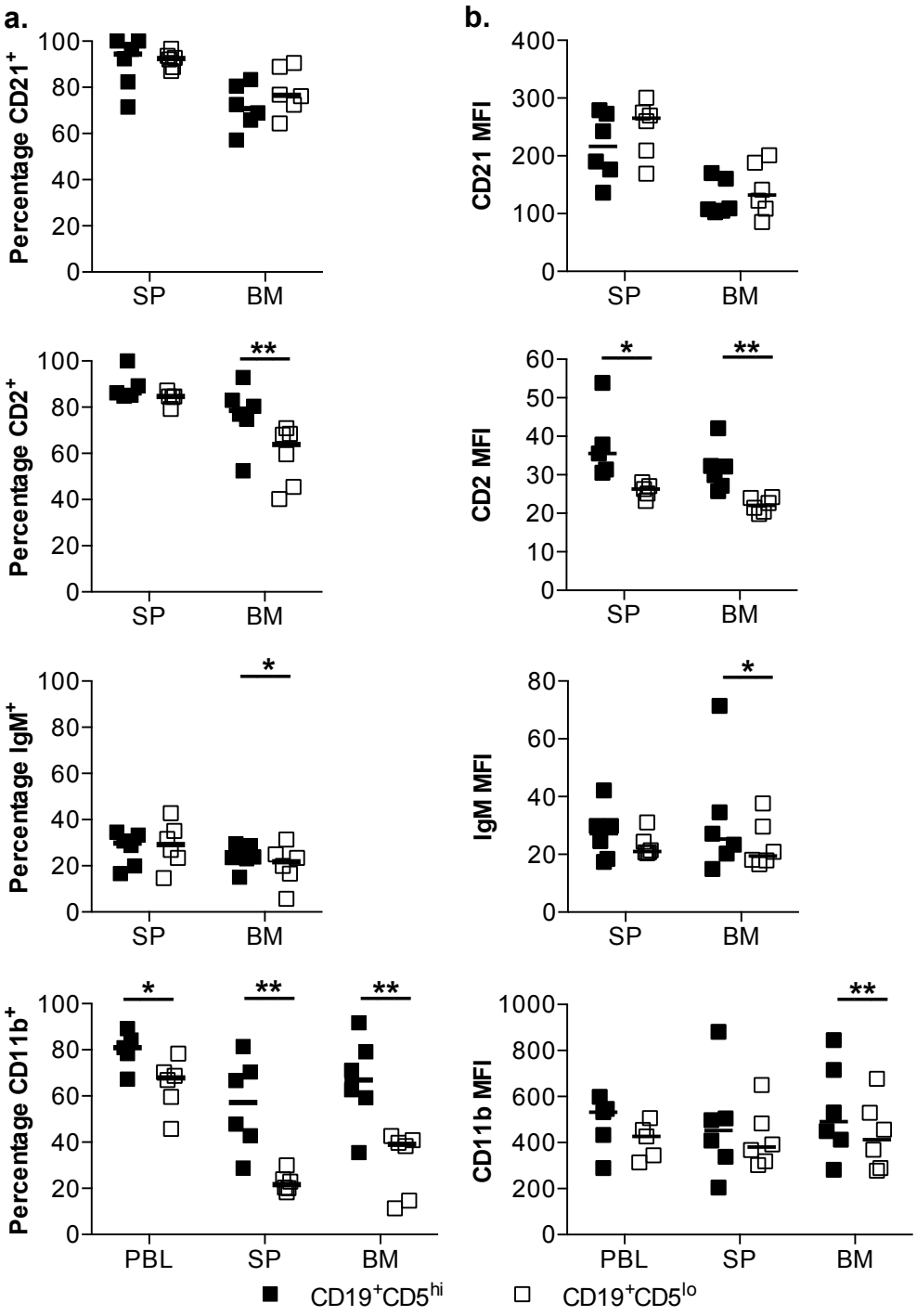


Figure 4.4 Co-expression of B cell markers in $CD5^{hi}$ and $CD5^{lo}$ B cells in different lymphoid tissues of adult horses. (a) The frequency of $CD5^{hi}$ (filled squares) and $CD5^{lo}$ B cells (open squares) co-expressing CD21, CD2, and IgM was measured in the spleen (SP) and bone marrow (BM). The frequency of $CD5^{hi}$ and $CD5^{lo}$ B cells co-expressing CD11b was measured in adult horse PBL, SP, and BM. (b) The geometric mean fluorescent intensity (MFI) of CD21, CD2, IgM, and CD11b in $CD5^{hi}$ and $CD5^{lo}$ in adult horse tissues. *p-value ≤ 0.10, **p-value ≤ 0.05

Table 4.2 Summary of the co-expression of B cell markers in $CD5^{hi}$ and $CD5^{lo}$ B cells

Marker	Tissue	B1 $CD19^{+}CD5^{hi}$	B2 $CD19^{+}CD5^{lo}$
CD2	PBL	++****	+***
	SP	++***	+***
	BM	++***	+[a]**
CD11b	PBL	++***	++**
	SP	++**	++
	BM	++**	++[b]*
CD21	PBL	+*	+*
	SP	++****	++****
	BM	+**	+***
IgM	PBL	++**	+**
	SP	++*	+[c]*
	BM	++	+
MHC II	PBL	++****	++****
	SP	ND	ND
	BM	ND	ND

++ = higher, + = lower relative geometric MFI of each marker for comparisons between $CD5^{hi}$ and $CD5^{lo}$ B cells within each tissue, $CD5^{hi}$ B cells between tissues, and $CD5^{lo}$ B cells between tissues, with PBL = peripheral blood leukocytes, SP = spleen, BM = bone marrow. The median percentage of $CD5^{hi}$ or $CD5^{lo}$ B cells positive for the marker is indicated: **** >90%, ***76-90%, **51-75%, *25-50%, or no star = <25%. ND indicates not determined.

a. Expression was lower than both $CD5^{hi}$ B cells within the BM and $CD5^{lo}$ B cells in other tissues

b. Expression was lower than $CD5^{hi}$ B cells within the BM but the same as $CD5^{lo}$ B cells in other tissues

c. Expression was the same as $CD5^{hi}$ B cells within the SP and $CD5^{lo}$ B cells in the BM, but less than $CD5^{lo}$ B cells in PBL

similar (p-values ≥ 0.11) to $CD5^{hi}$ B cells in the spleen and PBL, the MFI of CD2 expression in bone marrow $CD5^{lo}$ B cells was less (p-values ≤ 0.06) than $CD5^{lo}$ B cells from the spleen and PBL (Table 4.2). A greater (p-value = 0.03) percentage of $CD5^{hi}$ B cells co-expressed CD2 than $CD5^{lo}$ B cells within the bone marrow, while a similar (p-value = 0.2) percentage was measured within the spleen (Figure 4.4a). The MFI of CD2 expression was greater (p-values ≤ 0.06) in $CD5^{hi}$ B cells than in $CD5^{lo}$ B cells both within the bone marrow and within the spleen (Figure 4.4b and Table 4.2).

Fewer (p-values ≤ 0.001) $CD5^{hi}$ and $CD5^{lo}$ B cells in the spleen (medians 30 and 29%, respectively) and bone marrow (medians 24 and 22%, respectively) co-expressed IgM than in $CD5^{hi}$ and $CD5^{lo}$ B cells in the PBL. The MFI of IgM expression in $CD5^{hi}$ B cells was similar (p-values ≥ 0.5) for all pairwise comparisons between $CD5^{hi}$ B cells in the PBL, bone marrow, and spleen. The MFI of IgM in $CD5^{lo}$ B cells from the bone marrow was also similar (p-value ≥ 0.4) to $CD5^{lo}$ B cells in the PBL and spleen, though $CD5^{lo}$ B cells in the spleen had higher (p-value = 0.01) MFI of IgM expression than $CD5^{lo}$ B cells in the PBL. A greater (p-value = 0.09) percentage of $CD5^{hi}$ B cells co-expressed IgM with higher (p-value = 0.06) MFI than $CD5^{lo}$ B cells within the bone marrow, while no difference (p-values = 0.6) in IgM expression were observed between $CD5^{hi}$ and $CD5^{lo}$ B cells within the spleen (Figure 4.4 and Table 4.2).

The expression of CD11b, an integrin that is expressed in macrophages and B1 cells was also measured in $CD5^{hi}$ or $CD5^{lo}$ B cells of adult horse PBL, spleen, and bone marrow. A greater (p-values ≤ 0.06) percentage of $CD5^{hi}$ B cells co-expressed CD11b than $CD5^{lo}$ B cells within in the PBL (medians 81 and 67%, respectively), spleen (medians 57 and 22%, respectively), and bone marrow (medians 67 and 39%,

respectively) (Figure 4.4a). The MFI of CD11b expression was similar (p-values ≥ 0.13) in $CD5^{hi}$ and $CD5^{lo}$ B cells within the PBL and spleen, but greater (p-value = 0.03) in $CD5^{hi}$ than $CD5^{lo}$ B cells within the bone marrow (Figure 4.4b and Table 4.2). A similar (p-values ≥ 0.2) percentage of $CD5^{hi}$ B cells co-expressed CD11b in the bone marrow compared to $CD5^{hi}$ B cells in PBL and spleen, while a smaller (p-value = 0.05) percentage of $CD5^{hi}$ B cells in the spleen co-expressed CD11b compared to $CD5^{hi}$ B cells in PBL. A smaller (p-values ≤ 0.004) percentage of $CD5^{lo}$ B cells in the bone marrow and spleen co-expressed CD11b than in PBL. The MFI of CD11b expression was similar (p-values ≥ 0.5) in $CD5^{hi}$ and $CD5^{lo}$ B cells for all pairwise comparisons between tissues (Table 4.2).

mRNA expression of B1 and B2 signature genes

In addition to describing the phenotype of $CD5^{hi}$ B cells with surface molecule expression, I also wanted to test whether $CD5^{hi}$ and $CD5^{lo}$ B cells had distinct gene expression profiles. Since the B cells were sorted directly into RNA lysis buffer (Figure 4.5a) the mRNA expression of CD5 was measured to confirm that there was enrichment for $CD5^{hi}$ and $CD5^{lo}$ populations. As expected, B cells sorted for high surface protein expression of CD5 had a higher (p-value = 0.06) relative expression of CD5 at the mRNA level when compared to B cells that had low to no protein expression (Figure 4.5b). To calculate the fold difference in expression between $CD5^{hi}$ and $CD5^{lo}$ B cells, 2^-ΔΔCt was calculated with the CD5 mRNA expression calibrated to the $CD5^{lo}$ sample for each horse. $CD5^{hi}$ B cells had CD5 mRNA expression that ranged from 1.7 to 10.5

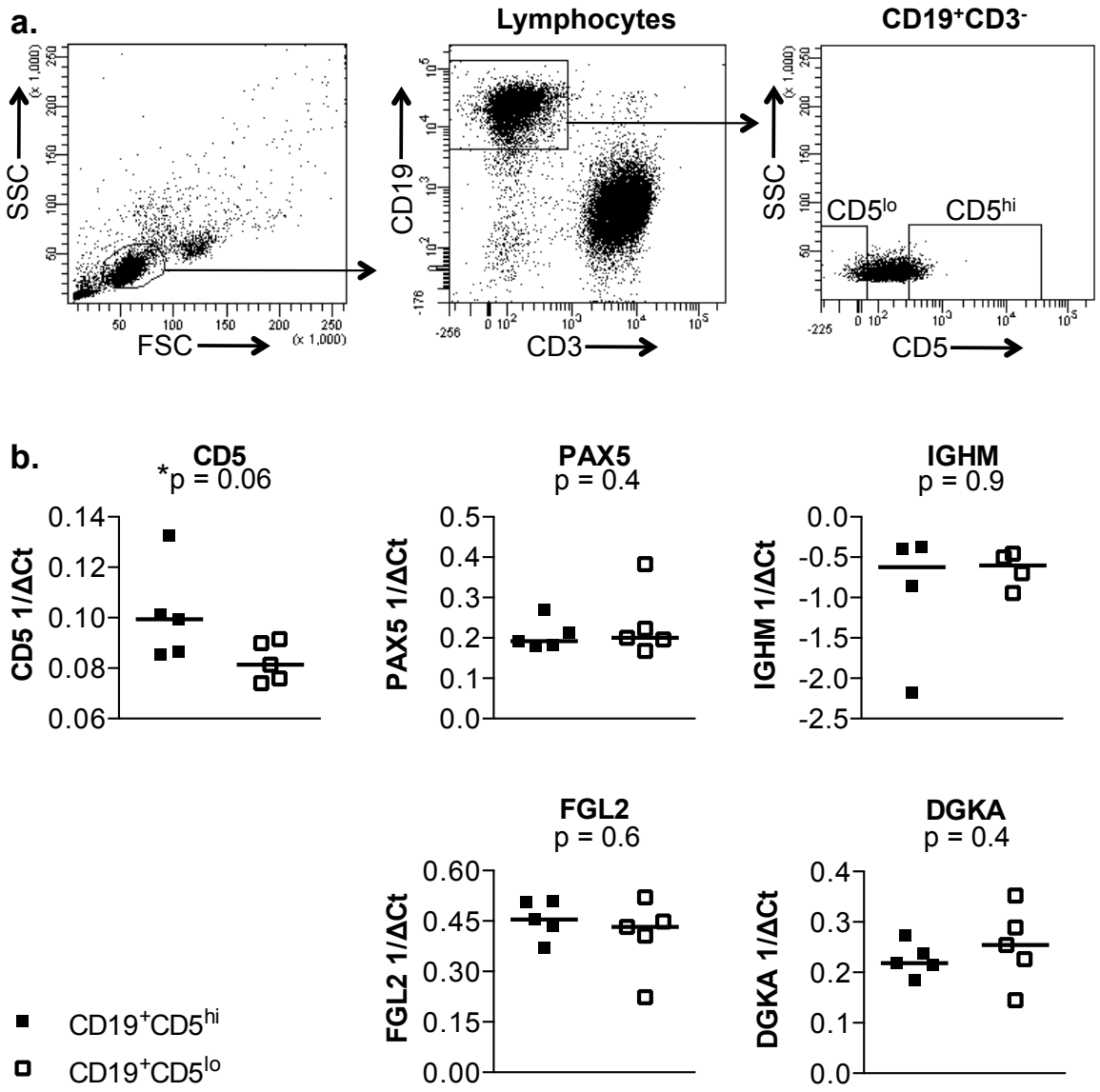


Figure 4.5 mRNA expression profile of $CD5^{hi}$ and $CD5^{lo}$ B cells.
(a) Representative dot plots showing gating strategy for FACS sorting of $CD19^{+}CD3^{-}CD5^{hi}$ and $CD19^{+}CD3^{-}CD5^{lo}$ B cells from adult horse PBL. Cells were first gated based on light scattering characteristics for lymphocytes; followed by FSC-H versus FSC-W doublet exclusion (not shown) and SSC-H versus SSC-W doublet exclusion (not shown); then B cells ($CD19^{+}CD3^{-}$ lymphocytes). Finally, $CD5^{hi}$ and $CD5^{lo}$ B cells (boxed areas) were sorted directly into RNA lysis buffer. (b) RNA was isolated from $CD19^{+}CD3^{-}CD5^{hi}$ (filled squares) and $CD19^{+}CD3^{-}CD5^{lo}$ B cells (open squares) from 5 adult horses (4 horses for IGHM) and the expression signature of genes for B1 or B2 cells was measured using real-time quantitative PCR. mRNA expression was normalized to the housekeeping gene B-ACTIN and expressed as the inverse of ΔCt.

fold higher than that in $CD5^{lo}$ B cells (median 3.5). This is comparable to the 2.5 and 2.8-fold difference of CD5 mRNA expression by B1 and B2 cells in humans and mice, respectively (Mabbott and Gray 2014). The mRNA expression of the remaining genes: PAX5, FGL2, DGKA, and IGHM were comparable (p-values ≥ 0.4) between $CD5^{hi}$ and $CD5^{lo}$ B cells (Figure 4.5b).

Diversity of the lambda light chain repertoire

The diversity of the lambda light chain repertoire of $CD5^{hi}$ and $CD5^{lo}$ B cells was examined as a molecular marker of function. The horse uses lambda light chains in more than 90% of circulating antibodies (Ford et al. 1994). Though murine B2 cells use lambda light chains in about 5% of immunoglobulins, peritoneal B1 cells have been shown to use lambda light chains in 20% of immunoglobulins (Hayakawa et al. 1986a). To test whether equine $CD5^{hi}$ and $CD5^{lo}$ B cells use similar ratios of lambda (IGL) and kappa (IGK) light chains, mRNA copy numbers of both immunoglobulin chains were measured using absolute quantitative RT-PCR. Higher mRNA copy numbers of IGL than IGK were observed, with similar (p-value = 0.8) median IGL/IGK ratios for $CD5^{hi}$ (74) and $CD5^{lo}$ B cells (82) (Figure 4.6a). I focused on measuring the diversity of the lambda light chain to learn if any differences in combinatorial diversity of the fetal repertoire previously observed (Tallmadge et al. 2014) could be attributed to biases in $CD5^{hi}$ B cells that are proportionally more frequent early in life. Twelve different IGLV segments were observed belonging to 3 different subgroups (Figures 4.6b and 4.7). Overall, IGLV8-128 and IGLV8-122 were the most frequently observed IGLV segments in this study. IGLV8-128 and IGLV8-122 are among the more frequently observed IGLV

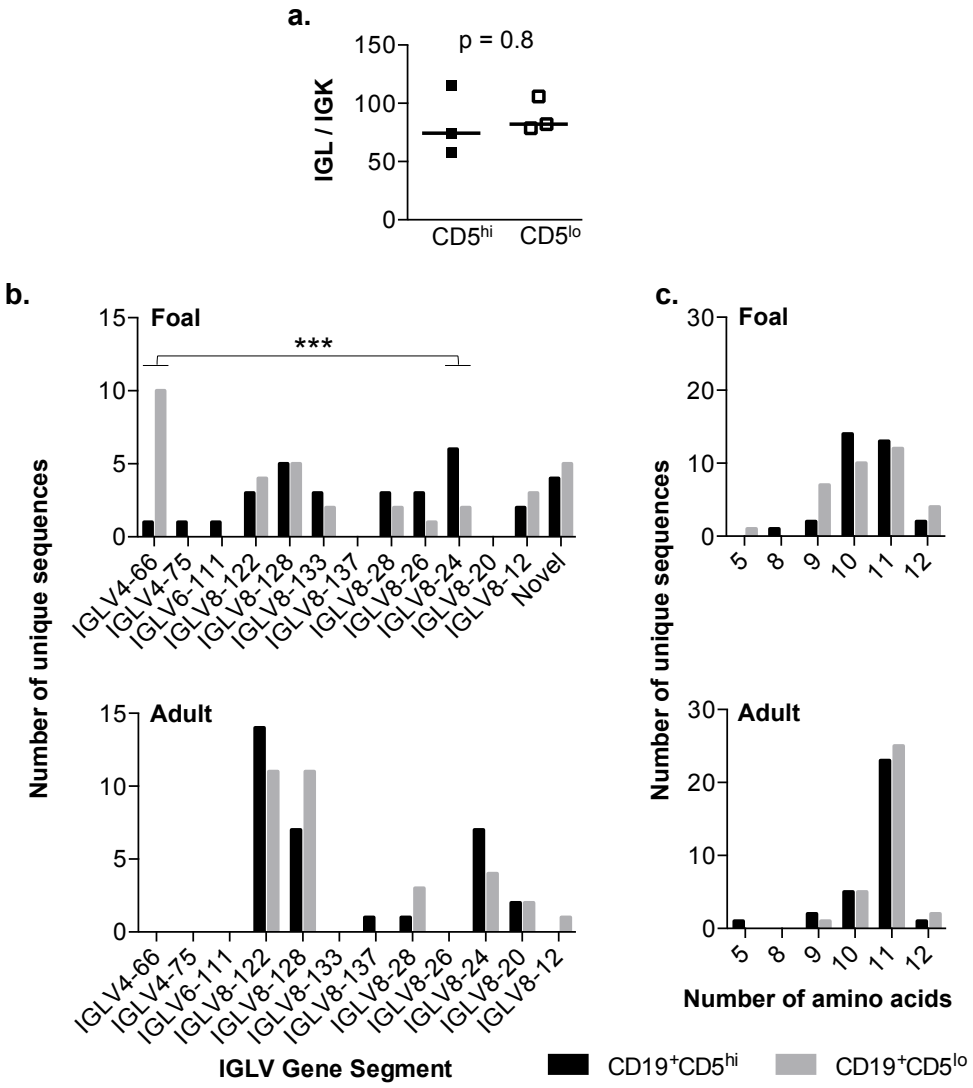
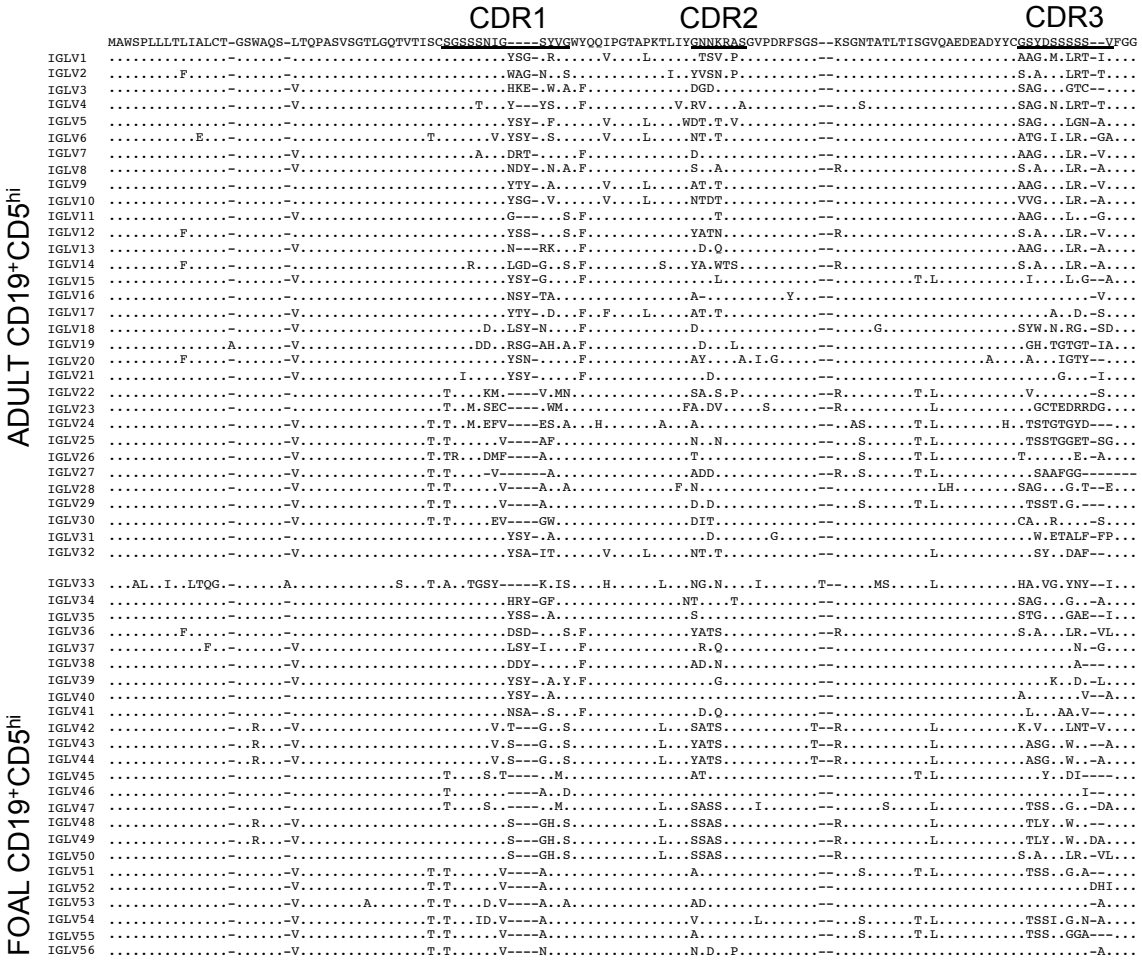


Figure 4.6 Diversity of immunoglobulin lambda light chain repertoire.
(a) Ratio of IGL/IGK mRNA transcripts measured with absolute quantitative RT-PCR in FACS-sorted $CD19^{+}CD3^{-}CD5^{hi}$ (filled squares) and $CD19^{+}CD3^{-}CD5^{lo}$ (open squares) peripheral blood B cells (n = 3 adult horses). Copy number of lambda transcripts were higher than the copy number of kappa transcripts, with similar IGL/IGK ratios for $CD5^{hi}$ and $CD5^{lo}$ B cells. (b) Immunoglobulin lambda light chain segment usage in foal and adult horse FACS-sorted $CD19^{+}CD3^{-}CD5^{hi}$ (black) and $CD19^{+}CD3^{-}CD5^{lo}$ (gray) peripheral blood B cells. The quantity of unique sequences utilizing each gene segment are shown. Significant differences (***p-values = 0.006) in usage of the two most common IGLV segments between $CD5^{hi}$ and $CD5^{lo}$ B cells in the foal (IGLV4-66, IGLV8-24) was observed, while no difference was found for the two most frequently observed IGLV segments in the adult horse (IGLV8-122, IGLV8-128, p-value = 0.4). (c) Distribution of CDR3 amino acid lengths for $CD19^{+}CD3^{-}CD5^{hi}$ (black) and $CD19^{+}CD3^{-}CD5^{lo}$ (gray) in the PBL of the foal and adult horse. No statistical differences (p-values ≥ 0.4) were measured between the median CDR3 lengths between $CD5^{hi}$ and $CD5^{lo}$ B cells in either the foal or adult horse.

Figure 4.7 Amino acid sequence alignment of immunoglobulin lambda light chain variable region in equine $CD5^{hi}$ and $CD5^{lo}$ B cells. Immunoglobulin lambda light chain nucleotide sequences and their respective translated amino acid sequences were obtained from equine foal and adult horse FACS-sorted $CD19^{+}CD3^{-}CD5^{hi}$ and $CD19^{+}CD3^{-}CD5^{lo}$ PBL. Alignment of the lambda chain variable region with amino acids identical to the consensus sequence are represented as dots, and residues different from the consensus noted. Gaps in the alignment are represented by dashes. The three CDR regions are indicated above the consensus sequence (Shimanuki et al. 2013, Sun et al. 2010). Sequences from each tissue are ordered based on the IGLV gene indicated to the right of each amino acid sequence.


CDR1
CDR2
CDR3
MAWSPLLLTLIALCT-GSWAQS-LTQPASVSGTLGQTVTISCSGSSSNIG----SYVGWYQQIPGTAPKTLIYGNNKRASGVPDRFSGS--KSGNTATLTISGVQAEDEADYYCGSYDSSSSS--VFGG IGLV
IGLV1 IGLV8-122
IGLV2 IGLV8-122
IGLV3 IGLV8-122
IGLV4 IGLV8-122
IGLV5 IGLV8-122
IGLV6 IGLV8-122
IGLV7 IGLV8-122*03
IGLV8 IGLV8-122*03
IGLV9 IGLV8-122*03
IGLV10 IGLV8-122*03
IGLV11 IGLV8-122*03
IGLV12 IGLV8-122*03
IGLV13 IGLV8-122*03
IGLV14 IGLV8-122*03
IGLV15 IGLV8-128
IGLV16 IGLV8-128
IGLV17 IGLV8-128
IGLV18 IGLV8-128
IGLV19 IGLV8-128
IGLV20 IGLV8-128
IGLV21 IGLV8-128
IGLV22 IGLV8-137
IGLV23 IGLV8-28
IGLV24 IGLV8-24
IGLV25 IGLV8-24
IGLV26 IGLV8-24
IGLV27 IGLV8-24
IGLV28 IGLV8-24
IGLV29 IGLV8-24
IGLV30 IGLV8-24
IGLV31 IGLV8-20
IGLV32 IGLV8-20
IGLV33 IGLV6-111
IGLV34 IGLV8-122*02
IGLV35 IGLV8-122*02
IGLV36 IGLV8-122*03
IGLV37 IGLV8-128
IGLV38 IGLV8-128
IGLV39 IGLV8-128
IGLV40 IGLV8-128
IGLV41 IGLV8-128
IGLV42 IGLV8-133
IGLV43 IGLV8-133
IGLV44 IGLV8-133
IGLV45 IGLV8-28
IGLV46 IGLV8-28
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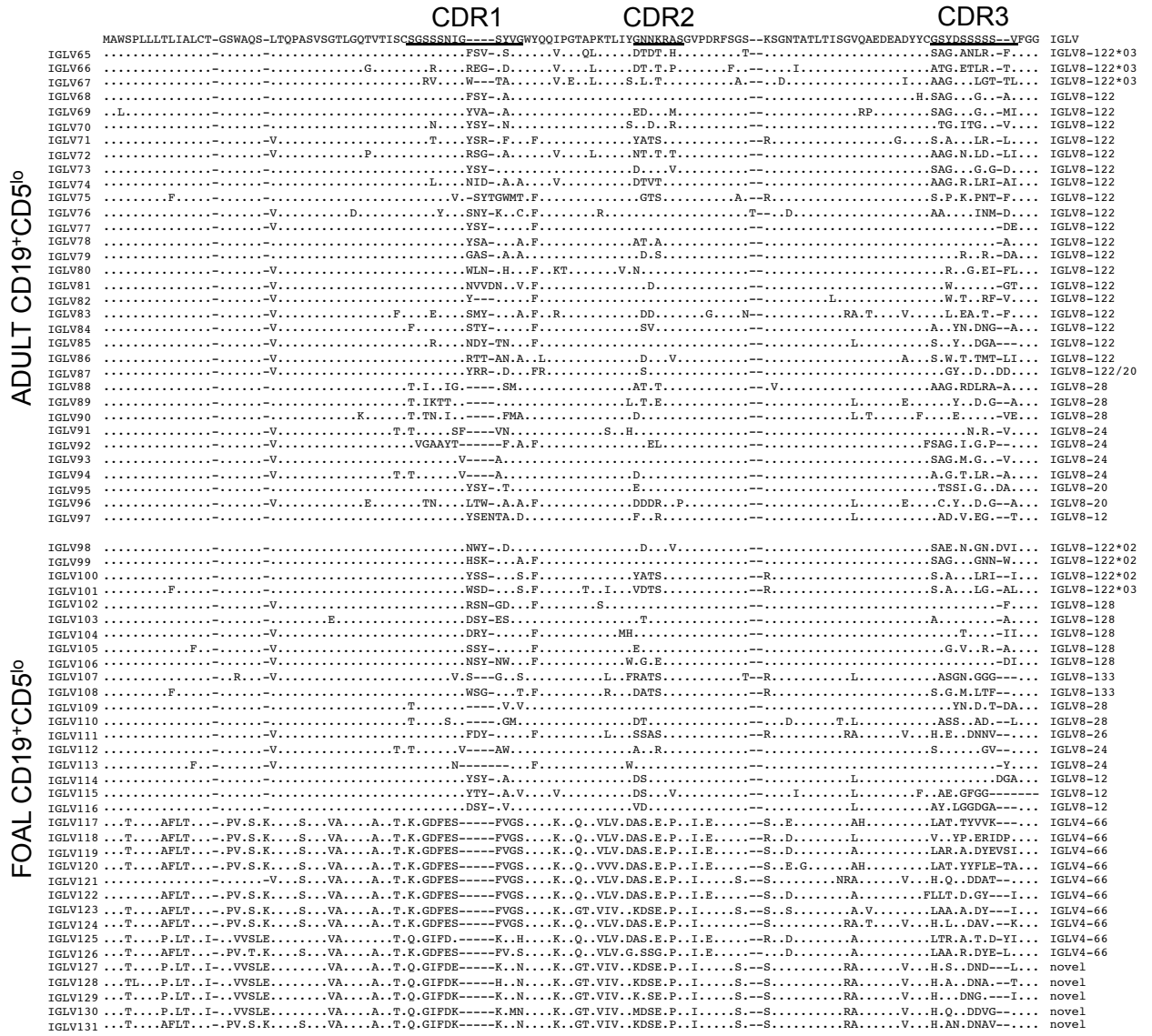
IGLV51 IGLV8-24
IGLV52 IGLV8-24
IGLV53 IGLV8-24
IGLV54 IGLV8-24
IGLV55 IGLV8-24
IGLV56 IGLV8-24
IGLV57 IGLV8-12
IGLV58 IGLV8-12
IGLV59 IGLV4-66
IGLV60 IGLV4-75
IGLV61 novel
IGLV62 novel
IGLV63 novel
IGLV64 novel
ADULT CD19+CD5hi
FOAL CD19+CD5hi

Figure 4.7 Continued



segments in other studies (Hara et al. 2012, Sun et al. 2010, Tallmadge et al. 2014). Nine IGLV segments expressed by foal CD5^{hi} (4 sequences) and CD5^{lo} (5 sequences) were designated as “novel”, since the best BLAST results comparing the expressed sequences to the equine EquCab2.0 genome were to unannotated sequences within the IGL locus. When these novel sequences were compared with the *Equus caballus* nr database using the BLAST tool, 7 sequences were found to be >96% similar to a novel sequence identified in a previous study (Tallmadge et al. 2014). One adult horse sequence (IGLV87) was equally similar to IGLV8-128 and IGLV8-20. Significant ($p < 0.0001$) differences in IGLV utilization were observed between the foal and adult horse, in contrast to a previous study in which no differences were observed between either the neonate (less than 1hr old) or foal (2 months of life) and the adult horse (Tallmadge et al. 2014). Therefore, foal and adult horse immunoglobulin sequences were analyzed separately. No differences (p-values ≥ 0.14) were observed for IGLV utilization between CD5^{hi} and CD5^{lo} B cells in the foal or adult horse when all annotated IGLV genes expressed in more than one sequence were compared. However, when the two most commonly used IGLV gene segments expressed in CD5^{hi} and CD5^{lo} B cells of the foal were compared (IGLV8-24 and IGLV4-66), a statistically significant difference (p-value = 0.006) in IGLV utilization was observed (Figure 4.6b). In the adult horse, there was no difference (p-value = 0.4) in the two most commonly used IGLV gene segments in CD5^{hi} and CD5^{lo} B cells (IGLV8-122 and IGLV8-128).

The next question was how different was the sequence diversity of expressed immunoglobulin sequences in CD5^{hi} and CD5^{lo} B cells. The germline alleles were cloned for the two most frequently observed IGLV genes in the dataset (IGLV8-122 and

IGLV8-128), and compared with the expressed sequences in $CD5^{hi}$ and $CD5^{lo}$ B cells from each donor. The adult horse had IGLV8-122 and IGLV8-122*03 alleles previously described (Sun et al. 2010, Tallmadge et al. 2014), and was homozygous for a novel IGLV8-128 allele named ILV8-128*04, which differs from the reference genome by one nucleotide within the intron sequence. The foal had previously described (Tallmadge et al. 2014) IGLV8-122*02 and IGLV8-122*03 alleles, and was also homozygous for the IGLV8-128*04 allele. For each individual, a second PCR reaction was performed to confirm the presence of only one IGLV8-128 allele. There was no statistically significant difference (p-value = 0.9) between nucleotide identity of IGLV gene segments expressed in $CD5^{hi}$ (median 95.3%, range 89.2-98.8, n=21 sequences) and $CD5^{lo}$ B cells (median 94.8%, range 88.3-99.7, n=22 sequences) of the adult horse PBL. In the foal, IGLV nucleotide identity to germline was also similar (p-value = 0.3) for $CD5^{hi}$ (median 97.4%, range 91.4-98.5, n=8 sequences) and $CD5^{lo}$ B cells (median 96.8%, range 91.1-98.3, n = 9 sequences).

Diversity of the complementary determining region 3 (CDR3), which comes in contact with antigen and contains the greatest degree of sequence diversity, is influenced by both combinatorial and junctional diversity. Increased CDR length has been associated with polyreactive antibodies (Ichiyoshi and Casali 1994); therefore, I wanted to test how different the CDR3 length would be in $CD5^{hi}$ and $CD5^{lo}$ B cells. The median CDR3 lengths were similar (p-values $\geq$ 0.4) for $CD5^{hi}$ (median 10 foal, 11 adult horse) and $CD5^{lo}$ B cells (median 10 foal, 11 adult horse) (Figure 4.6c).

BCR specificity for phosphorylcholine

Phosphorylcholine (PC) is the polar headgroup of the phospholipids phosphatidylcholine and sphingomyelin exposed on cellular membranes following oxidative damage or with phospholipid metabolism, respectively, and is expressed by different pathogens (Briles et al. 1982, Jenkins et al. 2009, Peters et al. 1999). B1 cells described in other species are enriched with BCRs that have specificity to PC (Griffin et al. 2011a, Lalor and Morahan 1990, Yammani and Haas 2013). A greater (p-value 0.008) percentage of $CD5^{hi}$ B cells (median 2.3%) in foal and adult horse PBL bound to the fluorescence-labeled PC than $CD5^{lo}$ B cells (median 1.1%). Foal $CD5^{hi}$ B cells more frequently (p-value = 0.03) bound to PC than $CD5^{hi}$ in adult horses, while the percentage of $CD5^{lo}$ B cells that bound to PC was similar in the foal and adult horse (p-value = 0.11) (Figure 4.8).

IL-10 secretion in vitro

B1 cells are known to secrete large amounts of the anti-inflammatory cytokine IL-10 (Griffin and Rothstein 2012b, Moore et al. 2001, O'Garra and Howard 1992). To compare IL-10 secretion between cell types, FACS-sorted $CD5^{hi}$ and $CD5^{lo}$ B cells from adult horse PBL were cultured for 3 days with medium alone or medium supplemented with pokeweed mitogen (PWM) (Figure 4.9). PWM has been previously been shown to stimulate IL-10 production in unsorted equine PBL (Flaminio and Antczak 2005, Wagner and Freer 2009). The magnitude of IL-10 secretion was variable between the three individuals. $CD5^{hi}$ B cells from 2 out of 3 horses increased IL-10 secretion with PWM stimulation (Figure 4.9b). Medium from cultured $CD5^{hi}$ B cells had greater IL-10

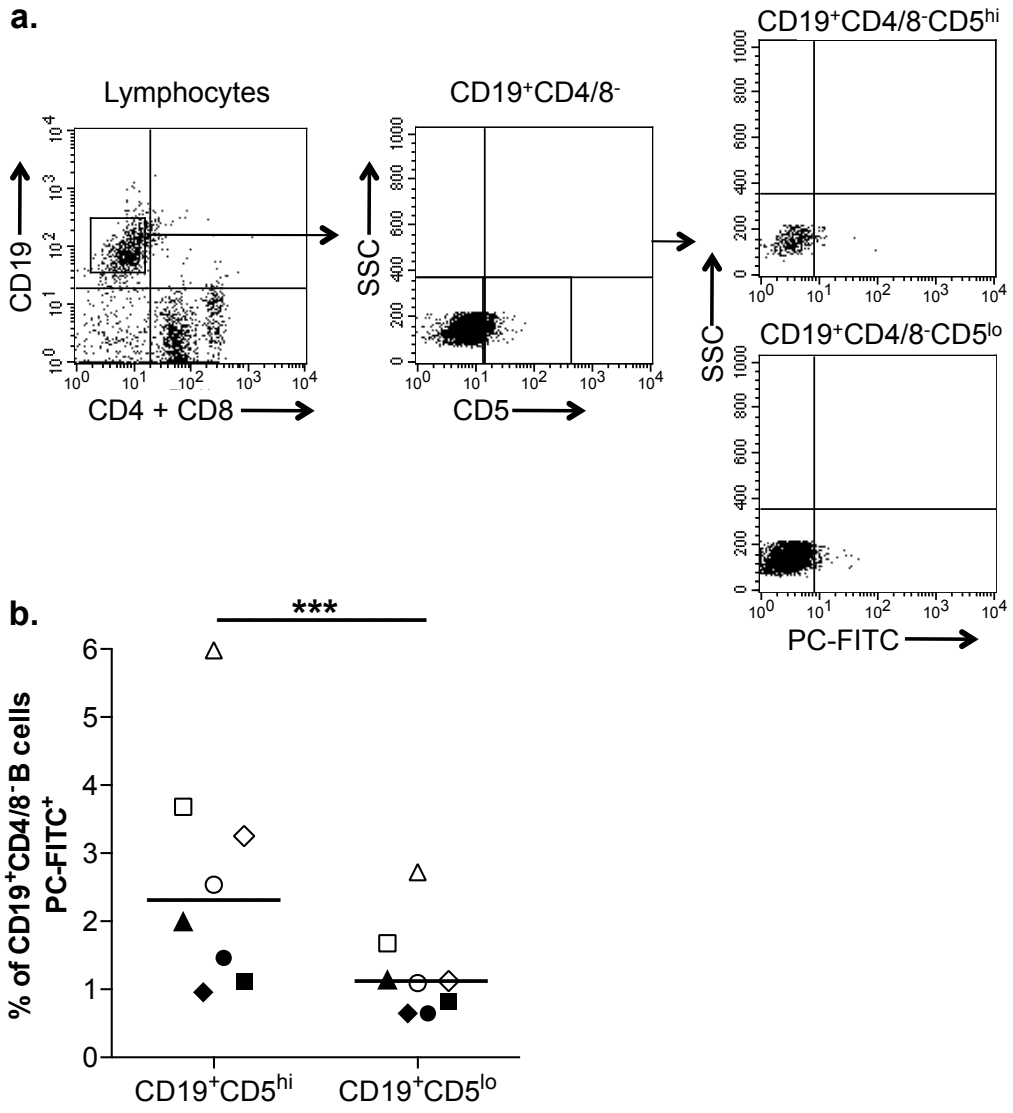


Figure 4.8 Frequency of BCR specificity for phosphorylcholine.
(a) Representative dot plots showing the gating strategy to measure the frequency of phosphorylcholine (PC)-FITC^{+}CD5^{hi} or PC-FITC^{+}CD5^{lo} B cells. Cells were first gated on on light scattering characteristics for lymphocytes, then B cells (CD19^{+}CD4^{-}CD8^{-}), then CD5^{hi} or CD5^{lo} B cells, and lastly PC-FITC. (b) Percentage of CD19^{+}CD4^{-}CD8^{-}CD5^{hi} and CD19^{+}CD4^{-}CD8^{-}CD5^{lo} B cells that are PC-FITC^{+}. Each horse is represented by a different symbol with filled symbols for adult horses (n=4) and open symbols for foals (n=4). Statistically significant more CD5^{hi} B cells bound to PC-FITC than CD5^{lo} B cells. ***p-value = 0.008

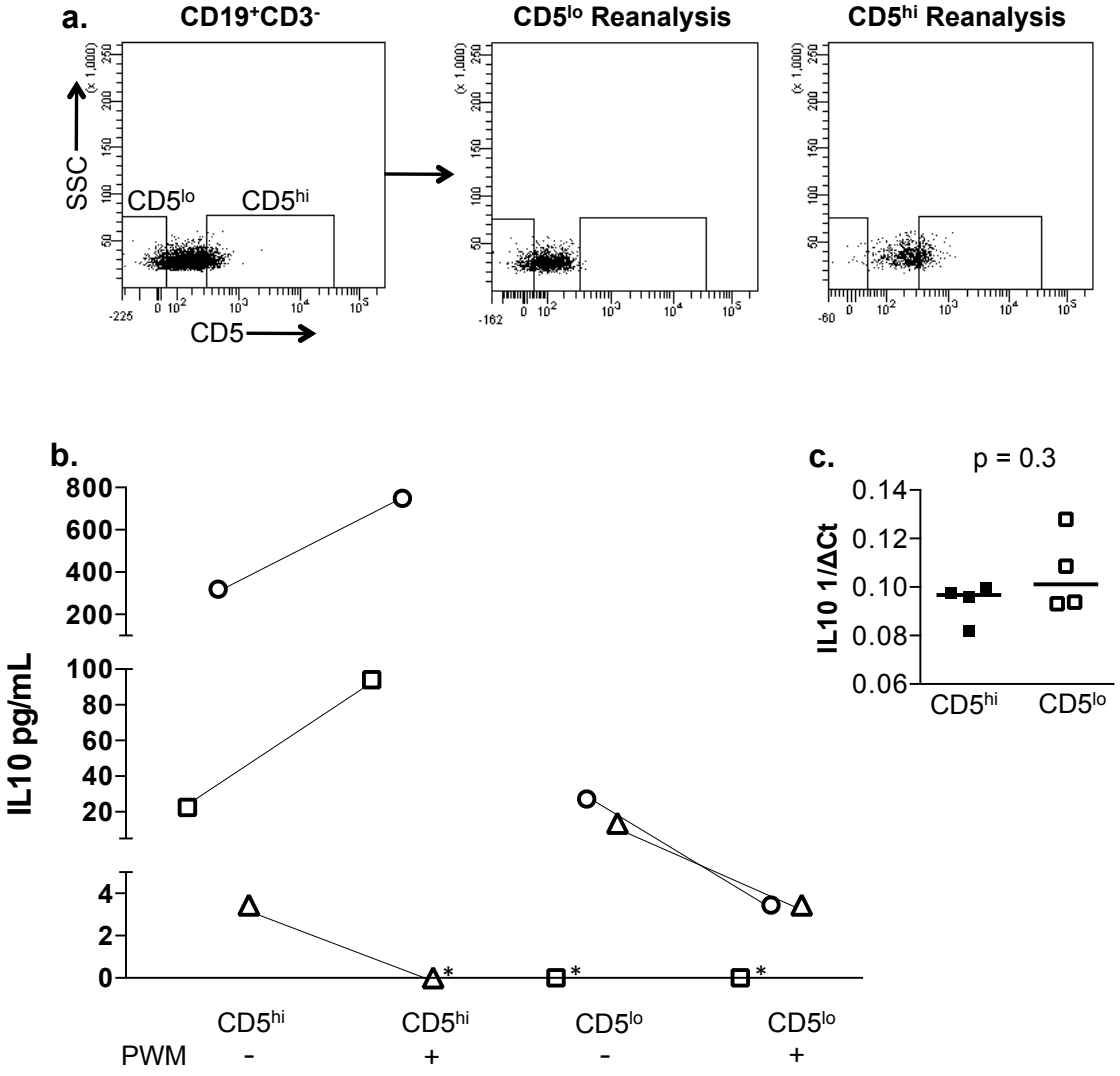


Figure 4.9 IL-10 secretion in vitro.
(a) Representative dot plots from FACS-sorted adult horse peripheral blood $CD19^{+}CD3^{-}CD5^{hi}$ or $CD19^{+}CD3^{-}CD5^{lo}$ B cells, with the $CD5^{hi}$ and $CD5^{lo}$ sorting gates shown. Reanalysis showed enrichment for $CD5^{hi}$ and $CD5^{lo}$ B cell populations. (b) IL-10 concentration measured with a Luminex assay in supernatants of $CD5^{hi}$ B cells (left) and $CD5^{lo}$ B cells (right) cultured for 3 days in medium alone or supplemented with pokeweed mitogen (PWM). Each horse is represented by a different symbol (n=3) showing that IL-10 production was variable between individuals. * Symbols were plotted with values of zero when the concentration was below the detectable limits of the assay; Comparison between $CD5^{hi}$ and $CD5^{lo}$ B cell populations for the IL-10 secretion showed no statistical significant difference (p-values = 0.5). (c) In a separate experiment, RNA was isolated from $CD19^{+}CD3^{-}CD5^{hi}$ (filled squares) and $CD19^{+}CD3^{-}CD5^{lo}$ B cells (open squares) from 4 adult horses and the mRNA expression for IL-10 was measured using real-time quantitative RT-PCR. mRNA expression was normalized to the housekeeping gene B-ACTIN and expressed as the inverse of ΔCt.

absolute concentration (medians 22.52 pg/ml media only, 94.15 pg/mL PWM stimulated) than $CD5^{lo}$ B cells (13.53 pg/mL media only, 3.45 pg/mL PWM) though this difference was not statistically significant (p-values = 0.5). Determining the IgM concentration in the same culture supernatants was also attempted with an equine specific IgM ELISA (Innovative Research, Novi, MI), though the concentrations were bellow the detectible limits of the assay (10ng/mL, however supernatants were diluted 1:20 to have the required volume for the assay). In addition, no quantitative difference (p-value = 0.3) in IL-10 mRNA expression was observed between FACS-sorted (without in vitro culture) $CD5^{hi}$ and $CD5^{lo}$ B cells from adult horse PBL (Figure 4.9c).

$CD5^{hi}$ B cells in horses with common variable immunodeficiency

Common variable immunodeficiency (CVID) is a late-onset primary humoral immunodeficiency in horses characterized by progressive loss of B cells in the primary and secondary lymphoid tissues, hypo- or agammaglobulinemia, and inability to respond to the tetanus toxoid protein vaccine, with consequent severe recurrent bacterial infections and death (Flaminio et al. 2009). Preliminary studies in our laboratory suggested that CVID-affected horses could maintain a population of B1 cells based on more sustainable serum IgA concentrations through time. Therefore, I performed flow cytometric analysis of the remaining peripheral blood B cells in a group of horses diagnosed with CVID, and found a greater (p-value = 0.01) median percentage of B cells were $CD5^{hi}$ (18%) when compared to healthy adult horses (14%) (Figure 4.10). CVID horses have a B lymphopenia and relative neutrophilia, and despite collecting up to 187,830 total events, in 2 out of the 7 horses as few as 16 events

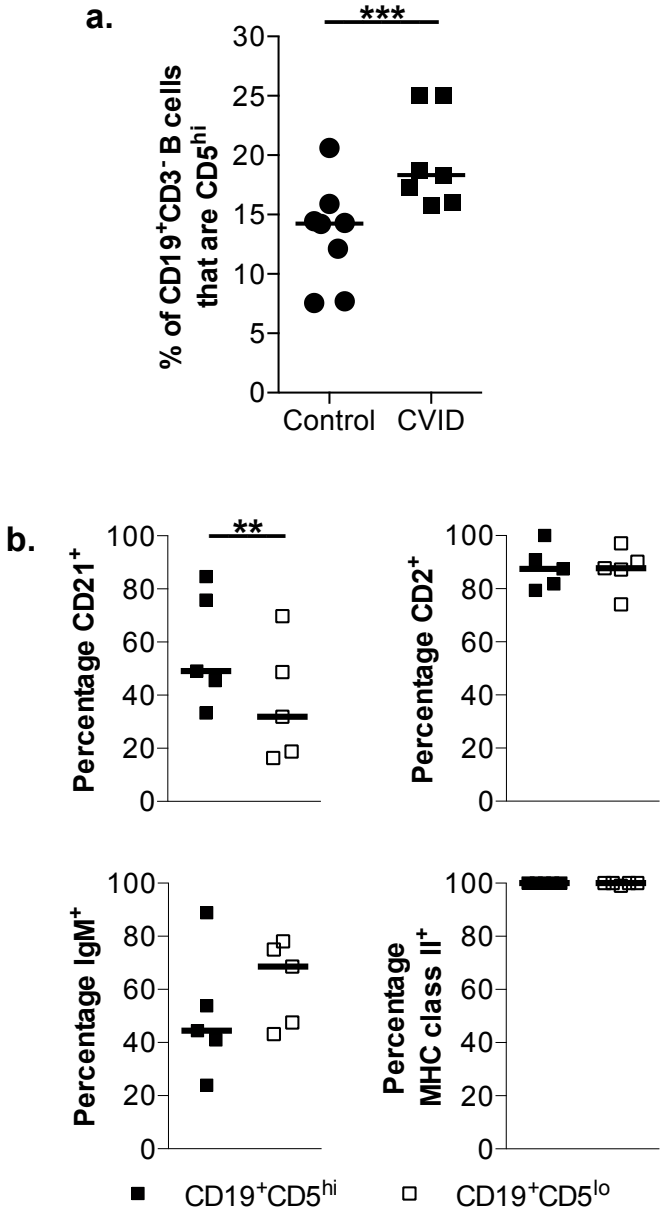


Figure 4.10 $CD5^{hi}$ B cells in horses with common variable immunodeficiency.
(a) Statistically significant greater proportion of remaining peripheral blood B cells in horses with CVID were $CD5^{hi}$ in comparison to healthy adult horses. The frequency of $CD5^{hi}$ B lymphocytes measured in adult horse PBL in figure 4.1 was included to facilitate the comparison. (b) Co-expression of CD21, CD2, IgM, and MHC class II in $CD5^{hi}$ and $CD5^{lo}$ B cells. Similar to healthy adult horses, a greater percentage of $CD5^{hi}$ B cells expressed CD21 in comparison to $CD5^{lo}$ B cells.
**p-value ≤ 0.05, ***p-value ≤ 0.01

were counted within the $CD19^{+}CD3^{-}$ B cell gate; samples from these two horses were not used to measure the co-expression of other B cell markers. A greater (p-value = 0.02) percentage of $CD5^{hi}$ compared to $CD5^{lo}$ B cells expressed CD21 (medians 49 and 32%, respectively) similar to healthy adult horses. The IgM expression in the B cell populations was variable, with no statistically significant difference (p-value = 0.2) in $CD5^{hi}$ (median 44%) or $CD5^{lo}$ B cells (median 69%) that were IgM positive. In contrast to healthy adult horses, there was no difference (p-values = 1) between $CD5^{hi}$ and $CD5^{lo}$ B cells that expressed CD2 (median 88% $CD5^{hi}$, 88% $CD5^{lo}$) or MHC Class II (median 100% $CD5^{hi}$, 100% $CD5^{lo}$). There were no statistically significant differences (p-values ≥ 0.13) between the median percentages of healthy adult and CVID-affected horse $CD5^{hi}$ B cells or $CD5^{lo}$ B cells expressing CD2, CD21, IgM, or MHCII.

Discussion

The studies herein show that a subset of equine B cells express higher levels of CD5, and these cells share some characteristics described for B1 cells in other species. Equine $CD5^{hi}$ B cells represent a greater proportion of B cells early in development in the fetal liver and peripheral blood of the foal. In the adult horse, $CD5^{hi}$ cells represent the greatest proportion of B cells in the peritoneal cavity, followed by peripheral blood, bone marrow, and spleen. The phenotype of equine $CD5^{hi}$ B cells can be more completely described as $CD19^{+}CD5^{hi}CD2^{hi}CD11b^{+}CD21^{int/-}IgM^{hi/-}MHCII^{+}$ in adult peripheral blood (Table 4.3). There is variation in the phenotype of $CD5^{hi}$ B cells in different lymphoid tissues; in the spleen $CD5^{hi}$ B cells are $CD21^{hi}$, while fewer $CD5^{hi}$ B cells in both the spleen and bone marrow express CD11b than in the peripheral blood.

Table 4.3 Phenotype of equine $CD5^{hi}$ and $CD5^{lo}$ B cells in different lymphoid tissues

Tissue		Phenotype
PBL	B1	$CD19^{+}$ $CD5^{hi}$ $CD2^{hi}$ $CD11b^{+}$ $CD21^{int/-}$ $IgM^{hi/-}$ $MHCII^{+}$
	B2	$CD19^{+}$ $CD5^{lo}$ $CD2^{lo}$ $CD11b^{+/-}$ $CD21^{int/-}$ $IgM^{lo/-}$ $MHCII^{+}$
BM	B1	$CD19^{+}$ $CD5^{hi}$ $CD2^{hi/-}$ $CD11b^{+/-}$ $CD21^{int/-}$ $IgM^{hi/-}$
	B2	$CD19^{+}$ $CD5^{lo}$ $CD2^{lo/-}$ $CD11b^{+/-}$ $CD21^{int/-}$ $IgM^{lo/-}$
SP	B1	$CD19^{+}$ $CD5^{hi}$ $CD2^{hi}$ $CD11b^{+/-}$ $CD21^{hi}$ $IgM^{int/-}$
	B2	$CD19^{+}$ $CD5^{lo}$ $CD2^{lo}$ $CD11b^{+/-}$ $CD21^{hi}$ $IgM^{int/-}$

$^{+}$indicates that > 80% of cells express the marker, with differential expression noted as low (lo), intermediate (int), or high (hi) when appropriate.
$^{+/-}$ indicates that < 80% of cells express the marker, and for the population of cells positive for the marker, differential expression is indicated as low (lo/-), intermediate (int/-) or high (hi/-).

Peripheral blood $CD5^{hi}$ B cells have diverse immunoglobulin lambda light chain repertoires, but more frequently express BCRs that bind to PC, and are capable of secreting IL-10 in vitro.

In the adult horse PBL, a median of 14% of B cells were $CD5^{hi}$. The percentage of B cells expressing CD5 in the adult peripheral blood varies by species: 10-20% in humans (Carsetti et al. 2004, Dalloul 2009), 10-20% in the pig (Appleyard and Wilkie 1998, Wilson and Wilkie 2007), 1% in the guinea pig (Dilwith and Wicher 1997), and 5-35% in cattle (Naessens and Williams 1992). In contrast, the majority of B cells in the rabbit are $CD5^{+}$ (Raman and Knight 1992), while no CD5 is expressed in rat B cells (Vermeer et al. 1994). In the horse, $CD5^{hi}$ B cells represented a larger proportion of B cells in the foal when compared to the adult horse, similarly to how B1 cells (Griffin et al. 2011a, Hayakawa et al. 1983, Hayakawa et al. 1986a) or $CD5^{+}$ B cells are more abundant early in ontogeny of other species. In humans, the $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ B1 cells are most abundant in umbilical cord blood (>80% of $CD27^{+}$ cells) (Griffin et al. 2011a), and between 70-90% of fetal spleen, cord blood, and neonatal B cells in the PBL are $CD5^{+}$ (Bhat et al. 1992, Carsetti et al. 2004). In the pig, the percentage of B cells that are $CD5^{+}$ is similar in fetal and neonatal life (15%), but decreases by a few percent in the young piglet, before returning to similar levels to fetal life in the adult pigs (Appleyard and Wilkie 1998). Though all B cells in the rabbit are $CD5^{+}$, B lymphopoiesis in the bone marrow peaks in the newborn and very few new B cells are generated in adult lymphoid tissues (Hayward et al. 1978, Lanning et al. 2000).

The greatest percentages of $CD5^{hi}$ B cells were found in the peritoneal cavity of the adult horse similarly to murine B1 cells, which can represent the majority of B cells

(35-70%) in this tissue (Baumgarth 2011). The second largest population of B1 cells in mice is found in the spleen (1-2%), and in pigs, a greater number of $CD5^+$ B cells are found in the spleen (11.6-27%) when compared to peripheral blood (Appleyard and Wilkie 1998). In contrast, the equine spleen contained the smallest proportion of B cells that were $CD5^{hi}$, the same as in cattle (2.9-10.7%) (Naessens and Williams 1992).

Murine and human B1 cells are distinguished from B2 cells by a combination of different surface markers. In mice, B1a cells have the phenotype $CD5^+CD45RA^{lo}IgM^{hi}$ $IgD^{lo}CD23^-CD43^+$, while B1b cells are $CD5^-$ (Herzenberg et al. 1987, Tung et al. 2004, Yenson and Baumgarth 2014). The population of human B1 cells described by Griffin et al. (2011a) is $CD20^+CD27^+CD43^+CD70^-$, which does not use the CD5 expression for B cell differentiation. Nevertheless, I chose to include the CD5 molecule because (1) our previous observation that the $CD5^+$ cells in equine PBL is greater than the $CD3^+$ cells suggested an uncharacterized population of $CD5^+$ B cells existed in the horse, (2) murine B1a and the majority of human B1 cells express CD5 (Griffin et al. 2011a) and (3) CD5 expression has been associated with B1-like characteristics such the secretion of poly-reactive natural antibodies and IL-10 (Dono et al. 2004, Gary-Gouy et al. 2002). In the horse, a greater percentage of $CD5^{hi}$ B cells than $CD5^{lo}$ B cells express CD21 in the PBL, CD2 in the PBL and bone marrow, IgM in the bone marrow, MHC class II in PBL, and CD11b in the PBL, bone marrow, and spleen. CD21 expression is also observed in human $CD11b^+$ B1 cells (Griffin and Rothstein 2011) and murine B1 cells (Rothstein et al. 2013). In addition, B1 cells in humans and mice have higher expression of another co-stimulatory molecule CD86, linked to their enhanced ability to stimulate T cells (Griffin and Rothstein 2011, Griffin and Rothstein 2012b, Tumang et al. 2004,

Zhong et al. 2007). Further studies are necessary to determine if the greater expression of CD21 in $CD5^{hi}$ B cells results in any functional consequences such as increased efficiency capturing immune complexes to transfer to follicular dendritic cells for antigen presentation.

To my knowledge, the expression of CD2 in equine B cells is a novel finding for this species. Positive CD2 expression in peripheral blood B cells has been reported in the pig (Sinkora et al. 1998) and mouse (Sen et al. 1989), but not in humans (Muraguchi et al. 1992). Expression on B cells in the pig is developmentally regulated, with more than 90% of B cells expressing CD2 in the fetus and neonate, and a subsequent decrease in expression to half of B cells by 6 months of age (Sinkora et al. 1998). CD2 expression also seems to be developmentally regulated in the horse with opposite results, with lower percentages of $CD5^{hi}$ and $CD5^{lo}$ B cells expressing CD2 in the 3-day-old foal when compared to the adult horse. CD2 had higher MFI of expression in $CD5^{hi}$ than $CD5^{lo}$ B cells in the PBL, spleen, and bone marrow, and therefore may be a useful marker in sorting more pure populations of $CD5^{hi}$ and $CD5^{lo}$ B cells in future studies.

A subset of human B1 cells, $CD5^{+}$ B cells in cattle, and murine B1 cells outside the spleen have been shown to express CD11b (Griffin and Rothstein 2011, Griffin and Rothstein 2012b, Naessens and Williams 1992, Stall et al. 1992). Similar to mice though with variable expression, proportionally fewer $CD5^{hi}$ B cells expressed CD11b in the equine spleen when compared to the other tissues tested. CD11b is more commonly expressed in myeloid cells and further studies are necessary to reveal other developmental and functional characteristics shared by the B lymphoid and myeloid lineages described in other species (Borrello and Phipps 1996).

Some investigators suggest that there are similarities between B1 and anergic B2 cells, pointing in part to the shared characteristics of CD5 expression and unresponsiveness to BCR ligation with anti-IgM (Wong et al. 2002). However, in contrast to anergic B cells, B1 cells have high expression of co-stimulatory molecules including MHC class II (Morris and Rothstein 1993), survive for extended periods in culture (Wong et al. 2002), and secrete immunoglobulin (Tumang et al. 2004). Similarly, equine $CD5^{hi}$ B cells had equal to or greater expression of MHC class II than $CD5^{lo}$ B cells, suggesting that they are not anergic B cells, but are capable of functioning as antigen presenting cells and are active participants in immune responses.

B1 and B2 cells in mice have distinct gene expression profiles including those coding for surface molecules, signaling molecules, transcription factors, and molecules involved in cell metabolism (Diehl et al. 2011, Mabbott and Gray 2014, Tumang et al. 2005). Yamagata et al. (2006) described a shared gene signature that distinguished innate-like lymphoid B1, NK, and CD8$\alpha\alpha$ cells from their adaptive counterparts, which included FGL2 and DGKA measured in this study. The panel of genes identified by Yamagata and colleagues also distinguished a population of human $CD21^{low}$ innate-like B cells identified in CVID patients shown to share functional characteristics with murine B1 cells, including immunoglobulin repertoires with few mutations, poor calcium mobilization and proliferation in response to BCR stimulation, high expression of the co-stimulatory molecule CD86, and preferential homing to respiratory mucosa (Rakhmanov et al. 2009). However, a subsequent meta-analysis of gene expression profiles of murine and human B1 and B2 cells described by Griffin et al. (2011a) revealed that only 3 genes were differentially expressed by B1 and B2 cells in both species: CD5, CCR1,

and SYT11 (Mabbott and Gray 2014). It is, therefore, not surprising that of the selected genes that have distinguished B1 and B2 cells in mice and humans, only CD5 was differentially expressed in $CD5^{hi}$ and $CD5^{lo}$ B cells in the horse. In mice and humans, CCR1 and SYT11 expression was only 2.0-2.9-fold higher in B1 than B2 cells with unclear biological significance in B cell function, so the expression of these genes was not tested in this study. B1 and B2 signature genes tested were chosen based on functional implications (PAX5, IGHM, IL-10) or greater magnitude of differential expression (7-fold) in B1 compared to B2 cells in mice and other innate-like human B cells (FGL2). Technical limitations preventing isolation of pure $CD5^{hi}$ and $CD5^{lo}$ B cells may also explain why differential gene expression was not observed. CD5 expression on B cells is low (dim) overall and not distinctly positive or negative, making it challenging to FACS sort pure $CD5^{hi}$ and $CD5^{lo}$ B cell fractions. One method to improve purity of FACS-sorted fractions is to perform two sequential sorts; this was not done for this study since a cost-prohibitive amount of reagents would be required to label enough cells to collect a sufficient number of $CD5^{hi}$ B cells for analysis, as these cells are relatively rare (0.11-1.44% of total cells) in adult PBL. Alternatively, a combination of multiple markers that are differentially expressed can be used to sort more pure populations of cells; this method is commonly used to isolate pure populations of B1 and B2 cells from mice (Yenson and Baumgarth 2014). Phenotypic analysis in this study revealed higher expression of CD2 and IgM in equine $CD5^{hi}$ B cells than $CD5^{lo}$ B cells, and using these markers in combination may facilitate the isolation of more pure populations in future studies.

B1 cells in mice use different V(D)J segments than B2 cells (Kantor et al. 1997). In addition, the antibodies classically produced by murine B1 cells against antigens including Thy-1, PtC (phosphatidylcholine), and PC are constructed with restricted immunoglobulin gene segments, such as $V_H11/V\kappa9$ or $V_H12/V\kappa4$ gene family members for PtC and PC antibodies, and these IGH gene families were found to be overrepresented in immunoglobulin repertoires of B1 compared to B2 cells (Hayakawa et al. 1990, Kantor et al. 1997, Pennell et al. 1989). In Chapter 3, I observed that though there was a bias for IGLV4-66 use in fetal life, the equine fetus used a broader range of IGLV segments than the adult horse. In this study, I wanted to learn (1) if there were differences in IGLV segment used between $CD5^{hi}$ and $CD5^{lo}$ B cells as described for murine B1 and B2 cells; and (2) if trends in IGLV segment use in $CD5^{hi}$ B cells could explain differences in combinatorial diversity previously observed between the equine fetus and adult horse (Tallmadge et al. 2014). I found that there was a statistically significant difference in IGLV segment use between $CD5^{hi}$ and $CD5^{lo}$ B cells in the foal, with IGLV8-24 used preferentially in $CD5^{hi}$ B cells, and IGLV4-66 in $CD5^{lo}$ B cells. Similarly to the fetal IGL repertoire, $CD5^{hi}$ B cells in the foal used a greater variety of IGLV gene segments to construct the lambda light chain. The dominant IGLV segment was present in 19% of sequences expressed in $CD5^{hi}$ B cells, but 29% of sequences expressed in $CD5^{lo}$ B cells; this trend was not observed in $CD5^{hi}$ and $CD5^{lo}$ B cells in the adult horse, and the dominant IGLV segment was present in 44% and 33% of sequences expressed in $CD5^{hi}$ and $CD5^{lo}$ B cells, respectively. Diversity of the murine B1 immunoglobulin repertoire has also been shown to change during developmental stages, as the number of N-nucleotide additions increases with age; and comparable

numbers of N-nucleotide additions with B2 cells are observed in immunoglobulin sequences of B1b cells by 8 weeks of age (Tornberg and Holmberg 1995), and of B1a cells by 6 to 10 months of age (Gu et al. 1990).

IGLV percent nucleotide identity to germline was similar between $CD5^{hi}$ and $CD5^{lo}$ B cells, indicating that these populations express IGL sequences that have similar degrees of mutations. This is in contrast to B1a cells in mice, which are nearly germline encoded with few mutations (Berland and Wortis 2002); however, B1b cells in particular express immunoglobulin sequences that contain mutations. Roy et al. (2009) showed that B1b cells that have class switched to IgA have immunoglobulin sequences with greater mutations than those of B1a or B1b IgM^+ B cells with evidence of somatic hypermutation. In one study, immunoglobulin sequences expressed by human cord blood $CD20^+CD27^+CD43^+$ B1 cells were observed to contain few mutations, though B2 cells from the same tissue were similarly unmutated (Griffin et al. 2011a). Analysis of human adult PC-specific $CD20^+CD27^+CD43^+$ B1 cells revealed immunoglobulin sequences that were extensively mutated (Fiskesund et al. 2014). Therefore the degree of sequence diversity in the immunoglobulin repertoires appears to differ between human B1 cells and murine B1a cells. Similar the immunoglobulin diversity observed in the human adult antigen specific B1 cells, equine $CD5^{hi}$ B cells express mutated IGL sequences allowing these cells the potential to respond to a greater range of different antigens for humoral protection.

No difference in CDR3 length was observed in IGLV sequences expressed in $CD5^{hi}$ and $CD5^{lo}$ B cells. CDR3 lengths were also similar between B1 and B2 cells in mice and humans (Griffin et al. 2011a, Kantor et al. 1997) and between equine fetal and

adult horse lambda light chain sequences (Tallmadge et al. 2014). However, increased heavy chain CDR3 length has been associated with autoreactivity (Wardemann et al. 2003) and polyreactivity (Ichiyoshi and Casali 1994) in some studies, but not all (Kasaian et al. 1994). Therefore though equine $CD5^{hi}$ B cells do not express immunoglobulins with longer CDR3 lengths than $CD5^{lo}$ B cells, this does not necessarily mean that they do not secrete polyreactive antibodies.

Phosphorylcholine is found on bacteria, helminths, apoptotic cell membranes, and oxidized lipids (Binder et al. 2002, Peters et al. 1999). In addition, anti-PC antibodies produced by B1 cells have a role in protection against pneumococcal infection (Briles et al. 1982), reduction of atherosclerotic lesions (Kyaw et al. 2011, Shaw et al. 2000), and clearing of apoptotic cells (Chen et al. 2009). Equine $CD5^{hi}$ B cells are enriched for BCRs that can bind PC, as described in B1 cells of the mouse (Lalor and Morahan 1990), human (Griffin et al. 2011a), and non-human primates (Yammani and Haas 2013). However, a study of human PC specific B cells revealed that equivalent percentages of PC-specific B cells were $CD5^{hi}$ and $CD5^{lo}$ (Fiskesund et al. 2014). $CD5^{hi}$ B cells in the foal were more frequently specific for PC than in the adult horse, perhaps due to differences in immunoglobulin repertoire between the developmental stages. The enrichment for BCRs that bind to PC suggests that equine $CD5^{hi}$ B cells, particularly in the foal, potentially play an important role in immunity to the wide variety of bacterial and helminth pathogens that express the antigen, and in maintaining homeostasis by facilitating the clearing of apoptotic cells (Chen et al. 2009, Clark and Weiser 2013).

B1 cells have been shown to secrete IL-10 with and without mitogen stimulation in vitro (Griffin and Rothstein 2012b, O'Garra and Howard 1992). Equine $CD5^{hi}$ B cells had comparable IL-10 expression with $CD5^{lo}$ B cells at the protein level after stimulation with PWM in vitro and, similar to the pig, at the mRNA level immediately post-sorting (Wilson and Wilkie 2007). IL-10 secretion is not unique to B1 subsets; regulatory B cells with the phenotype $CD1d^{hi}CD5^{+}$ in mice and $CD38^{hi}CD24^{hi}$ cells in humans have also been shown to be important IL-10 secreting cells (Bouaziz et al. 2010, Yanaba et al. 2008, Yanaba et al. 2009). IL-10 has widespread anti-inflammatory effects on many different hematopoietic cells, and autocrine IL-10 secretion by B cells has also been shown to promote their survival (Gary-Gouy et al. 2002, Moore et al. 2001). The ability of equine $CD5^{hi}$ B cells to secrete IL-10 suggests that they may have important immunoregulatory functions and have the potential to promote their own long-term survival.

A greater proportion of the remaining B cells were $CD5^{hi}$ in horses affected with CVID when compared to healthy adult horses. The thought of investigating B1 cells in CVID-affected horses came from the observation of normal serum IgA concentrations in these patients when IgG and IgM are below protective levels. Though murine B1 cells primarily produce IgM, they preferentially class switch to IgA (Kaminski and Stavnezer 2006, Tarlinton et al. 1995). The B1b subset of B1 cells more frequently expresses IgA than B2 cells at the mRNA and protein levels (Roy et al. 2009), and B1 cells contribute with half of the total intestinal IgA (Kroese et al. 1989). Importantly, a median of only 58% of $CD5^{hi}$ B cells co-expressed IgM in the peripheral blood of healthy adults suggesting that these cells are capable of class switch recombination and express other

immunoglobulin isotypes. Further studies are necessary to determine which immunoglobulin isotypes are produced by equine $CD5^{hi}$ B cells, and how they may be responsible for maintaining IgA levels in CVID patients. B1 cells are known to respond to T-cell independent antigens, such as lipopolysaccharides, and play an important role in the immunity to *Streptococcus* pneumonia (Berland and Wortis 2002). Equine $CD5^{hi}$ B cells in this study were enriched for BCRs that can bind to PC, and further studies are needed to determine if they are responsible for the response to pneumococcal polysaccharide vaccines. CVID is the most common clinically relevant primary immunodeficiency in human patients, with a prevalence of 1:50,000 to 1:200,000 people in the general population (Kumar and Bhatia 2013). In contrast to equine CVID patients described in my study, a study in humans found that the percentage of $CD27^+$ B cells with the $CD20^+CD27^+CD43^+$ B1 phenotype was similar in CVID patients and healthy controls (Suchanek et al. 2012). The percentage of $CD20^+CD27^+CD43^+$ B1 cells that were $CD5^+$ were also similar between human CVID patients and healthy controls, though CD5 expression was more variable on B cells from CVID patients (Suchanek et al. 2012).

In total, the studies herein show that equine $CD5^{hi}$ B cells share some similarities described for B1 cells in mice and humans including: (1) representing a greater proportion of B cells early in ontogeny, (2) greater proportion of B cells in the peritoneal cavity, (3) enrichment for BCRs specific for PC, and (3) the ability to secrete IL-10. Unlike murine B1 cells but similarly to human B1 cells, equine $CD5^{hi}$ B cells have diverse immunoglobulin repertoires. Further studies are needed to determine other functional similarities attributed to B1 cells, primarily the ability to spontaneously secrete

immunoglobulin, the ability to efficiently stimulate T cells, response to BCR-crosslinking, and down stream signaling events such as tonic intracellular signaling. The greater distribution of $CD5^{hi}$ B cells in horses with CVID is intriguing, and further studies are also necessary to reveal whether these cells contribute to the prolonged maintenance of serum IgA, can respond to pneumococcal polysaccharide, and, ultimately, be targeted for protection in immunodeficient patients.

CHAPTER FIVE

FINAL COMMENTS

B lymphopoiesis and the generation of an immunoglobulin repertoire capable of recognizing the antigenic universe have been topics of curiosity and investigations in the field of immunology for more than a century. And the horse has been associated with some landmark discoveries in the field. At the end of the 19th and beginning of the 20th century, horse serum was used for passive therapy in human patients to treat bacterial infections (e.g. diphtheria, pneumococcal pneumonia), and the observation that anti-toxin serum titers were higher in horses that had infections at the site of the toxin injection inspired Dr. Gaston Ramon to perform the initial experiments that led to the discovery of adjuvants (Graham and Ambrosino 2015). Today, the horse continues to be proposed as a model organism to study the immune responses associated with infectious diseases (e.g. influenza viruses, herpes viruses), allergic disease (e.g. asthma), immunodeficiencies, vaccinology, oncology, pregnancy, aging, osteoarthritis, and regenerative medicine (Horohov 2015). The studies of my thesis describing B lymphopoiesis in the primary lymphoid tissues of the equine fetus, generation of the pre-immune immunoglobulin repertoire, and CD5^{hi} B cells contribute to the knowledge of humoral immunity in the horse and may facilitate the use of this species as a model organism to study B1-like cells and common variable immunodeficiency (CVID).

B lymphopoiesis begins during embryonic development. It remains unknown when progenitors with B cell potential arise in the equine fetus; however, by 90 DG of gestation, they can be detected at the molecular level (Tallmadge et al. 2009, Tallmadge et al. 2013, Tallmadge et al. 2014), and the studies in my thesis bring

evidence of B cell surface protein expression by 100 DG (Chapter 1). B cells in the fetal liver include both $CD5^{hi}$ and $CD5^{lo}$ B cell populations; and MHC class II protein expression is positive at this stage and throughout development, in contrast to T cells, which follows age-dependent expression after birth (Lunn et al. 1993) (Chapter 4). B lymphopoiesis is one of the best-studied hematopoietic pathways in species such as mice and humans, though many key questions remain: Are B1 cells in humans derived from a distinct B cell progenitor as described for murine B1 cells? Does the increased plasticity of fetal CLPs for differentiation into myeloid cells observed in vitro also occur in vivo? And naturally occurring immunodeficiencies in the horse, including foal immunodeficiency syndrome and CVID raise additional questions regarding the molecular mechanisms of hematopoiesis, and offer unique opportunities to study aspects of B lymphopoiesis that may be common to many different species.

Foal immunodeficiency syndrome (aka Fell Pony Syndrome) is a fatal disease characterized by the rapid disappearance of both erythrocytes and B cells during the first few weeks of life (Tallmadge et al. 2012a). Though less severe, T cells and myeloid cells may also be developmentally impaired. The progression of this disease suggests that fetal B lymphopoiesis and erythropoiesis occur but the ontological switch to produce these hematopoietic lineages in the foal fails (Tallmadge et al. 2012a). Fell Pony pedigree analysis suggests an autosomal recessive inheritance of the defective genetic mechanism, and an associated mutation with unknown functional consequence has been identified (Fox-Clipsham et al. 2011). No single mutation has been identified in humans or mice that severely affects both erythroid and B lymphoid lineages, and further study of the molecular mechanisms of this disease could reveal novel genes or

regulatory mechanisms essential for the development of adult erythrocytes and B cells. CVID is another primary immunodeficiency disease of horses that involve progressive loss of the B lymphoid lineage with a late-onset disease manifestation. Decreased mRNA expression and differential methylation patterns of the B cell transcription factor PAX5 in horses with CVID suggests involvement of epigenetic disturbance in the impaired progression of pre-pro B cells to the pro-B cell stage (Tallmadge et al. 2012b, Tallmadge et al. 2015). Understanding the defect in CVID horses may help further explain how epigenetic modifications, together with gene regulatory networks and cytokine signaling regulate B cell hematopoiesis. Much of what is known about mammalian B lymphopoiesis is based on murine and human studies, with similarities and differences, and studies in other domestic species elucidate the complexity and alternative pathways of cell differentiation that can be used to explain mechanisms of disease or searched for treatment.

My studies suggest that the B cells produced in the liver and bone marrow of the equine fetus generate a wide repertoire of pre-immune immunoglobulins for protection. The B cells that develop in the primary lymphoid tissues during gestation have already elements of combinatorial, junctional, and sequence diversity in the immunoglobulin repertoire observed in the adult horse, and indicates that the equine fetus is equipped with the molecular machinery necessary to generate a diverse antibody repertoire (Chapter 3). The biased use of IGHV2S2, IGHV2S3, and IGHV2S4 in the adult is already established in the fetal liver and bone marrow at 100 DG. It is unknown why these IGHV genes are most frequently used to construct the heavy chain. The entire annotated equine IGHV locus is accessible throughout development so preferential

usage of IGHV2S2, IGHV2S3, and IGHV2S4 is likely independent of genomic position (Tallmadge et al. 2013). Perhaps these IGHV gene segments better associate with a surrogate light chain or have been selected for over evolutionary time for immune protection against common pathogens of the horse.

The immunoglobulin repertoire of the equine fetus has limited sequence diversity, perhaps reflecting the sterile environment of the womb. Curiously, despite limited heavy chain diversity, the fetus uses a greater number of different lambda variable gene segments than the adult horse. The increased combinatorial diversity of IGLV genes may help the neonate to respond to a wide range of antigens at birth. Between neonatal and adult life, IGLV genes from the subgroup 8, in particular IGLV8-122, IGLV8-128, and IGLV8-24 are the most frequently used gene segments to build the lambda light chain. The same biases occur in horses of different breeds and geographical locations (Hara et al. 2012, Sun et al. 2010, Tallmadge et al. 2014). The rationale for a switch to more restricted use of light chain genes is puzzling, and whether these IGLV genes are favored at level of IGL rearrangement, are less likely self-reactive and therefore avoid clonal deletion, or are more frequently positive-selected following antigen exposure can only be speculated.

Different populations of B cells develop during fetal life: B1, B2, and marginal zone B cells. Soon after the identification of B1 cells in mice, $CD5^+$ B cells in humans were described, and found to share characteristics of increased frequency early in development (Bhat et al. 1992) and secretion of polyreactive natural antibodies and autoantibodies (Casali et al. 1987, Hardy et al. 1987, Schutte et al. 1991). CD5 expression on human B cells, however, is more heterogeneous than in mice (Carsetti et

al. 2004), and different populations of B cells have been proposed to be the human equivalent of the murine B1 cell (Griffin et al. 2011a, Kruetzmann et al. 2003, Rakhmanov et al. 2009). The most convincing evidence is for the $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ population, the majority of which are also $CD5^{+}$, as these share four major functional similarities with murine B1 cells: spontaneous secretion of IgM and IL-10, efficient stimulation of T cells, and tonic BCR signaling (Griffin et al. 2011a, Griffin and Rothstein 2012b).

In my thesis, equine $CD5^{hi}$ B cells were identified and isolated, and aspects of their ontogeny, phenotype, and function where compared with those attributed to B1 cells in humans and mice (Chapter 4). Equine $CD5^{hi}$ B cells represent a greater proportion of B cells early in ontogeny in the fetal liver and peripheral blood of the foal, and in adult horse PBL are described as $CD19^{+}CD5^{hi}CD2^{hi}CD11b^{+}CD21^{int/-}IgM^{hi/-}MHCII^{+}$, while $CD5^{lo}$ B cells are $CD19^{+}CD5^{lo}CD2^{lo}CD11b^{+/-}CD21^{int/-}IgM^{lo/-}MHCII^{+}$. The phenotypic characterization of $CD5^{hi}$ B cells provides clues as to their biological function in the horse. Expression of the complement receptor CD21 suggests $CD5^{hi}$ B cells could participate in innate immunity by responding to complement bound pathogens. $CD5^{hi}$ B cells also have the potential to influence the adaptive humoral response; the expression of CD21 may allow the $CD5^{hi}$ B cells to capture and transfer immune complexes to follicular dendritic cells, and the expression of MHC class II suggests $CD5^{hi}$ B cells have the potential to act as antigen presenting cells. Expression of the integrin CD11b, commonly expressed in myeloid cells, may suggest $CD5^{hi}$ B cells share other functional characteristics with the myeloid lineage. The observation that not all $CD5^{hi}$ B cells express IgM implies they undergo class switch recombination and express other

immunoglobulin isotypes. $CD5^{hi}$ and $CD5^{lo}$ B cells can now be distinguished not only by CD5 expression, but also the expression of CD2, CD21, IgM, and CD11b, depending on the tissue. It remains unknown whether the differential expression of these molecules results in any functional distinctions between $CD5^{hi}$ and $CD5^{lo}$ B cells; however, the use of these additional markers may facilitate the isolation of more pure populations in future studies.

Peripheral blood $CD5^{hi}$ B cells have diverse immunoglobulin lambda light chain repertoires, indicating their potential to respond to a wide range of antigens. $CD5^{hi}$ B cells more frequently express BCRs that bind to phosphorylcholine (PC) than $CD5^{lo}$ B cells, and may play a role in the production of natural antibodies that can bind to apoptotic cells or the variety of pathogens that express this antigen. The observation that $CD5^{hi}$ B cells are capable of secreting IL-10 is suggestive of an immunoregulatory function. Though these observations are intriguing, $CD5^{+}$ B cells are relatively rare in adult peripheral blood leukocytes (< 1.5%), and it remains unknown to what extent they may contribute to local or systemic immune function.

Are equine $CD5^{hi}$ B cells the horse equivalent of B1 cells? In Chapter 4, I found that equine $CD5^{hi}$ B cells share some similarities described for B1 cells in mice and humans, including: (1) representing a greater proportion of B cells early in ontogeny, (2) representing a greater proportion of B cells in the peritoneal cavity, (3) enrichment for BCRs specific for PC, and (4) ability to secrete IL-10. Unlike murine B1 cells but more similar to human B1 cells, equine $CD5^{hi}$ B cells have diverse immunoglobulin repertoires. Based on these findings, I believe equine $CD5^{hi}$ B cells are putative B1 cells; however, two other major functional aspects need to be studied to more

convincingly determine whether they are indeed B1 cells. The first is spontaneous secretion of immunoglobulin, as this function characterizes the role of B1 cells in immunity and homeostasis. Quantification of equine IgM and IgA protein requires the optimization of existing assays (e.g. ELISA) or development of others (e.g., ELISPOT) with improved sensitivity and specificity for measuring low concentrations of these proteins (Chapter 4). In addition, optimization of culture conditions for FACS sorted $CD5^{hi}$ and $CD5^{lo}$ B cells may promote greater immunoglobulin secretion. Other important questions include differences in BCR signaling between $CD5^{hi}$ and $CD5^{lo}$ B cells (e.g. intracellular calcium mobilization, tonic BCR signaling), and how $CD5^{hi}$ B cells stimulate allogeneic T cell proliferation. In mice, it appears that B1 and B2 cells develop from distinct progenitors (Montecino-Rodriguez et al. 2006) while it remains unknown whether the same is true in humans. The developmental origin of equine $CD5^{hi}$ B cells is also unknown. The differences detected in phenotype and function of $CD5^{hi}$ and $CD5^{lo}$ B cells were not great in magnitude; for example both $CD5^{hi}$ and $CD5^{lo}$ B cells expressed CD21, CD2, IgM, and MHCII to some degree and only a few percent bound to the fluorescently labeled phosphorylcholine. Therefore it is tempting to speculate that these two populations share a common progenitor and developmental history.

A primary motivation for studying equine $CD5^{hi}$ B cells as putative B1 cells is the possibility of their presence in horses with B2 cell deficiency, such as in common variable immunodeficiency (CVID). I found that a greater percentage of the remaining circulating B cells in CVID-affected horses are $CD5^{hi}$ compared to healthy controls, even though B cells were rare in the blood (Chapter 4). The CVID-affected horses in this study had circulating B cells that comprised from <1 to 5% of total lymphocytes, and it

would be intriguing to follow these patients over time to test whether the proportion of $CD5^{hi}$ B cells increased as the disease progressed. A question that remains unanswered is if CD5 expression can be induced on equine B2 cells, and if a greater proportion of $CD5^{hi}$ B cells may be found in horses with infectious or inflammatory conditions.

CVID-affected horses maintain IgA levels within the low normal range for a much longer time than IgG and IgM. Murine B1 cells are known to secrete significant amounts of IgA and it is possible that B1 cells in CVID-affected horses keep the production of IgA near normal titers (Kroese et al. 1989). In this study, IgM expression at the mRNA level was similar between $CD5^{hi}$ and $CD5^{lo}$ B cells (Chapter 4), though the expression of other isotypes, including IgA, was not examined, and the possibility that $CD5^{hi}$ B cells preferentially secrete this isotype remains. Horses with CVID can also respond to pneumococcal polysaccharide, but not to vaccination using proteins, and B1 cells are known to participate in the response to T cell-independent antigens (Berland and Wortis 2002). In this study, $CD5^{hi}$ B cells were enriched for their binding to PC, an epitope expressed by pneumococci. Whether these $CD5^{hi}$ B cells are also responsible to the pneumococcal polysaccharide specific antibody production in vivo, however, is another unanswered but consequential question.

If further functional studies demonstrate that equine $CD5^{hi}$ B cells are definitive homologues of B1 cells in mice and humans, then CVID-affected horses may present a unique and natural model for in vivo functional studies (e.g. response to vaccination), since humoral response is not confounded by the function of B2 cells. Lifelong periodic IgG replacement therapy is the mainstay of treatment for human patients with CVID to

prevent reoccurring infections, although adverse reactions and incomplete protection are limitations (Abolhassani et al. 2013). While transfusion of plasma products is possible in CVID-affected horses, it is cost-prohibitive, and no purified IgG product is available for frequent use (Flaminio et al. 2009). Murine B1b cells have been shown to produce antigen-specific immunoglobulins and generate memory to infections with certain pathogens, such as *Borrelia Hermsii* (Alugupalli et al. 2004) and *Streptococcus pneumonia* (Haas et al. 2005). If equine B1-like cells have similar function, then they could be targeted for immunization and protection in the CVID-affected horses, and open a new line of investigations for alternative and complementary treatments for human and animal humoral immunodeficiencies.

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GLOSSARY

Aiolos	A member of the Ikaros family of transcription factors, encoded by the gene IKZF3, with important functions in B cell differentiation at the pre-B cell stage, and later stages of B cell maturation, proliferation, and germinal center formation.
AGM/P-SP	The aorta-gonad-mesonephros/para-aortic splanchnopleura regions are embryonic tissues where hematopoiesis initiates. The aorta-gonad-mesonephros is the mesodermal tissue that develops from the para-aortic splanchnopleura.
B1 B cell	B1 cells are the first B lymphoid lineage to arise during ontogeny. These cells are important in innate immunity, with functions including spontaneous secretion of natural immunoglobulins with restricted diversity, secretion of IL-10, efficient stimulation of T cells, tonic BCR signaling, and are maintained largely by self-renewal.
B2 B cell	Conventional or follicular B2 cells are the second B lymphoid lineage to arise during ontogeny. B2 cells have important functions in adaptive immunity with diverse immunoglobulin repertoires, produce antigen specific antibodies, and are continuously replenished from bone marrow precursors.
BAFF	B cell activating factor is a cytokine necessary for the survival and maturation of transitional and immature B cells, and is important in selecting germinal center B cells with high affinity for particular antigens.
BCR	The B cell receptor is the transmembrane form of the immunoglobulin molecule non-covalently associated with CD79a and CD79b. The immunoglobulin is composed of 2 heavy and 2 light chains joined by disulfide bonds. The variable regions come in contact with the antigen and are produced by somatic rearrangement of the V(D)J gene segments.
BLIMP1	B-lymphocyte-induced maturation protein 1 is the master transcriptional regulator of B cell differentiation into antibody secreting cells, encoded by the PRDM1 gene.
BLNK	B-cell linker is an adaptor protein important in the pre-BCR and BCR signal transduction pathways. After being phosphorylated by Syk, BLNK binds to PLCγ2 and BTK, facilitating the phosphorylation and activation of PLCγ2.

BTK	Bruton's tyrosine kinase is important in pre-BCR and BCR signaling, as it binds to the adaptor protein BLNK and phosphorylates PLCγ2. Mutations in this gene cause X-linked agammaglobulinemia.
CDRs	Complimentary determining regions are the sequences that come in contact with antigen and the most diverse regions of the immunoglobulin molecule; CDR1 and CDR2 are located in the IGHV segment while CDR3 contains the greatest degree of sequence variability and spans the junction of the IGHV, IGHD, and IGHJ gene segments in the heavy chain, and IGLV and IGLJ gene segments in the light chain.
CCR1	Chemokine (C-C-motif) receptor 1 is a member of the beta chemokine receptor family with ligands belonging to the C-C chemokine family including macrophage inflammatory protein 1 alpha (MIP-1 alpha). In the B lymphoid lineage, CCR1 expression has been observed in human tonsil crypt intraepithelial B cells.
CLP	Common lymphoid progenitors are derived from ELPs within the LMPP population, and have the potential to differentiate into B, T, NK, and DC cells in the adult, and additionally macrophages in the fetus.
CEBPA	CCAAT/enhancer binding protein, alpha, is a basic region-leucine zipper transcription factor important in myelopoiesis.
CMP	Common myeloid progenitors are derived from ST-HSCs that can differentiate into myeloid, megakaryocyte, and erythroid lineages.
CVID	Common variable immunodeficiency is a late onset primary humoral immunodeficiency characterized in horses by progressive B lymphopenia, hypo to agammaglobulinemia, inability to respond to protein vaccination, and clinically manifests with reoccurring infections. The disease is similarly characterized in humans, however, while some patients have a severe lymphopenia, others have decreased switched memory cells, or an expansion of transitional B cells.
DGKA	Diacylglycerol kinase, alpha is important in attenuating BCR signaling by inhibiting the second messenger diacylglycerol, and is highly expressed by B2 cells.
CXCL12	CXC-chemokine ligand 12 is an important chemokine for the colonization of the bone marrow by HSCs, the differentiation of pre-pro and pro-B cells, and the homing of plasma cells back to the bone marrow. CXCL12 is produced, at least in part, by bone marrow reticular cells and its main receptor is CXC-chemokine receptor 4.

DWEYS	The consensus sequence of peptides that are molecular mimics of the double stranded DNA epitopes recognized by anti-DNA antibodies.
EBF	Early B cell factor is a transcription factor encoded by the EBF1 gene that binds to DNA via an amino terminal zinc coordination motif, and is expressed beginning at the pre-pro B cell stage, with important functions specifying the B cell fate.
ELP	Early lymphoid progenitors are derived from LMPPs and differentiate into CLPs.
E2A	A basic helix-loop-helix transcription factor composed of E12 and E47 proteins encoded by the E2A gene, important in differentiation of B cell progenitors and specification of the B cell fate.
FCRL4	Fc-receptor-like-4 is a member of the immunoglobulin superfamily that inhibits BCR signaling by recruiting phosphatases to its intracellular immunoreceptor tyrosine-based inhibitory motifs. FCRL4 is expressed primarily by a subpopulation of memory B cells.
FGL2	Fibrinogen-like protein 2 is a member of the fibrinogen-related protein superfamily, with the secreted form of the protein having immunosuppressive functions and possibly inducing the differentiation of regulatory B cells. B1 cells have higher FGL2 mRNA expression than B2 cells.
Flt3	FMS-like receptor tyrosine kinase-3 is a tyrosine kinase receptor with signaling important in differentiation of early hematopoietic progenitors from the multi-potent progenitors through the common lymphoid progenitor stages.
FOXO	Forkhead box transcription factors of the O class with members including FOXO1 and FOXO3a, have a winged helix-binding motif. In the B lymphoid lineage these transcription factors promote differentiation of B cell progenitors.
GALT	Gut associated lymphoid tissues such as Peyer's patches in the ileum, the sacculus rotundus, and the appendix, are important sites of B cell expansion and diversification in rabbits and sheep.
GATA1	GATA binding protein 1 is a member of the GATA family of transcription factors and is essential for erythropoiesis.

Ikaros	Ikaros is the founding member of a family of zinc finger transcription factors, encoded by the IKZF1 gene, with important functions in differentiation of early hematopoietic progenitors (such as LMPPs), and later stages of B cell differentiation, maturation, proliferation, and germinal center formation.
IL-10	Interleukin 10 is an anti-inflammatory cytokine that has widespread effects on hematopoietic cells including inhibiting inflammatory cytokine secretion and MHC class II expression by monocytes and macrophages, inhibiting differentiation of Th1 and Th17 cells, promoting development of $FoxP3^+$ Tregs, and promoting B cell survival.
IGLL1	Immunoglobulin lambda-like polypeptide 1 forms the surrogate light chain heterodimer with VpreB that associates with the rearranged heavy chain to form part of the Pre-BCR.
IL-7R	The interleukin 7 receptor is composed of a heterodimer of the IL-7α chain and IL-2γc chain expressed from the CLP to pre-B cell stage, with signaling important in differentiation and proliferation of pre-pro B and pro-B cells.
Immature B cell	A B cell that has completed V(D)J rearrangements expressing IgM as part of the BCR. Immature B cells egress from the bone marrow and complete their maturation in peripheral lymphoid tissues.
IRF4, IRF8	Interferon regulatory factors 4 and 8 are transcription factors that contain a tryptophan pentad repeat DNA-binding domain and are important in the transition of pre-B cells to immature B cells.
KIT	c-KIT is a receptor tyrosine kinase expressed by HSCs and B cell progenitors through the pro-B cell stage. Binding to its ligand, stem cell factor, activates the receptor and promotes survival of HSCs and development of B cell progenitors.
LEF1	Lymphoid enhancer-binding factor 1 is a HMG box transcription factor that mediates Wnt signaling and is important in the development of pro-B cells.
LIN28B	The Lin-28 homologue B is a transcription factor expressed in fetal and neonatal hematopoietic cells and fetal derived hematopoietic lineages (B1 B cells, MZ B cells $\gamma\delta$T cells, erythrocytes expressing fetal hemoglobin).
LT-HSC	Long-term reconstituting HSCs are capable of both self-renewal and multi-lineage differentiation.

LMPP	Lymphoid-primed multipotent progenitors are derived from ST-HSCs, and can differentiate into lymphoid and myeloid, but not erythroid, megakaryocyte, or granulocyte lineages.
Lyn	Lyn is a Src-family protein tyrosine kinase important in pre-BCR and BCR signal transduction cascades. Along with Syk, Lyn is recruited to the phosphorylated immunoreceptor activating motifs of CD79a and CD79b following BCR stimulation and phosphorylates downstream targets that promote (CD19, Syk) and inhibit (CD22) BCR signaling.
MZ B cell	Marginal Zone B cells reside mainly in the marginal zone between the red and white pulp of the spleen, and share functional characteristics with both B1 and B2 cells.
N-nucleotide	Non-template nucleotides are sequence independent nucleotide additions to the regions flanking immunoglobulin gene segments during V(D)J recombination by the enzyme terminal deoxynucleotidyl transferase (TdT).
NOTCH1	The Notch1 transmembrane receptor is important in specifying the T lymphoid lineage.
PC	Phosphorylcholine is the polar headgroup of some phospholipids expressed by bacterial and helminth pathogens, oxidized lipids, and cellular membranes following oxidative damage.
PtC	Phosphatidylcholine is a phospholipid commonly expressed by bacteria, exposed on bromelain-treated red blood cells, and is an antigen frequently recognized by B1 cell BCRs.
PAX5	Paired-box 5 is a B cell specific transcription factor that binds to DNA with a paired domain (box). PAX5 regulates commitment to the B cell lineage in pro-B cells and maintains the B cell identity by promoting transcription of B cell specific genes and repressing lineage inappropriate genes.
PLCγ2	Phospholipase C gamma 2 is a transmembrane enzyme important in pre-BCR and BCR signaling. Upon phosphorylation, PLCγ2 converts phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate and diacylglycerol, activates protein kinase C beta, ultimately activating nuclear factor κB (NFκB) and nuclear factor of activated T cells (NFATc).

P-nucleotide	Palindromic-nucleotides are template dependent dinucleotide additions palindromic to the flanking ends being joined from hairpin intermediates during V(D)J recombination events.
Pre-B cell	Pre-B cells are B cell progenitors that express productively rearranged heavy chains with the surrogate light chains as part of the pre-BCR, and in later stages undergo rearrangement of light chain variable gene segments.
Pre-BCR	The pre-B cell receptor is composed of the productively rearranged heavy chain associated the surrogate light chain (consisting of VpreB and λ5 proteins), CD79a, and CD79b, and is expressed on the membrane of pre-B cells.
Pre-Pro B cell	The pre-pro B cell is the earliest B cell specific progenitor that expresses the transcription factors EBF and E2A, but has not completed rearrangement of the immunoglobulin heavy chain variable gene segments.
Pro-B cell	Pro-B cells are B cell progenitors that have committed to the B cell lineage. These cells express PAX5 and have rearranged the immunoglobulin heavy chain gene segments.
PU.1	PU.1 is a transcription factor encoded by the SPI1 gene with an Ets binding domain, is expressed in multi-potent hematopoietic progenitors, and has important functions in the differentiation of myeloid and B lymphoid lineages.
RAG1, RAG2	The recombination-activating proteins encoded by the RAG1 and RAG2 genes are essential for V(D)J rearrangement of the immunoglobulin molecule in B cells, and T cell receptor in T cells.
RSSs	Recombination signal sequences are conserved nanomer and heptamer sequences separated by either a 12 or 23bp spacer that flank each V(D)J segment; Segments with different length spacers are recombined ensuring that only IGHD to IGHJ, or IGHV to IGHD-IGHJ recombination occurs according to the 12/23 rule.
SHP-1	SHP-1 is a member of the protein tyrosine phosphatase family that binds to immunoreceptor tyrosine-based inhibitory motifs on receptors such as CD22, FcγRIIB, FCRLs, and dephosphorylates a variety of targets inhibiting BCR signaling.

ST-HSC	Short-term reconstituting HSCs have limited capabilities for self-renewal, but have the same multi-lineage differentiation potential as LT-HSCs.
STAT3	Signal transducer and activator of transcription 3 is a signaling molecule that is activated by phosphorylation by the Janus-family tyrosine kinases allowing it to translocate into the nucleus, bind to DNA, and activate a variety of different genes.
SOX4, SOX5	Sex determining region Y – box 4 and 5 are HMG box transcription factors with widespread effects determining cell fate. SOX4 important in the proliferation of pro-B cells in response to IL-7 signaling and transitioning to the pre-B cell stage. SOX5 is upregulated in human innate-like B1 cell populations ($FCRL4^{+}$ and $CD21^{lo}$).
Syk	Spleen tyrosine kinase is a cytoplasmic non-receptor tyrosine kinase that is recruited to the phosphorylated immunoreceptor tyrosine activating motifs of CD79a and CD79b following BCR stimulation, and subsequently phosphorylates different substrates downstream of the receptor including BLNK and the Rho family guanine nucleotide exchange factor Vav.
SYT11	Synaptotagmin XI is a member of the synaptotagmin proteins, and in macrophages has been shown to negatively regulate phagocytosis and cytokine secretion.
TSLP	Thymic stromal-derived lymphopoietin is a cytokine important in fetal liver pro-B cell survival and differentiation, the receptor of which is composed of the IL-7α chain along with at least one other protein named TSLP receptor.
TI antigens	T cell-independent antigens can elicit an antibody response without T cell stimulation. TI type 1 (TI-1) antigens stimulate B cells by binding to both the BCR and a second receptor (such as a TLR), while TI type 2 (TI-2) antigens have repeated epitopes that induce multivalent crosslinking of the BCR. In contrast for T cell-dependent antigens, peptides are presented to T cells on MHC class II, and the T cell delivers activating signals to the B cells.
VPREB	VPREB encodes the VpreB protein that forms the surrogate light chain heterodimer with the immunoglobulin lambda-like polypeptide 1. In humans there are 2 VpreB genes (VPREB1, VPREB2) and in mice there are 3 (VPREB1, VPREB2, VPREB3).

YS	The yolk sac is an extra-embryonic tissue where hematopoiesis initiates.