DEVELOPMENTAL ADAPTATIONS OF γδ T CELLS AND B CELLS IN BLOOD AND INTESTINAL MUCOSA FROM BIRTH UNTIL WEANING IN HOLSTEIN BULL CALVES

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ABSTRACT

This study aimed to characterize the development of systemic and colon tissue resident B and γδ T cells in newborn calves from birth until weaning. At birth, calves have limited capacity to initiate immune responses, and the immune system gradually matures over time. Gamma delta (γδ) T cells are an important lymphocyte subset in neonatal calves that confer protection and promote immune tolerance. A total of 36 newborn calves were enrolled in a longitudinal study to characterize how systemic and colon tissue resident B and γδ T cells develop from birth until weaning. Blood and colon biopsy samples were collected on d 2, 28, and 42 to determine the proportions of various B and γδ T cell subsets by flow cytometry. We classified γδ T cells into different functional subsets according to the level of expression intensity of the coreceptors WC1.1 (effector function) and WC1.2 (regulatory function). Furthermore, naïve B cells were classified based on the expression IgM receptor, and activation state was determined based on expression of CD21 and CD32, 2 receptors with opposing signals involved in B cell activation in early life. Additional colon biopsy samples were used for 16S sequencing, and microbial diversity data is reported. At birth, γδ T cells were the most abundant lymphocyte population in blood, accounting for 58.5% of the lymphocyte pool, after which the proportions of these cells declined to 38.2% after weaning. The proportion of γδ T cells expressing WC1.1 decreased by 50% from d 2 to d 28, whereas no change was observed in the expression of WC1.2. In the colon, there was a 50% increase of γδ T cells after weaning and the proportion of WC1.2+ γδ T cells doubled from d 28 to 42. The proportion of IgM+ B lymphocytes in blood increased from 23.6% at birth to 30% after weaning, were the proportion of B cells expressing CD21 increased by 25%, while the proportion of B cells expressing CD32 decreased by 30%. While no changes were observed for the overall proportion of IgM+ B lymphocytes in the colon, there was a 6-fold increase in the proportion of CD21+ B cells from pre (d 28) to postweaning (d 42). Microbial diversity increased from d 2 of life to 28 and declined abruptly after weaning. The reduction in microbial diversity during weaning was negatively correlated with the increase in all γδ T cell subsets and CD21+ B cells. These data suggest that developmental adaptations after birth coordinate expansion of γδ T cells to provide early systemic protection, as well as to steer immune tolerance, while B cells mature over time. Additionally, the increase of colonic γδ T cells on d 42 suggests a protective role of these cells during weaning.

Keywords: Neonatal immunology, Mucosal Immunology, Calf health, Gut health, Weaning

INTRODUCTION

At birth, neonatal calves depend exclusively on the successful transfer of passive immunity from colostrum to confer protection against environmental pathogens. Failure of transfer passive immunity can result in increased calf morbidity and mortality (Urie et al., 2018). During the first weeks of life, calves have limited capacity to initiate immune responses, and the immune system gradually matures over time. A combination of increased fetal cortisol and decreased maternal progesterone before parturition collectively modify cell-mediated effector function in CD4 T helper cells that persists into the early post-natal period to promote a Th2 tolerogenic biased response (Harris and Barletta, 2001; Chase et al., 2008). In addition, maternal immunoglobulins in colostrum decrease B cell activation in a process mediated by the interaction of the FC portion of IgG with the receptor CD32 in naïve B cells, limiting its activation until maternal Ig concentrations
start to decrease in circulation (Chattha et al., 2009). Furthermore, colostrum seems to mediate the expansion of a group of non-traditional T cells called gamma delta T cells (γδ T cells) as colostrum deprived calves have lower levels of γδ T cells in circulation (Krueger et al., 2016).

In cattle, γδ T cells are a major lymphocyte subset and in early life they account for over 50% of all circulating lymphocytes, providing early defense against intracellular infections during a period when other branches of the adaptive immune system are not fully responsive (Hein et al., 1991; Guerra-Maupome et al., 2019). These unconventional T cells possess a set of characteristics that set them apart from the traditional αβ T cells and are starting to emerge as a critical lymphocyte population in early life, endowed with both adaptive and innate-like characteristics, serving as a bridge between the 2 arms of the immune system (Guerra-Maupome et al., 2019). Unlike traditional αβ T cells, γδ T cells do not require antigen presentation in the context of major histocompatibility complexes (MHC) I and II, and alternatively, they can recognize unprocessed antigens directly via the γδ TCR (Baldwin et al., 2021). Furthermore, developmental pre-programming in the thymus dictates the phenotype and function of γδ T cells (Baldwin et al., 2021), and in cattle, functional subtypes can be classified based on the expression of 2 different classes of the transmembrane glycoprotein workshop cluster 1 (WC1). The WC1 receptor serves as a costimulatory molecule similar to CD4 and CD8 in traditional αβ T cells (Wang et al., 2011; Hsu et al., 2015), and can be subdivided into WC1.1+ and WC1.2+. Gamma delta T cells that express the WC1.1+ receptor are implicated in clearing viral infections, and secrete interferon-gamma (IFN-γ) and interleukin 17 (IL-17; Rogers et al., 2005; McGill et al., 2013). Gamma delta T cells that constitutively express the coreceptor WC1.2+ have an immunomodulatory function (Guerra-Maupome et al., 2019; Baldwin et al., 2021), and produce anti-inflammatory cytokines, such as IL-10 and TGF-β, upon stimulation (Hoek et al., 2008; Guzman et al., 2014; Guerra-Maupome et al., 2019).

Of all the immune compartments, the intestinal immune system is the largest in the body and is separated from the lumen, which is densely populated with microorganisms, by only one layer of epithelial cells (Vancamelbeke and Vermeire, 2017). In early life, the intestine is rapidly colonized by microorganisms that provide necessary signals to promote the development of both the intestinal barrier and the mucosal immune system. The intestinal microbiota promotes intestinal health by stimulating epithelial cell proliferation and mucus production, thereby promoting immune exclusion by avoiding direct contact of the intestinal milieu with the intestinal epithelium (Mantis et al., 2011). However, abrupt changes in microbial composition, particularly during early life, can induce aberrant immune function, impacting the progression and pathogenesis of many intestinal disorders (Wood et al., 2015; Abuamjahieh et al., 2016; and Kvidera et al., 2017).

Collectively, it appears that a combination of pre-and post-natal maternal and environmental factors affect the activation and function of the adaptive immune system, driving immune education of the naïve immune system and ensuring proper immune responses against microbial encounters later in life. However, to date, there is paucity of research regarding early-life developmental adaptations of the adaptive immune system in cattle, and how changes in intestinal microbiota around weaning influence immune function. The objectives of this study were to characterize how systemic and colon tissue resident B and γδ T cells develop from birth until weaning. We hypothesized that pre-programmed ontological adaptations during early life stimulate the expansion of γδ T cells, particularly the WC1.2 subset, to promote a tolerogenic phenotype of the naïve immune system to prevent unnecessary immune activation and excessive inflammation in response to early microbial encounters while providing an early mechanism of defense against intracellular infections.

**MATERIALS AND METHODS**

**Animals, Housing, and Treatments**

The experiment was conducted as per the guidelines of the Canadian Council of Animal Care (CCAC, 2009) at the Ponsonby Research Station, University of Guelph (Guelph, ON, Canada). The animal use protocol was approved by Animal Care Committee at the University of Guelph (Animal Use Protocol #4470). A power analysis was conducted to calculate the number of replicates based on the % of γδ T cells in circulation in preweaning calves reported by on Krueger et al. (2016), with a standard error of the mean of 4.8% and an expected difference of 10% between treatments (α = 0.01 and β = 0.8). Based on this, a total of 36 single and naturally delivered Holstein bull calves were included in a longitudinal study to evaluate changes in the proportions and receptor expression of B cells and γδ T cell populations in blood and colon tissue from birth until one week after weaning on d 42. The calves were distributed across 3 experimental periods, born from June 2021 to May 2022, with a birth body weight (BW) between 42 and 55 kg. Animals were sourced from the Elora Dairy Research Station and transported 8 km to temperature-controlled rooms at the Ponsonby Research Station right after the first meal of colostrum.
All calves included in this study were healthy at birth based on general appearance, rectal temperature, umbilical appearance, and nasal discharge; none of the calves were treated with antimicrobials during the study. Calves did not receive maternal colostrum but received 2 meals of a 26% IgG standardized colostrum replacer (HeadStart, Saskatoon Colostrum Co. Ltd., Saskatoon, SK, Canada), fed at 750 g of colostrum replacer diluted to 3.5 L with water at 45°C to deliver 195 g of IgG in each meal. At 24 ± 2 h after the first colostrum meal, a blood sample was taken to assess serum IgG by radial immunodiffusion, as described by Lopez et al. (2023). All calves had successful transfer of passive immunity with an average serum IgG of 28.2 ± 6.6 g/L. Using the 4 IgG categories developed by Lombard et al. (2020), 72% of calves were on the excellent category (>25 g/L of IgG), 22% on the good category (18–24.9 g/L of IgG), and 6% were on the fair category (10–17.9 g/L of IgG).

The first feeding was within the first 2 h after birth and the second fed at 12 h after birth. The milk replacer used in this study contained 42% lactose, 26% CP, and 20% crude fat (MR; 150 g of MR powder/L; Grober, ON, Canada). During the first week of life, calves were offered 6 L of milk split over 2 meals and then increased to 9 L/day in the second week of life. Calves were offered texturized starter fed ad libitum (40% starch, and 20% NDF; Shurgain) starting on d 35. Calves were vaccinated against infectious Bovine Rhinotracheitis (IBR) virus, Bovine Respiratory Syncytial Virus (BRSV), Parainfluenza 3 virus, Mannheimia haemolytica and Pasteurella multocida using an intranasal vaccine administered at 7 d of life (Bovilis Nasalgen 3-PMH, Merck). Calves included in this experiment were part of a larger complete randomized study looking at the effect of supplementation of a tyndalized Lactobacillus Helveticus postbiotic (n = 18) or control without supplementation of the postbiotic (n = 18). Due to the absence of treatment differences and treatment by interaction for all the variables discussed herein, we focused our analysis solely on the developmental adaptation through time from birth to weaning without discussing treatment effects.

**Blood Sampling, Colon Biopsies, and Lymphocyte Isolation**

Blood samples were taken on d 2, 28, and d 42. Peripheral blood mononuclear cells (PBMCs) were isolated according to Chattha et al. (2009). Briefly, blood samples were centrifuged at 1500 g for 15 min at room temperature, and the buffy coat was harvested using a 10mL transfer pipette into a 50 mL sterile tube and diluted to 15 mL with phosphate buffered saline (PBS). PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich) and washed twice with PBS. Red blood cells were removed by hypotonic lysis using sterile water. Live PBMCs were counted with an automated cell counter (MOXI Z, ORFLO) to ensure at least 1 × 10^6 live cells viable cells, and then aliquoted into 500 μL microcentrifuge tubes with a PBS solution containing 0.1% sodium azide and 0.5% bovine serum albumin (PBA solution). Colon tissue biopsies were taken on a subset of 28 calves on d 2, 28, and d 42, according to procedures previously described by van Neerk et al. (2018). Briefly, calves were restrained in a calf chute, and lubricant was applied to an endoscope before insertion. The endoscope (160 cm length, 9.8 mm diameter; GIF-Q140, Olympus, Tokyo, Japan) connected to a light source and processor (CLV-U40 and CV-140, Olympus) was slowly inserted through the calf’s anus until 70–80 cm past the anus. Endoscopic biopsy forceps (Multi-QROC® biopsy forceps, 2.4 mm diameter; Primed Instruments, Ontario, Canada) were used to collect 20 colon tissue samples. The samples were placed in PBS, then quickly transferred to 4°C Hank’s balanced salt solution (HBSS, calcium, magnesium, and phenol red-free; SH30588.02, Cytiva, Marlborough, USA) for transportation back to the laboratory.

Intestinal epithelial lymphocytes (IELs) were isolated from the colon biopsies according to Lamers et al. (2023). Briefly, the biopsy samples were transferred into 10 mL of isolation solution, made up of HBSS supplemented with 10% HEPES (B299–1, Fisher, Hampton, NH), 4% heat-inactivated fetal bovine serum (26010066, Fisher), and 5 mM EDTA (V4233; Promega, Madison, WI) and incubated for 20 min at 37°C. The tubes were vortexed thoroughly after incubation and the solution was filtered through a 100 μm cell strainer (352360, Fisher) and diluted to 15 mL with phosphate buffered saline (PBS). PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich) and washed twice with PBS. Red blood cells were removed by hypotonic lysis using sterile water. Live PBMCs were counted with an automated cell counter (MOXI Z, ORFLO) to ensure at least 1 × 10^6 live cells viable cells, and then aliquoted into 500 μL microcentrifuge tubes with a PBS solution containing 0.1% sodium azide and 0.5% bovine serum albumin (PBA solution). Colon tissue biopsies were taken on a subset of 28 calves on d 2, 28, and d 42, according to procedures previously described by van Neerk et al. (2018). Briefly, calves were restrained in a calf chute, and lubricant was applied to an endoscope before insertion. The endoscope (160 cm length, 9.8 mm diameter; GIF-Q140, Olympus, Tokyo, Japan) connected to a light source and processor (CLV-U40 and CV-140, Olympus) was slowly inserted through the calf’s anus until 70–80 cm past the anus. Endoscopic biopsy forceps (Multi-QROC® biopsy forceps, 2.4 mm diameter; Primed Instruments, Ontario, Canada) were used to collect 20 colon tissue samples. The samples were placed in PBS, then quickly transferred to 4°C Hank’s balanced salt solution (HBSS, calcium, magnesium, and phenol red-free; SH30588.02, Cytiva, Marlborough, USA) for transportation back to the laboratory.

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**Antibody staining of PBMCs and IELS**

The PBMCs and IELs were divided into 2 different tubes, one for a γδ T cell and another for a B cell panel. Table 2.1 shows the different monoclonal antibodies used, along with the fluorochromes used for detection by flow cytometry. Briefly, cells in the γδ T cell panel tube were incubated for 30 min with a solu-
tion containing 1:400 (stock concentration 1 μg/μL) of a GB21A anti-bovine γδ T cell antibody (Washington State University, Washington, USA) conjugated with APC (1090–11S; Southern Biotech, Birmingham, AB), followed by incubation with a solution containing 1 μg of CACTB32A anti-bovine WC1.1 antibody (Washington State University) conjugated to RPE (Z25055, ThermoFisher), and 1 μg BAQ159A anti-bovine WC1.2 antibody (Washington State University) conjugated to AlexaFluor 488. For the B cell panel cells were incubated with 1 μg of BM-23 anti-bovine IgM antibody (Sigma) conjugated to AlexaFluor 647 (Z25008, ThermoFisher) for 30 min, followed by incubation with a 1:100 solution (stock concentration 1 μg/μL) of CC21 anti-bovine CD21 antibody (Stock Bio-Rad), conjugated with FITC, and 1 μg CCG36 anti-bovine CD32 antibody (Bio-Rad) conjugated with RPE (Z25055, ThermoFisher). A third incubation was done with a fixable Live/Dead staining kit (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, ThermoFisher), to effectively exclude non-viable cells from our analysis, and to ensure that our subsequent data analysis solely focused on live, functionally active cell populations. After each incubation, 300 μL of PBA was added and centrifuged at 700 g for 5 min at room temperature. The supernatant was discarded, and the cells were washed twice with PBA. The stained cells were re-suspended in 200 μL of PBS before running on a BD FACSCanto (BD Biosciences, San Jose, CA). A minimum of 50,000 events for PBMCs and 10,000 events for IELs were collected per sample. Flow cytometry data was analyzed with FlowJo software (Version 10.0.7, Treestar, Palo Alto, CA) according to the gating strategy depicted in Figure 2.1. To generate t-distributed stochastic neighbor embedding (tSNE) plots, data were downsampled (Downsample v3 plugin) to 1 million cells per plot so that all samples had the same number of cells. We then concatenated cells using an equal number of cells per group of animals. The tSNE FlowJo feature was used to generate clusters according to each event’s Ab expression.

**DNA Extraction, Library Preparation, and 16s Amplicon Sequencing**

In addition to taking colon biopsy samples for isolation of IELs, additional samples were taken, snap frozen in liquid nitrogen, and stored at −80°C for extraction of epimural microbial DNA according to protocols reported by Yu and Morrison, (2004) and Villot et al. (2020). Briefly, colon biopsy samples (~0.3 g ± 0.1 g) were washed twice with Tris-EDTA buffer. After the addition of cell-lysis buffer containing 4% SDS, samples were subjected to physical disruption at 5,000 rpm for 3 min using TissueLyser II (Qiagen) with 0.1mm Zirconia and silica beads, followed by incubation at 70°C for 15 min and centrifugation for 5 min at 16,000 × g at 10°C. The bead beating, incubation, and centrifugation were repeated once and impurities were removed from the supernatant using 10 M ammonium acetate, followed by DNA precipitation using isopropanol. After precipitation, DNA was further purified using QIAamp fast DNA stool mini kit (Qiagen Inc., Germantown, MD). The quantity of DNA were evaluated using Qubit as per manufacturer recommendations (Invitrogen, Thermo Fisher, MA, USA) and DNA was stored at −20°C until further use. The concentration of isolated DNA ranged from 8.15 to 220 ng/μL with an average of 89.5 ng/μL.

Library preparation and sequencing were conducted at Genome Quebec (McGill University, Montreal, QC, Canada). Briefly, a fragment of the 16 S rRNA gene spanning the V3–V4 hypervariable region was amplified by qRT-PCR using dual index (forward and reverse primers), with the forward primer 341F (5′-CCTACGGGNGGCWGCAG-3′) and the reverse primer 805R (5′-GACTACHVGGGTATCTAATCC-3′). The quantity of purified PCR products was evaluated using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) to ensure that the concentration of DNA from all samples was higher than 25 ng/μL. A 16S V3–V4 PCR product library was then prepared using the Nextera XT Index (Illumina) and sequenced to generate paired-end 2 × 300 bp on the Illumina MiSeq platform according to the manufacturer’s instructions. The adapter sequences were trimmed from the raw fastq files, and the trimmed reads were demultiplexed according to the samples using the bcl2fastq2 conversion software version 2.20.0. (Illumina).

**Bioinformatic and Statistical Analysis**

For flow cytometry data from both PBMCs and IELs, Kruskal–Wallis non-parametric analysis was used for comparing the receptor expression of γδ TCR+, WC1.1+, and WC1.2+ in the γδ T cell panel, and IgM+.
CD32+ and CD21+ in the B cell panel on d 2, d28, and d 42 of life as indicated by the log expression of median fluorescence intensity (MFI). In addition, proportions of lymphocytes, γδ T cells, and B cell populations were calculated based on receptor expression in FlowJo, and data were analyzed using a generalized linear mixed-effect model (SAS version 9.4, SAS Institute Inc., Cary, NC, USA). Initially, we included the effects of the tyndalized Lactobacillus Helveticus postbiotic supplementation in the model, but since no differences were observed for any of the variables measured in this study, we removed the treatment effect from the analysis and focused solely on the changes over time. The final model included the fixed effects of day and the random effect of calf nested within day. The covariance structure with the lowest Akaike’s information criterion was selected for each variable. We used the first-order autoregressive structure for equally spaced measurements or spatial power for unequally spaced measurements. The Kenward–Roger method was used to calculate the approximate denominator degrees of freedom for the F tests in the statistical models. Continuous data were examined for normal distribution of residuals after fitting the statistical models using Shapiro-Wilk and homogeneity of
variance by plotting residuals against predicted values. Non-normally distributed data were subjected to Box-Cox transformation using the TRANS-REG procedure of SAS to achieve normality before analyses. Statistical significance was considered at $P \leq 0.05$ and a tendency was considered at $0.05 < P \leq 0.10$.

For bioinformatic analysis of the 16s sequencing data, the sorted reads were imported and processed using the Quantitative Insight into Microbial Ecology (QIIME2) package version 2021.2 (Boylen et al., 2019). First, low-quality reads (Phred score < 20) and short (<100 bp) reads were filtered out. This was followed by denoising and merging using the plugin DADA2 to generate an amplicon sequence variant (ASV) feature table. Chimeric sequences and singleton ASVs were excluded from further analyses. Alpha-diversity analyses were conducted with standard diversity metrics accessed via QIIME2, including Chaol, Shannon index, and Phylogenetic diversity (PD) index. A non-parametric ANOVA (Kruskal-Wallis test) was used to test differences in α-diversity among treatment groups and to calculate $P$-values. Beta diversity was calculated based on the weighted UniFrac distances and non-metric multidimensional scaling (NMDS) was applied to the resulting distance matrix. Analysis of similarity (ANOSIM) was used to calculate $P$-values and to test differences in β-diversity among the different days for significance. Statistical significance was declared at $P \leq 0.05$ and a tendency was considered at $0.05 < P \leq 0.10$.

Lastly, to understand if the changes of lymphocyte populations in the colon during weaning were partially accounted for by changes in colon microbial diversity, we perform non-parametric Spearman rank correlation analysis using the data from d 28 and d 42. This analysis was used to test if the changes in the proportion of total γδ T cells, γδ T cell subsets, IgM+ lymphocytes, and proportion of IgM+ B cells expressing CD21+ and CD32+ from colon IEL were correlated with changes in the colon epimural microbial diversity in samples from d 28 and d 42. The resulting correlation matrix was visualized in a heatmap format generated by the corrplot package of R [Corrplot: visualization of a correlation matrix; R package version 4.1.2. 2021].

### RESULTS

#### γδ T Cell Development from Birth to Weaning in Peripheral Blood Mononuclear Cells

The proportion of lymphocytes from total PBMCs increased with age from 25.2% on d 2 to 31.7% on d 28 ($P = 0.02$; Table 2; Figure 2). Furthermore, γδ T cells were detected based on the expression of the TCR-γ chain of the γδ TCR (γδTCR$^+$). On d 2 of life, γδ T cells accounted for 58.5% of the total circulating lymphocytes and gradually decreased until d 42 to 38.2% ($P < 0.01$; Table 2; Figure 2). Additionally, the expression of the γδTCR as indicated by MFI also decreased with age ($P < 0.01$; Figure 6a). Moreover, we further classified γδ T cells into different functional subsets according to the

### Table 1: Antibodies used for flow cytometric analysis of peripheral blood and colonic intra-epithelial lymphocytes.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Target receptor</th>
<th>Primary antibody dilution (μg/μL)</th>
<th>Antibody clone</th>
<th>Mouse isotype</th>
<th>Function</th>
<th>Fluorochromes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cell</td>
<td>IgM</td>
<td>1/50</td>
<td>BM-23</td>
<td>IgG1</td>
<td>General B cell marker</td>
<td>AF 647</td>
<td>Sigma</td>
</tr>
<tr>
<td>B cell</td>
<td>CD21</td>
<td>1/100</td>
<td>CC21</td>
<td>IgG1</td>
<td>Increase B cell activation</td>
<td>FITC</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>B cell</td>
<td>CD32</td>
<td>1/50</td>
<td>GB21A</td>
<td>IgG1</td>
<td>Reduce B cell activation</td>
<td>RPE</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>γδ T cell</td>
<td>TCR-γ chain</td>
<td>1/400</td>
<td>GB21A</td>
<td>IgG1</td>
<td>General γδ T cell marker</td>
<td>APC</td>
<td>WSU</td>
</tr>
<tr>
<td>γδ T cell</td>
<td>WC1.1</td>
<td>1/50</td>
<td>CACTB32A</td>
<td>IgG1</td>
<td>Co-receptor, effector function</td>
<td>RPE</td>
<td>WSU</td>
</tr>
<tr>
<td>γδ T cell</td>
<td>WC1.2</td>
<td>1/50</td>
<td>BAQ159A</td>
<td>IgG1</td>
<td>Co-receptor, regulatory function</td>
<td>AF 488</td>
<td>WSU</td>
</tr>
</tbody>
</table>

1Proportions of lymphocytes from peripheral blood mononuclear cells. 2Proportion of lymphocytes expressing the gamma delta T cell (γδ T cell) populations in peripheral blood of Holstein calves at d 2, 28, and 42 of age. Means ± SE 3Proportion of lymphocytes expressing γδTCR$^+$ and WC1.2+. 4Proportion of lymphocytes expressing γδTCR$^+$ or WC1.2$^+$. 5Proportion of γδ T cells expressing either WC1.1$^+$ or WC1.2$^+$ at high or low levels.
level of expression intensity (low, high) of the coreceptors WC1.1 (effector function) and WC1.2 (regulatory function). The proportion of lymphocytes co-expressing \( \gamma^sTCR^+ \) and either WC1.1\(^{+\text{high}} \) or WC1.1\(^{+\text{low}} \) decreased from d 2 to d 28 \((P < 0.01; \text{Table 2})\). The proportion of \( \gamma^s \) T cells expressing high levels of WC1.1 (WC1.1\(^{+\text{high}} \) \( \gamma^s \) T cells) did not change over time \((P = 0.24; \text{Table 2})\), whereas the proportion of \( \gamma^s \) T cells that expressed low levels of WC1.1 (WC1.1\(^{+\text{low}} \) \( \gamma^s \) T cells) decreased from d 2 to d 28 \((P < 0.01; \text{Table 2})\). The proportion of lymphocytes co-expressing \( \gamma^sTCR^+ \) and WC1.2\(^{+\text{high}} \) significantly decreased from d 2 to d 42 \((P < 0.01; \text{Table 2})\), whereas the proportion of \( \gamma^s \) T cells expressing the coreceptor WC1.2 at high or low levels did not change over time \((P > 0.11; \text{Table 2})\). A graphical representation of the changes in the proportion of lymphocytes expressing \( \gamma^sTCR^+ \), and either WC1.1 or WC1.2 is depicted in the tSNE plots in Figure 2 without distinction between low and high expression. Lastly, the expression of both WC1.1\(^+ \) and WC1.2\(^+ \) in \( \gamma^s \) T cells decreased with age \((P < 0.01; \text{Figure 6a})\).

**B Cell Development from Birth to Weaning in Peripheral Blood Mononuclear Cells**

Immature B cells were identified based on the expression of the membrane-bound antigen receptor IgM (IgM\(^+\)). The proportion of IgM\(^+\) cells within the lymphocyte pool increased with age from 23.6\% on d 2 to 31.2\% on d 28 \((P = 0.02; \text{Table 3; Figure 3})\). The expression of the IgM receptor also increased with age \((P < 0.01; \text{Figure 6b})\). The proportion of lymphocytes expressing IgM\(^+\)CD21\(^+\) increased with age \((P < 0.01; \text{Table 3; Figure 3})\), whereas no changes were observed for the proportion of lymphocytes expressing IgM\(^+\)CD32\(^+\) \((P = 0.18; \text{Table 3; Figure 3})\). Furthermore, the proportion of IgM\(^+\) cells expressing CD21, a receptor that enhances B cell activation, increased with age \((P < 0.01; \text{Table 3})\). In contrast, the propor-

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**Figure 2.** T-distributed stochastic neighbor embedding (tSNE) plots of calves’ peripheral blood mononuclear cells. tSNE plots display the proportions of lymphocytes, proportion of lymphocytes expressing the gamma delta T cell (\( \gamma^sTCR^+ \)), and proportion of lymphocytes co-expressing \( \gamma^sTCR^+ \) and, workshop cluster (WC) coreceptor WC1.1\(^+\) or WC1.2\(^+\). Downsampled data was used before concatenating files to generate the plots so that equal numbers of cells were acquired for each group. Manually gated populations were overlayed on the tSNE plot and assigned different colors. Heatmap plots were performed on the previously gated populations to visualize expression intensity of \( \gamma^s \) T cell receptor (\( \gamma^sTCR^+ \)), and \( \gamma^s \) T cell coreceptors WC1.1\(^+\), and WC1.2\(^+\) using the multigraph color mapping option of FlowJo. Heat colors show overall expression levels (red, high expression; Green-Yellow, median expression; dark blue, no expression).
tion of IgM+ cells expressing CD32, a receptor that reduces B cell activation, decreased with age (P < 0.01; Table 3). Additionally, almost 75% of IgM+ cells co-expressed both CD21 and CD32 on d 2 of life, whereas by d 28 there was a significant reduction in the % IgM+ cells co-expressing both receptors (P = 0.01; Table 3). Lastly, the expression of CD21 on IgM+ cells gradually increased with age (P < 0.01; Figure 6b), whereas no change was observed for CD32 expression on IgM+ cells (P = 0.21; Figure 6b).

**γδ T Cell and B cell Development from Birth to Weaning in Colon Intra-epithelial lymphocytes**

The proportion of lymphocytes from colon IELs increased significantly with age from 3.6% on d 2 to 16.7% after weaning on d 42 of life (P < 0.01; Table 4; Figure 4). The proportions of lymphocytes expressing the γδTCR+ increased from d 28 to d 42 (P = 0.02; Table 4; Figure 4), and the expression of the γδTCR doubled from d 2 to 42 (P ≤ 0.01; Figure 6c). The proportion of lymphocytes co-expressing γδTCR+ WC1.1+ increased by 2.5-fold from d 28 to 42 (P = 0.01; Table 4; Figure 4), and the proportion of lymphocytes co-expressing γδTCR+ WC1.2+ increased by 2.7-fold from d 28 to 42 (P ≤ 0.01; Table 4; Figure 4). Furthermore, there was an increase in the proportion of γδ T cells expressing either the coreceptor WC1.1, or WC1.2 from d 28 to d 42 (P ≤ 0.03; Table 4; Figure 4) Lastly, the expression of WC1.2 in γδ T cells increased over time (P = 0.02; Figure 6c), with no changes in the expression of WC1.1 (P = 0.23; Figure 6c).

The proportion of IgM+ cells from colon IELs did not change with age (P = 0.69; Table 4; Figure 5), and no changes were observed in the expression of the IgM receptor (P = 0.77; Figure 6d). No changes were observed in the proportion of lymphocytes co-expressing IgM+CD21+ or IgM+CD32+ lymphocytes over time (P
However, the proportion of IgM+ cells that expressed CD21 increased from d 28 to d 42 ($P = 0.02$; Table 4), while the proportion of IgM+ cells that expressed CD32 tended to gradually decrease with age ($P = 0.08$; Table 4). Lastly, the expression of CD21 in IgM+ cells did not change with age ($P = 0.97$; Figure 6d), whereas the expression of CD32 in IgM+ cells decreased over time ($P = 0.02$; Figure 6d).

**Microbial Diversity**

The species richness and evenness of the epimural microbial community increased from d 2 to d 28, as denoted by the chao1 ($P = 0.03$; Figure 7) and Shannon ($P < 0.01$; Figure 7) indexes. During weaning, a severe reduction in microbial diversity was observed between d 28 and d 42, as demonstrated by the reduction in Chao1, Shannon, and Phylogenetic Diversity indices ($P < 0.01$; Figure 7). Weighted UniFrac distance matrixes were calculated, and analysis of similarity was used to understand if the bacterial profiles generated from colon epimural samples separated into distinct clusters according to day, and visualized using an NMDS plot (Figure 8). These results revealed the colon epimural associated communities separated into distinct clusters according to day of life (d 2 vs d 28, $P < 0.01$, R = 0.17; and d 28 vs d 42, $P < 0.01$, R = 0.41).

**Correlations between Lymphocyte Populations and Microbial Diversity in the Colon**

Spearman rank correlation analysis comparing the proportions of $\gamma\delta$ T cells and B cell subsets from colon intra epithelial lymphocytes were correlated with changes in the colon epimural microbial diversity between d 28 and d 42. The proportion of lymphocytes expressing $\gamma\delta$ TCR$^+$ was negatively correlated with phylogenetic diversity ($P = 0.01$, Spearman$\rho = 0.42$, $P = 0.02$; Table 4). However, the proportion of IgM+ cells that expressed CD21 increased from d 28 to d 42 ($P = 0.02$; Table 4), while the proportion of IgM+ cells that expressed CD32 tended to gradually decrease with age ($P = 0.08$; Table 4). Lastly, the expression of CD21 in IgM+ cells did not change with age ($P = 0.97$; Figure 6d), whereas the expression of CD32 in IgM+ cells decreased over time ($P = 0.02$; Figure 6d).
The development of the calf’s adaptive immune system is a complex and highly dynamic process coordinated by several maternal and environmental factors (Chase et al., 2008; Chattha et al., 2009). During these developmental adaptations, γδ T cells, a major subset of the lymphocyte pool, seem to play the dual role of conferring early protection against intracellular infections while preventing exacerbated immune responses by promoting tolerance (Hoek et al., 2008; Guzman et al., 2014). This current study confirms previous findings (Hein et al., 1991; Davis et al., 1996), showing that γδ T cells are the most abundant circulating lymphocyte population in neonatal calves. We showed that on d 2 of life γδ T cells account for almost 60% of the lymphocyte pool in the blood of Holstein calves and then decline in their proportion along with the amount of receptor expression until weaning. Furthermore, we identified populations of WC1.1+ and WC1.2+ γδ T cells that express either low or high levels of the WC1 coreceptor (indicated by MFI). However, further research is required to elucidate if the differences in receptor expression translate into functional differences between these subsets. The γδ T cells expressing low and high levels of WC1.2 were the most abundant subset in PBMCs, accounting for 55% of all γδ T cells shortly after birth, with high WC1.2+expressing populations declining in receptor expression after weaning. Interestingly, we observed an opposite trend in colon intra-epithelial lymphocytes, showing a 50% increase of γδ T cells from d 28 to d 42 of which the majority expressed either WC1.2+ or WC1+. In a series of in vitro studies, Hoek et al. (2008) and Guzman et al. (2014) demonstrated that WC1.2+ and WC γδ T cells serve as the principal immunoregulatory T cell subset in circulation and at mucosal sites, respectively. Furthermore, the regulatory role of γδ T cells has been demonstrated in vivo where depletion of WC1+ γδ T cells results in enhanced antibody responses in animals challenged with ovalbumin (Howard et al., 1989). In mice, the depletion of γδ T cells after severe infection with Listeria monocytogenes induces septicemia and death (Skeen et al., 2001). Our data suggest that there was a migration of regulatory γδ T cells to colon intra-epithelial sites during weaning to promote tolerance during this challenging adaptation.

In this study, calves were abruptly transitioned from milk to solid feed at d 42 of life. As a result of the rapid stressful shift of diet, calves experienced a steep decline in intake (M.F. Olmeda et al., data not published) that resulted in a marked reduction in microbial diversity in the colon and increased intestinal permeability (M.F. Olmeda et al., data not published). The impact of abrupt weaning on microbial diversity has been previously reported to happen in the rumen and fecal microbiota of calves (Meale et al., 2016). The commensal intestinal microbiota induces tolerance by the intestinal immune system by promoting mechanisms that decrease direct contact of epithelial cells with microorganisms in a process referred as “immune exclusion.” This includes stimulation of intestinal epithelial cell proliferation and differentiation, mucin

<table>
<thead>
<tr>
<th>Population, %</th>
<th>Day 2</th>
<th>Day 28</th>
<th>Day 42</th>
<th>SEM</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>Lymphocytes 1</td>
<td>25.2</td>
<td>31.7</td>
<td>31.4</td>
<td>1.91</td>
<td>0.02</td>
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<td>IgM+</td>
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<td>31.2</td>
<td>30.0</td>
<td>2.06</td>
<td>0.02</td>
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<tr>
<td>IgM+CD21+</td>
<td>18.9</td>
<td>28.6</td>
<td>27.7</td>
<td>1.85</td>
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</tr>
<tr>
<td>IgM+CD32+</td>
<td>22.2</td>
<td>16.3</td>
<td>15.7</td>
<td>2.77</td>
<td>0.18</td>
</tr>
<tr>
<td>IgM+ cells expressing:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD21+</td>
<td>82.5</td>
<td>91.9</td>
<td>92.3</td>
<td>1.87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD32+</td>
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<td>55.5</td>
<td>7.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD32+CD21+</td>
<td>81.3</td>
<td>68.8</td>
<td>80.5</td>
<td>5.43</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1Proportions of lymphocytes from peripheral blood mononuclear cells.
2Proportion of lymphocytes expressing the membrane bound immunoglobulin M (IgM+) cell receptor (IgM+).
3Proportion of lymphocytes expressing IgM+ and cluster of differentiation 21 (CD21).
4Proportion of lymphocytes expressing IgM+, and cluster of differentiation 32 (CD32).
5Proportion of lymphocytes expressing IgM+, and co-expressing CD 21, and CD32.

Figure 9). Furthermore, the proportion of lymphocytes co-expressing WC1.1+, and γδTCR+ was negatively correlated with Shannon (P = 0.02, Spearman ρ = 0.40, Figure 9) and phylogenetic diversity indices (P < 0.01, Spearman ρ = 0.47, Figure 9) and the proportion of γδ T cells expressing WC1.1+ was negatively correlated with phylogenetic diversity (P = 0.03, Spearman ρ = 0.38, Figure 9). The proportion of lymphocytes co-expressing γδTCR+ and WC1.2+ was negatively correlated with phylogenetic diversity (P = 0.02, Spearman ρ = 0.41, Figure 9) and the proportion of γδ T cells expressing WC1.2+ was negatively correlated with phylogenetic diversity (P = 0.04, Spearman ρ = 0.37, Figure 9). The proportions of B cells expressing CD32+, which express the Fc gamma receptor II, which is part of a receptor complex that reduces B-cell activation,positively correlated with phylogenetic diversity (P < 0.01, Spearman ρ = 0.47, Figure 9) and Chao1 (P = 0.01, Spearman ρ = 0.41, Figure 9). Lastly, the proportions of B cells expressing CD21+, which lowers the threshold of activation in B cells, were negatively correlated with Shannon index (P = 0.04, Spearman ρ = 0.33, Figure 9).
production, and antimicrobial peptides and secretory IgA production (Malmuthuge et al., 2015; Seifert et al., 2021). When microbial diversity is affected in response to a challenge, the stimulatory signals that promote gut barrier function can be compromised (Malmuthuge et al., 2017; Meale et al., 2017) leading to increased intestinal permeability, inflammation, and tissue damage (Cangiano et al., 2022). In the current study, it appears that certain adaptations during weaning promoted the migration of γδ T cells to the colon epithelium, and the activation of IgM+ B cells. The decrease in microbial diversity possibly attributed to the abrupt weaning model was negatively correlated with the increase in γδ T cell populations. This may suggest that the reduction in microbial diversity during the weaning challenge partially stimulated local proliferation or the migration of γδ T cells possibly to promote tolerance, prevent further tissue damage, and help support a rapid response against invading microorganisms. In addition, while no changes were observed for the proportions IgM+ lymphocytes, there was a gradual decrease in the proportion of IgM+ B cells expressing CD32+ from birth to weaning, and a 6-fold increase in the proportion of CD21+ after weaning, suggesting a lower threshold for these B cells to become activated. Lastly, the reduction in microbial diversity during weaning was moderately correlated with the changes in B cell receptor expression, as indicated by the reduction of CD32+ and increase in CD21+ expressing B cells. Therefore, it appears that the reduction in microbial diversity during weaning promoted the activation of B lymphocytes colon.

During the first weeks of life, calves exclusively rely on successful transfer of passive immunity from colostrum to provide early protection against pathogens (Weaver et al., 2000; Godden et al., 2019). In addition, maternal prenatal and postnatal factors promote immune tolerance and reduce effector function of CD4 T cells, compromising the ability of the newborn’s

Figure 5. T-distributed stochastic neighbor embedding (tSNE) plots of calves’ colon intra-epithelial lymphocytes. tSNE plots display populations of lymphocytes, proportion of lymphocytes expressing the membrane bound immunoglobulin M (IgM+) cell receptor, and the proportion of lymphocytes expressing IgM+, and cluster of differentiation 21 (CD21+), or CD32+. Downsampled data was used before concatenating files to generate the plots so that equal numbers of cells were acquired for each group. Manually gated populations were overlayed on the tSNE plot and assigned different colors. Heatmap plots were performed on the previously gated populations to visualize expression intensity of B cell membrane bound IgM, and of receptors CD21, and CD32 within IgM+ B cells using the multigraph color mapping option of FlowJo. Heat colors show overall expression levels (red, high expression; Green-Yellow, median expression; dark blue, no expression).
Figure 6. Expression of γδTCR+ (Gamma delta T cell receptor) in gamma delta T cells (γδ T cells) indicated by log-transformed median fluorescence intensity (MFI), and expression of the coreceptors WC1.1+, and WC1.2+ within γδ T cells from peripheral blood mononuclear cells (A) and colon intra-epithelial lymphocytes (C). Expression of the B cell membrane bound IgM receptor, and of receptors CD21, and CD32 within IgM+ B cells from peripheral blood mononuclear cells (B) and colon intra-epithelial lymphocytes (C). Data is depicted as box plots overlayed with violin plots to show data distribution. The lines, boxes, and whiskers in the box plots represent the median, and 25th, and 75th percentiles, and the min-to-max distribution of replicate values, respectively. * Indicates a significant difference between days (P < 0.05).

Figure 7. Alpha diversity index in colon epimural samples of calves taken on d 2, 28, and 42 of life. Species richness and diversity index were measured with 3 different matrices: Chao1, Shannon index, and Faith phylogenetic diversity (PD index). * Indicates a significant difference between days (P < 0.05). The lines, boxes, and whiskers in the box plots represent the median, and 25th, and 75th percentiles, and the min-to-max distribution of replicate values, respectively.
immune system to mount effective responses against intracellular infections. Immature humoral immune responses and limited seroconversion in response to vaccination are a hallmark of neonatal immune responses (Windeyer et al., 2019; Kolmann et al., 2020). In cattle, maternal antibodies present in colostrum reduce B cell activation via interaction of the Fc region of IgG with CD32 receptor that becomes cross-linked with membrane-bound IgM. In this study, we observed that at birth IgM+ B cells in PBMCs represented 20% of circulating lymphocytes and constitutively expressed both CD32 and CD21, a receptor for complement c3 that partially mediates B cell activation (Nielsen et al., 2002; Firth et al., 2005). As calves aged, there was an increase in the proportion of IgM+ lymphocytes with an increased proportion of CD21+IgM+ B cells and a decreased proportion of CD32+IgM+ B cells, which would be anticipated as maternal antibodies wane as the immune system of the calf matures. While no changes were observed in the expression of CD32 in IgM+ B cells over time, there was an increase in the expression of CD21 and of IgM suggesting that a reduction of the CD32-mediated inhibitory effects of maternal IgG started to decrease as early as 28 d of life. The activation of B cell lymphocytes seems to depend, at least in part, on the outcome of opposing signals between CD32
and CD21 setting a threshold for activation (Nielsen et al., 2002), suggesting that the reduction of CD32 expression in combination with an increase in CD21 observed ultimately promoted an increase in B cell activation. Similar results were observed by Kampen et al. (2006), who reported a rapid increase in the number of CD21⁺ lymphocytes during the first 3 mo of life, and Chatta et al. (2009), who observed greater expression of CD21 in IgM⁺ B cells for adult cows than for calves of less than 90 d of life. However, Chatta et al. (2010) observed that CD32 expression increased over time. These discrepancies might be attributed to differences in the age, sex, transfer of passive immunity, and source of colostrum used in each of the studies. Lastly, CD21 requires complement c3 for its activation, and its concentrations are low during early life and gradually start to increase with age (Pihlgren et al., 2004; Firth et al., 2005). The current study and others (Chatta et al., 2009; Chatta et al., 2010) suggest that CD21 expression is not a limiting factor for B cell activation and rather an insufficient activity of the complement c3 system in combination with high CD32 expression are the 2 major limiting factors for B cell activation in neonatal calves.

While protection against extracellular infections in early life is primarily mediated by maternal antibodies, the calf’s immune system is poorly equipped to deal with intracellular infections via traditional mechanisms.

Figure 9: Spearman rank correlations of changes in the proportion of total γδ T cells and γδ T cell subsets, WC1.1⁺ and WC1.2⁺, and proportions of IgM⁺ lymphocytes and proportion of B cells expressing the receptors CD21⁺ and CD32⁺ from colon intra epithelial lymphocytes were correlated with changes the colon epimural microbial diversity in samples from d 28 and d 42. Data is graphically presented in a dot plot heatmap. Correlations are indicated by a color scale denoting whether the correlation is positive (closer to 1, blue squares) or negative (closer to −1, red squares). Statistically significant correlations are indicated by a *, **, and *** levels of significance.
in early life, particularly if fresh maternal colostrum containing functional T-cells and other leukocytes is not provided (Harris and Barletta, 2001; Chase et al., 2008). A subset of γδ T cells called WC1.1+ γδ T cells has been suggested to provide early protection to the neonatal calf and promote clearance of intracellular infections during a period where effector functions of traditional αβ T cells are blunted. Mice studies have shown that developmental pre-programming of γδ T cells in the thymus prepares these cells to swiftly respond to infections without the need to go through the steps of activation, differentiation, and clonal expansion that conventional T cells must undergo first (Baldwin et al., 2014; Guerra-Maupome et al., 2019).

Upon stimulation, WC1.1+ γδ T cells express perforin, IFN-γ, and IL-17, and secrete chemokines MCP-1 and MIP-1α to attract monocytes and neutrophils to the site of infection (Brown et al., 1994; Toka et al., 2011; McGill et al., 2013). In our study, at birth, 44% of γδ T cells expressed WC1.1+, of which the majority were in the low expression group (WC1.1low γδ T cells), and as the calf aged, the proportion of WC1.1+ γδ T cells, as well as the receptor expression WC1.1+, gradually declined.

In conclusion, these data suggest that developmental adaptations during early life coordinate immune function and development, promoting γδ T cell expansion possibly to provide early systemic protection and colonic immune tolerance. In addition, the reduction in microbial diversity during weaning experienced by the calves in this study seems to promote either local proliferation or migration of γδ T cells into the colon to provide both protection and tolerance in response to the challenge. Further research is required to determine if the increase and shift in γδ T cell populations in the colon during weaning is a direct consequence of the reduction in microbial diversity and if the changes observed in these populations over time are representative of the overall immune status of the intestine.

REFERENCES


