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Developmental expression of B cell molecules in equine lymphoid tissues

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Abstract

Identification and classification of B cell subpopulations has been shown to be challenging and inconsistent among different species. Our study tested aspects of ontogeny, phenotype, tissue distribution, and function of equine CD5^{hi} B cells, which represented a greater proportion of B cells early in development and in the peritoneal cavity. CD5^{hi} and CD5^{lo} B cells differentially expressed B cell markers (CD2, CD21, IgM) measured using flow cytometry, but similar mRNA expression of signature genes (DGKA, FGL2, PAX5, IGHM, IL10) measured using quantitative RT-PCR. Sequencing lambda light chain segments revealed that CD5^{hi} B cells generated diverse immunoglobulin repertoires, and more frequently bound to fluorescence-labeled phosphorylcholine. This study shows developmental characteristics and tissue distribution of a newly described subpopulation of B cells in the horse.

Keywords

Equine; B cell; Development; CD5; Immunoglobulin repertoire

Introduction

In mice, the development of the humoral immune system begins during gestation with the production of three main populations of B cells: 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

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greater abundance near the end of fetal life and in the adult (Hayakawa et al., 1983); 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). In mice, B1 cells are produced in the fetal liver and populate mainly the peritoneal and pleural cavities, with a small population in the spleen (Montecino-Rodriguez and Dorshkind, 2006 and Baumgarth, 2011). In contrast, B2 cells are abundant in peripheral blood and other lymphoid tissues, including the bone marrow, spleen, and lymph nodes.

B1 cells in the mouse were initially identified based on the expression of CD5, and the unique functional characteristic of spontaneous secretion of IgM (Hayakawa et al., 1983, Hayakawa et al., 1984, Hardy et al., 1989, and Hayakawa et al., 1990). 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 to raise 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 B1 cells were further classified as B1a (CD5⁺) or B1b (CD5⁻), and are best distinguished from B2 cells with a combination of different markers (Herzenberg et al., 1987, Tung et al., 2004 and Yenson and Baumgarth, 2014).

In human cord blood and neonates, 70–90% of B cells are CD5⁺ (Carsetti et al., 2004), and this percentage decreases to 10–20% in peripheral blood with age (Hardy et al., 1987, Gary-Gouy et al., 2002 and Carsetti et al., 2004). In humans, CD5 is expressed by many different B cell subsets from immature to memory B cell stages in addition to B1a-like B cells (Carsetti et al., 2004, Dalloul, 2009 and Lee et al., 2009). CD5 expression can be induced in response to certain stimuli (Appleyard and Wilkie, 1998, Berland et al., 2010, Yanaba et al., 2009 and Dalloul, 2009), and the simultaneous stimulation of both the BCR and CD40 promotes CD5 upregulation (Gagro et al., 2000). Griffin et al. (2011a) recently identified a human equivalent to the murine B1 cells in umbilical cord blood with the phenotype CD20⁺CD27⁺CD43⁺CD70⁻, the majority also co-expressing CD5, though the unique phenotypic and functional identity of these cells continues to be debated (Descatoire et al., 2011, Griffin et al., 2011b, Perez-Andres et al., 2011 and Bemark, 2015).

B lymphopoiesis can be readily detected at the molecular level in the equine fetus around 90 to 120 days of gestation (DG) but potentially earlier in the yolk sac of the embryo (Tallmadge et al., 2009, Tallmadge et al., 2013 and 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 90–120DG (Tallmadge et al., 2009). The expression of CD19, CD21, immunoglobulin, and major histocompatibility complex I and II have been described in equine B cells using flow cytometry (Kydd et al., 1994, Lunn et al., 1998 and Ibrahim and Steinbach, 2012). Although CD2 and CD5 have been considered equine T cell markers, our research laboratory observed throughout the years that the percentage of equine peripheral blood lymphocytes expressing CD2 or CD5 is greater than that of CD3⁺ T lymphocytes. Taken the fact that natural killer cells do not express CD5 (Dalloul, 2009), the unaccounted for CD2⁺CD5⁺CD3⁻ population would possibly represent a subset of B cells. CD5⁺ B cells have been observed in different

species, including pigs (Appleyard and Wilkie, 1998), cattle (Naessens and Williams, 1992), rabbits (Raman and Knight, 1992), and guinea pigs (Dilwith and Wicher, 1997).

The purpose of this study was to test how aspects of ontogeny, phenotype, tissue distribution, and function characterize equine B cells. Our hypothesis was that subpopulations of B cells exist in the horse according to age and tissue. We found that CD5 expression on equine B cells was not distinctly present or absent; instead, expression was variable from CD5^{hi} to CD5^{lo}, similar to observations described in other species, and that the frequency of equine CD5^{hi} B cells varied with development and between tissues.

Materials and methods

2.1 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. Different types of lymphoid tissues were sampled by age groups because of their availability and accessibility. Fetal liver leukocytes were obtained as previously described (Battista et al., 2014). In brief, 2 adult mares (1 Thoroughbred and 1 Warmblood) at the Cornell Equine Park were bred, 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 each of 4 healthy Warmblood foals belonging to the Cornell Equine Park at five different time points (same foals) including 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 obtained from 8 healthy adult horses belonging to the Cornell Equine Park (6 Warmbloods, 1 Thoroughbred-cross, and 1 Quarter Horse, ages 8–20yrs). 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 research programs at Cornell University College of Veterinary Medicine (10 Thoroughbreds, 2 Quarter Horses, 1 Arabian, 1 breed unknown, ages 1–25 yrs). 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 cell suspensions using Ficoll gradient centrifugation (density 1.077, GE Healthcare, Piscataway, NJ) as previously described (Flaminio et al., 2000). For analyses not performed immediately, cells were stored 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.

2.2 Flow cytometric lymphocyte immunophenotyping

One million cells per sample were blocked with 10% normal goat serum in phosphate buffered solution (PBS) and labeled with an equine-specific B cell monoclonal antibody (CD19-like, clone cz2.1, D.F. Antczak, Cornell University, Ithaca, NY) (Kydd et al., 1994) 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] (Lunn et al., 1998), 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), or major histocompatibility complex (MHC) class II-Alexa Fluor 647 (cz11, D.F. Antczak), for a 4-color cytometric analysis. 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, and IgG1-APC (MOPC-21, BD Biosciences). Cells were fixed with 2% paraformaldehyde in PBS, and fluorescence 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 for each fluorophore confirmed that non-specific staining was at least less than 2% of lymphocyte-gated cells.

2.3 Sorting of CD19⁺CD3⁻CD5^{hi} and CD19⁺CD3⁻CD5^{lo} B cells

Using the gate strategies in Fig. 1A, our flow cytometric data revealed that B cells expressed the CD5 molecule with high (CD5^{hi}), intermediate and low (CD5^{lo}) intensity in all age groups and tissue types studied, and CD19⁺CD3⁻CD5^{hi} and CD19⁺CD3⁻CD5^{lo} cells were used to further characterize B cell populations during development and in different tissues. A custom mouse anti-equine CD19 antibody was made by Abmart (Shanghai, China) to replace the Cz2.1 CD19-like hybridoma cells that became no longer viable. The monoclonal antibody production included design and generation of multiple peptide epitopes based on the extracellular domain 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 calf serum (FCS), penicillin and streptomycin (Thermo Fisher Scientific) until medium exhaustion. Supernatants were collected and centrifuged at 300 × g for 5 min to remove cells and debris, and aliquots stored at 4 °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 when compared with the equine anti-CD19-like (clone cz2.1) monoclonal antibody. Two-step labeling (primary antibody followed by fluorophore-conjugated secondary antibody) was performed for this marker since quality and fluorescence intensity were superior when compared to the fluorophore-conjugated product (Supplemental Fig. 1).

Between 20 to 30×10⁶ isolated PBL were labeled as described above with the custom monoclonal antibody anti-equine CD19 followed by 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% FCS (Thermo Fisher Scientific). Leukocytes were resuspended in RPMI with 5% FCS for cell sorting. CD19⁺CD3⁻CD5^{hi} and CD19⁺CD3⁻CD5^{lo} were sorted with the BD FACSAria III Cell Sorter at the Cornell Biomedical Sciences Flow Cytometry Core Lab (Cornell University, Ithaca, NY).

2.4 Quantitative RT-PCR measurement of gene expression profiles

Peripheral blood CD19⁺CD3⁻CD5^{hi} or CD19⁺CD3⁻CD5^{lo} leukocytes from 5 equine healthy donors (four adult horses, one 21-day-old foal) were sorted as described above directly into RNA lysis buffer, and RNA was isolated with the Quick-RNATM MicroPrep kit with DNase I treatment to destroy genomic DNA (Zymo Research, Irvine, CA) according to manufacturer's instructions. The concentration of RNA was quantified using a NanoDrop (Thermo Fisher 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 genes are part of the differential B1 and B2 cell signature described by Yamagata et al. (2006): DGKA that attenuates BCR signaling is highly expressed in B2 cells (Wheeler et al., 2013), and FGL2 with roles of immunosuppression (Wang et al., 2014) is 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 similar expression in B1 when compared with B2 cells in 2 different studies (Tumang et al., 2005 and Fuxa and Busslinger, 2007). IL-10 and IGHM have greater expression in B1 cells (O'Garra and Howard, 1992 and Rothstein et al., 2013) and were measured as molecular markers of function. Finally, Ig kappa light chain (IGK) and Ig lambda light chain (IGL) mRNA expression was measured in peripheral blood CD19⁺CD3⁻CD5^{hi} and CD19⁺CD3⁻CD5^{lo} B cells from 3 adult horse donors. Since the B cells were sorted directly into RNA lysis buffer, the mRNA expression of CD5 was measured to confirm that there was enrichment for CD5^{hi} and CD5^{lo} populations (Supplemental Fig. 2A).

Ten microliter qRT-PCR reactions were performed in triplicate with 500nM of primer and iTaqTMUniversal SYBR Green One-Step kit (Bio-Rad, Hercules, CA) in a CFX96 Real-Time PCR Detection System (Bio-Rad). Cycling parameters were: 50 °C × 10 min, 95 °C × 5 min, [95 °C × 10 sec, 60 °C × 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 were 63 °C for IGK, 61 °C for CD5, 58 °C for IL-10, and 57 °C for FGL2. SYBR primers (Table 1) were designed with Beacon Designer 7.91 software (PREMIER Biosoft International, Palo Alto, 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, La Jolla, CA). In addition, to measure the fold difference in expression between CD5^{hi} and CD5^{lo} B cells, $2^{-\Delta\text{Ct}}$ was calculated with the CD5 mRNA expression calibrated to the CD5^{lo} sample.

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 Igs, peritoneal B1 cells have been shown to use lambda light chains in 20% of Igs (Hayakawa et al., 1986). Therefore, absolute quantification was performed for IGL and IGK, and RNA standard curves were prepared for each of these genes (Badial et al., 2015) to determine whether equine CD5^{hi} and CD5^{lo} B cells use similar ratios of IGL and IGK light chains. A region that included the SYBR primers was amplified from whole peripheral blood cDNA (Table 1) with iProof polymerase (iProof™ High-Fidelity PCR kit, Bio-Rad) for IGL or with 5'-rapid amplification of cDNA ends (RACE, SMARTer™ RACE cDNA Amplification Kit, Clontech, Mountain View, CA) for IGK according to manufacturer's instructions (Badial et al., 2015) cloned into pJET1.2 vector (CloneJET™ PCR Cloning Kit, Thermo Fisher 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 Fisher Scientific). RNA transcripts were purified with the RNA Clean & Concentrator™ kit (Zymo Research) and quantified using the NanoDrop (Thermo Fisher Scientific). Ten-fold serial dilutions were made, and absolute quantification of mRNA transcripts was determined from the RNA standard curve with the CFX Manager software (Bio-Rad).

2.5 Immunoglobulin lambda light chain sequencing

RNA was isolated from peripheral blood CD19⁺CD3⁻CD5^{hi} or CD19⁺CD3⁻CD5^{lo} B cells sorted from 2 healthy equine donors (one adult horse, one 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 1× 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 Ig sequences previously described (Tallmadge et al., 2014). The RT-PCR reaction was performed with the iProof™ High-Fidelity PCR kit (Bio-Rad) in a 50μl reaction including 10ng cDNA, and 1× iProof HF buffer with 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 × 3 min, [denaturing 98 °C × 10 sec, annealing 60 °C × 10 sec, extension 72 °C × 10 sec] repeated 40 times, and final extension 72 °C × 10 min. Up to 40ul 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 *Escherichia 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 Ig lambda variable genes (IGLV), 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 1× 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.

2.6 Immunoglobulin lambda light chain analysis

Sixty-four CD19⁺CD3⁻CD5^{hi} (32 from each donor) and sixty-seven CD19⁺CD3⁻CD5^{lo} (34 from the foal and 33 from the adult horse) unique and productive 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 (<http://www.genious.com>, (Kearse et al., 2012)); the first 8 nucleotides that overlapped with the forward primer were not included in the analysis to avoid potential bias. The lambda light chain complementary determining region 3 (CDR3) was measured between the YCC motif in the IGLV and FGG motif in the Ig lambda joining (IGLJ) gene segments as previously described (Ford et al., 1994 and Sun et al., 2010).

2.7 BCR specificity for phosphorylcholine

B1 cells are enriched with BCRs that have specificity to phosphorylcholine (Lalor and Morahan, 1990, Griffin et al., 2011a and Yammani and Haas, 2013). Phosphorylcholine-specific B cells were identified by incubating 1×10^6 cells with $40 \mu\text{g/ml}$ 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, RPE-conjugated (AbDSerotec) CD4 (HB61A, WSU Monoclonal Antibody Center), and RPE-conjugated CD8 (HT14A, WSU Monoclonal Antibody Center). CD4 and CD8 monoclonal antibodies were used to exclude the T cell population in the analysis, since they are available in purified form for RPE conjugation. 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).

2.8 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 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 for increased stringency. 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 ≤ 0.05 was considered significant.

The liver is the main hematopoietic organ in early fetal life, and the bone marrow takes over this role later in gestation and after birth. In addition, in the species described, the liver is the main site for B1 cell lymphopoiesis, markedly during fetal life. Therefore, in this study, we included the equine fetal liver as the source of leukocytes for the fetal stage of life, but not for the foal and adult horse since hepatic hematopoietic activity is decreased in these groups. In addition, access was limited to adequate amount of peripheral blood from the 90–120d fetus, and flow cytometric studies were not possible for this stage. Consequently, direct statistical comparisons between the fetal liver and peripheral blood leukocytes of the foal or adult horse were not pursued; instead, values were described independently for the fetus leukocytes, and comparisons made between foal and adult horse variables.

Results

3.1 Expression of B cell molecules during development

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 (Fig. 1B). The median percentage of CD5^{hi} B cells was the greatest in the fetal liver (20%) and foal peripheral blood leukocytes at days 21–28 of life (20–21%), and the lowest in the adult horse peripheral blood leukocytes (14%), with a statistically significant difference between the 21-day-old foal and adult horse (p-value = 0.02) (Fig. 1). Overall, CD5 expression in CD19⁺CD3[−] B cells was dimmer than in CD3⁺ T cells for all age groups, 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, and CD5^{hi} B cells accounted for 0.2–3.2% of total CD5⁺ lymphocytes; the remaining were negative for CD19 and CD3 or excluded as potential T:B cell doublets.

3.2 Distribution of B cells expressing CD5 in different tissues

To test for variation of the distribution of B cells expressing CD5 in different body sites housing immune cells, multicolor flow cytometric analysis was used to measure the percentage of CD5^{hi} B cells in the peritoneal cavity, spleen, and bone marrow samples. When compared to PBL, a statistically significant greater (p-value = 0.03) median percentage of B cells were CD5^{hi} in the peritoneal cavity (26%), similar proportion in the bone marrow (10%, p-value = 0.11), and less in spleen (7%, p-value = 0.008) (Fig. 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), but not between bone marrow and spleen (p-value = 0.06) samples (Fig. 2).

3.3 Co-expression of surface molecules in B cells during development

The co-expression of CD5, CD21, CD2, IgM, and MHC class II in B cells was measured using multicolor flow cytometric analysis in 3- to 42-day-old foal and adult horse PBL (Fig. 3A). 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 and Molnar et al., 2008). Greater (p-values = 0.02) percentages of foal CD5^{hi} (medians 79–89%) and CD5^{lo} B cells (medians 64–88%) expressed CD21 when compared to adult horse CD5^{hi} (median 48%) and CD5^{lo} B cells (median 41%), respectively, for all time points measured (Fig. 3B). 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 (Fig. 3).

CD2 is involved in cell adhesion and activation (Moingeon et al., 1989), and has traditionally been considered a T cell marker in the horse. CD2 expression in peripheral blood 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 were greater (p-values = 0.02) than for CD5^{lo} B cells (Fig. 3).

The percentage of CD5^{hi} and CD5^{lo} peripheral blood 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 adult horse (medians for CD5^{hi} 59%, CD5^{lo} 61%); in contrast, a greater (p-values = 0.03) percentage of CD5^{hi} (medians 78–85%) and CD5^{lo} B cells (medians 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) (Fig. 3B). However, the MFI of IgM expression in adult horse CD5^{hi} B cells was greater (p-value = 0.05) than that of CD5^{lo} B cells (Fig. 3C).

The expression of MHC class II by CD5^{hi} and CD5^{lo} B cells and CD3⁺ T cells was measured in fetal liver leukocytes and PBL from foal and adult horses. Greater than 98% (medians = 99) of CD5^{hi} and CD5^{lo} B cells in the fetal liver and foal PBL, and 92% (medians = 99) in the adult horse PBL expressed MHC class II. In contrast, median percentage of PBL 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 (Fig. 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 for CD5^{hi} B cells, CD5^{lo} B cells, and CD3⁺ T cells in the foal (p-values = 0.01) and adult horse (p-value = 0.04) PBL (Fig. 3B); however, no difference (foal p-values = 0.4, adult horse p-value = 0.06) was observed for the pairwise comparison of the percentage of CD5^{hi} and CD5^{lo} B cells expressing MHC class II at any life stage (Fig. 3B). The MFI for MHC class II expression was similar (p-value = 0.1) in adult horse CD5^{hi} and CD5^{lo} B cells (Fig. 3C).

3.4 mRNA expression of selected B cell subpopulation signature genes

In addition to describing the phenotype of CD5^{hi} B cells with surface molecule expression, CD5^{hi} and CD5^{lo} B cells were tested for genes known to be differentially expressed by subpopulations of B cells (Supplemental Fig. 2). B cells sorted for high surface protein expression of CD5 had a higher relative expression of CD5 at the mRNA level that approached statistical significance (p-value = 0.06) when compared to B cells that had low protein expression (Supplemental Fig. 2B). To measure the fold difference in expression between CD5^{hi} and CD5^{lo} B cells, $2^{-\Delta C_t}$ 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 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, IGHM, and IL10 were comparable (p-values = 0.4) between CD5^{hi} and CD5^{lo} B cells (Supplemental Fig. 2B).

3.5 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. 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 Ig chains were measured using absolute quantitative RT-PCR. Higher mRNA copy numbers of IGL than IGK were observed in CD5^{hi} and CD5^{lo} B cells, with similar (p-value = 0.8) median IGL/IGK ratios (74 and 82, respectively) (Fig. 4A). The diversity of the lambda light chain was also measured in order 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 (Fig. 4B and Supplemental Fig. 3). Overall, IGLV8-128 and IGLV8-122 were the most frequently observed IGLV segments in this study. 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, therefore, foal and adult horse Ig 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 (Fig. 4B). 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 Ig 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 and Tallmadge et al., 2014), and was homozygous for a novel IGLV8-128 allele named IGLV8-128*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) between 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 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, CDR3 length was tested in CD5^{hi} and CD5^{lo} B cells. The median CDR3 amino acid lengths were similar (p-values = 0.4) between CD5^{hi} (median 10 foal, 11 adult horse) and CD5^{lo} B cells (median 10 foal, 11 adult horse) (Fig. 4C).

3.6 BCR specificity for phosphorylcholine

Phosphorylcholine (PC) is the polar headgroup of the phospholipids phosphatidylcholine and sphingomyelin exposed on cellular membranes following oxidative damage or phospholipid metabolism, respectively, and is expressed by different pathogens (Briles et al., 1982 and Jenkins et al., 2009). B1 cells described in other species are enriched with BCRs that have specificity to PC (Lalor and Morahan, 1990, Griffin et al., 2011a and 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) (Fig. 5 and Supplemental Fig. 4).

Discussion

The studies herein show that equine B cells express low to high levels of CD5, and co-expression of CD21, CD2, and IgM varies with age. Equine CD5^{hi} B cells represent a greater proportion of B cells early in development; a marked proportion of B cells in the fetal liver are CD5^{hi} and a greater proportion of B cells in the peripheral blood of foals are CD5^{hi} compared to adult horses. 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}CD21^{int/-}IgM^{hi/-}MHCII⁺ in adult horse peripheral blood (Table 2). Peripheral blood CD5^{hi} B cells have diverse Ig lambda light chain repertoires similar to CD5^{lo} B cells but more frequently express BCRs that bind to PC. CD5^{lo} B cells represent the majority of the B cell population at all developmental stages and increase in proportion in the peripheral blood with development, consistent with B2 cells described in other species.

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 horse peripheral blood varies by species: 10–20% in humans (Carsetti et al., 2004 and Dalloul, 2009), 10–20% in the pig (Appleyard and Wilkie, 1998 and Wilson and Wilkie, 2007), 1% in the guinea pig (Dilworth 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, the presence of a small population of B cells expressing CD5 has also been observed but not fully characterized using another monoclonal antibody (Blanchard-Channell et al., 1994). By using multi-color flow cytometry to rigorously exclude potential T:B cell doublets, it became apparent that B cells expressed the CD5 molecule with high (CD5^{hi}), intermediate and low (CD5^{lo}) intensity. Due to the low level of expression of CD5 by B cells compared to T cells, initial studies characterizing CD5 expression by equine B cells may have interpreted the CD5^{lo} B population identified in our study as CD5⁻. In our

study, CD5^{hi} B cells represented a larger proportion of B cells in the PBL of the foal when compared to the adult horse, similarly to how B1 cells (Hayakawa et al., 1983, Hayakawa et al., 1986 and Griffin et al., 2011a) 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 and Carsetti et al., 2004). The percentage of B cells that are CD5⁺ in the pig (Appleyard and Wilkie, 1998) and rabbit (Hayward et al., 1978 and Lanning et al., 2000) do not significantly change with development.

Similar to B1 cells in mice, the greatest percentages of CD5^{hi} B cells were found in the peritoneal cavity of the adult horse (Baumgarth, 2011). In contrast to B1 cells in mice and CD5⁺ B cells in pigs (Appleyard and Wilkie, 1998, and Baumgarth, 2011), the equine spleen contained the smallest proportion of B cells that were CD5^{hi}. It remains unknown whether the larger proportion of CD5^{hi} B cells is unique to the B cell population of the PBL early in development, or whether their frequency in the peritoneal cavity and lymphoid tissues are developmentally regulated as well. Another potential limitation of this study includes that all of the foals and the majority of adult horses PBL samples were from Warmbloods, and it is unknown whether there is variation in the proportion of CD5^{hi} B cells in different breeds

To our knowledge, the expression of CD2 in a small percentage of equine B cells is a novel finding for this species, despite the fact that the antibody used in our assays (monoclonal antibody clone HB88a) was partially characterized in the Second International Workshop on Equine Leukocyte Antigens (Lunn et al., 1998). 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). In the pig, expression on B cells 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 (81%) when compared to the adult horse (94%) (Fig. 3). CD2 plays important roles in adhesion and activation of T cells (Moingeon et al., 1989) but its function is less understood in B cells.

In the horse, a greater percentage of CD5^{hi} B cells than CD5^{lo} B cells express CD21 in the PBL. 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, 2012; 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 roles, such as increased efficiency capturing immune complexes.

CD5 was historically used to identify murine B1a cells. B1 cells are considered components of innate immunity and have important developmental, phenotypic, and functional differences from B2 cells (Berland and Wortis, 2002, Baumgarth, 2011 and Montecino-

Rodriguez and Dorshkind, 2012). B1 cells play important roles in immunity to pathogens by secreting natural antibodies (Boes et al., 1998, Ochsenbein et al., 1999, Baumgarth et al., 2000 and Haas et al., 2005) and acting as effective antigen presenting cells (Zhong et al., 2007). B1 cells contribute to homeostasis by promoting efferocytosis (Chen et al., 2009) and secreting the anti-inflammatory cytokine IL-10 (O'Garra and Howard, 1992 and Griffin and Rothstein, 2012), although they 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 (Rothstein and Kolber, 1988, Morris and Rothstein, 1993, Tumang et al., 2004 and Hardy and Hayakawa, 2015b) tonic BCR signal transduction events (Karras et al., 1997 and Holodick et al., 2009), gene expression profiles (Yamagata et al., 2006, Rothstein et al., 2013 and Mabbott and Gray, 2014), and limited immunoglobulin (Ig) repertoires (Tornberg and Holmberg, 1995, Kantor et al., 1997 and Roy et al., 2009)

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 (Tumang et al., 2005, Yamagata et al., 2006, Diehl et al., 2011 and Mabbott and Gray, 2014), though 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). The lack of observed differential gene expression between CD5^{hi} and CD5^{lo} B cells in the horse may therefore be explained by species differences in differential expression of genes by B1 and B2 B cells, and technical limitations preventing isolation of pure CD5^{pos} or CD5^{neg} B cell populations.

Critical to B lymphopoiesis from the pro-B cell through the immature B cell developmental stage is the generation of a functional immunoglobulin (Ig) molecule, which requires somatic recombination of the heavy and lambda or kappa light chain gene segments. The pre-immune Ig receptor repertoire develops during fetal life in the absence of exogenous antigens in the primary lymphoid tissues (Sun et al., 1998 and Liljavirta et al., 2014). B1 B cells in mice use different V(D)J segments than B2 B cells (Kantor et al., 1997). The antibodies classically produced by murine B1 cells against antigens, including Thy-1, PtC (phosphatidylcholine), and PC are constructed with restricted Ig gene segments, and these IGH gene families were found to be overrepresented in Ig repertoires of B1 compared to B2 cells (Pennell et al., 1989, Hayakawa et al., 1990, and Kantor et al., 1997). However, immunoglobulin diversity is generated differently among species. Previously, we observed that although there was a bias for IGLV segment use in fetal life, the equine fetus used a broader range of IGLV segments than the adult horse (Battista et al., 2014 and Tallmadge et al., 2014). In this study, we wanted to learn (1) if there were differences in IGLV segment used between CD5^{hi} and CD5^{lo} B 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). IGLV8-128 and IGLV8-122 were the most frequently observed IGLV segments in this study, indicating that IGLV bias of the individuals in this study is comparable to previous reports (Sun et al., 2010, Hara et al., 2012 and Tallmadge et al., 2014). However, we 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. These trends in IGL diversity were not observed in CD5^{hi} and CD5^{lo} B cells in the adult horse. Future studies are needed to determine whether other biases are observed in a larger number of individuals, including different breeds.

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, murine B1b cells and human antigen specific B1-like cells have been shown to express Ig sequences that contain mutations (Roy et al., 2009 and Fiskesund et al., 2014). The expression of mutated IGL sequences allows equine CD5^{hi} B cells the potential to respond to a greater range of different antigens for humoral protection. It is unknown whether the CD5^{hi} cells are naïve B cells or include a subset of memory B cells. Between 49–85% CD5^{hi} B cells expressed IgM in the peripheral blood of foal and adult horses; therefore, it seems likely that a proportion of the IgM^{negative} cells have undergone class switch recombination. Further studies are necessary to confirm and determine which other immunoglobulin isotypes these cells express.

Phosphorylcholine is found on bacteria, helminths, apoptotic cell membranes, and oxidized lipids (Peters et al., 1999 and Binder et al., 2002). 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 (Shaw et al., 2000 and Kyaw et al., 2011), 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, equivalent percentages of PC-specific human B cells have been shown to be CD5^{hi} and CD5^{lo} (Fiskesund et al., 2014). CD5^{hi} B cells in the foal PBL were more frequently specific for PC than in the adult horse, perhaps due to differences in Ig repertoire between the developmental stages. B cells derived from precursors originating in fetal and adult life in mice and humans are known to have functional differences, particularly with response to antigenic stimulation and Ig repertoire (Hardy and Hayakawa, 2015a). 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 a 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 and Clark and Weiser, 2013).

Despite the published heterogeneity of B1 cell phenotypic and functional classification among species, the studies reveal the presence of equine CD5^{hi} B cells, which 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) and enrichment for BCRs specific for PC. Unlike murine B1 cells but similarly to proposed human B1 cells, equine CD5^{hi} B cells have diverse Ig lambda chain repertoires. Further studies are needed to determine other functional similarities attributed to equine CD5^{hi} B cells, primarily self-renewal, the ability to spontaneously secrete Ig, isotype-switch,

secrete IL-10, efficiently stimulate T cells, respond to BCR-crosslinking, and develop downstream signaling events such as tonic intracellular signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CDR	complementary determining region
DG	days of gestation
DGKA	diacylglycerol kinase alpha
FCS	fetal calf serum
FGL2	fibrinogen-like protein 2
Ig	immunoglobulin
IGK	Ig kappa light chain
IGL	Ig lambda light chain
IGLJ	Ig lambda joining gene
IGLV	Ig lambda variable gene
IGHM	Ig mu heavy chain
IL-10	interleukin-10
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
N-nucleotide	non-template nucleotide
PAX5	paired box 5
PBS	phosphate buffered solution
PC	phosphorylcholine
PBL	peripheral blood leukocytes
RACE	rapid amplification of cDNA ends

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Fig. 1. Frequency of B cells expressing CD5 molecule during development

(A) Representative dot plots showing the flow cytometric 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, with symbols indicating the frequency measured for a particular individual and medians indicated. These data show that these cells represent a greater proportion of B cells early in life..*p-value = 0.05

Fig. 2. Frequency of B cells expressing CD5 molecule in different tissues

The frequency of B cells expressing CD5 was measured in the peritoneal cavity (PerC, n=5), spleen (SP, n=6), and bone marrow (BM, n=6) of adult horses. The frequency of CD5^{hi} B lymphocytes measured in adult horse PBL in Fig. 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 0.05, **p-value 0.01

Fig. 3. Co-expression of B cell markers in CD5^{hi} and CD5^{lo} B cells in the peripheral blood during development

(A) Gating strategy to measure the co-expression of B cell markers in the peripheral blood during development. Representative dot plots showing the gating strategy to measure 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 0.05, **p-value 0.01

Fig. 4. 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 was higher than the copy number of kappa transcripts, with similar IGL/IGK ratios for CD5^{hi} and CD5^{lo} B cells. (B) Ig 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 is 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.

Fig. 5. Frequency of BCR specificity for phosphorylcholine

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

Table 1

Primer sequences for B cell subpopulation 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 ATGGCAAATGTTCTCTAAG	132
		Reverse GTGTAACCTCTGTAGGTCTCA	
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 GTCATCGATTCTGCCCTGT	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 TCCTCACCTCATCGCTCTC	423
		Reverse CTCAGAGGAGGGCGGGAA	
IGK ^b	XM_003362951	Forward RACE UPM ^d	737
		Reverse AATGTCTATGCCTGCGAGGTCTCCC	

^a(Tallmadge et al., 2012)^b(Badial et al., 2015)^c(Sanchez-Matamoros et al., 2013)^dSMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA)

Table 2Phenotype of equine CD5^{hi} and CD5^{lo} B cells in different lymphoid tissues

Phenotype	
CD5 ^{hi}	CD19 ⁺ CD2 ^{hi} CD21 ^{int/-} IgM ^{hi/-} MHCII ⁺
CD5 ^{lo}	CD19 ⁺ CD2 ^{lo} CD21 ^{int/-} IgM ^{lo/-} MHCII ⁺

⁺ 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/-).