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Blood immune transcriptome analysis of artificially fed dairy calves and naturally suckled beef calves from birth to 7 days of age

Key external stakeholders:
Beef and dairy farmers; Teagasc KT, Department of Agriculture, Food and the Marine (DAFM), veterinary surgeons, Animal Health Ireland (AHI), Bord Bia.

Practical implications for stakeholders:

- These data provide a greater understanding of the molecular control of the early development of the neonatal immune system of dairy and beef calves from birth to 168h post-birth. The new information highlights some of the molecular mechanisms regulating the development of immune -competence, likely due to variations in colostrum ingestion. Dairy calves initially demonstrate a surge in pro-inflammatory cytokines with major differences observed between beef and dairy calves at 168 h post-birth including increased abundance of *IG* in beef CL calves.

Main results:

Neonatal calves possess an immature and naïve immune system and are reliant on the intake of maternal colostrum for passive transfer of immunoglobulins. Variation in colostrum management of beef and dairy calves is thought to affect early immune development. Therefore, the objective of this study was to examine changes in gene expression and investigate molecular pathways involved in the immune-competence development of neonatal Holstein dairy calves and naturally suckled beef calves using next generation RNA-sequencing during the first week of life. Jugular whole blood samples were collected from Holstein (H) dairy calves (n=8) artificially fed 5% B.W. colostrum, and from beef calves which were the progenies of Charolais-Limousin (CL; n=7) and Limousin-Friesian beef suckler cows (LF; n=7), for subsequent RNA isolation. In dairy calves, there was a surge in pro-inflammatory cytokine gene expression possibly due to the stress of separation from the dam. LF calves exhibited early signs of humoral immune development with observed increases in the expression genes coding for Ig receptors, which was not evident in the other breeds by 7 days of age.

Opportunity / Benefit:

Immune and health related DEGs identified as up-regulated in beef calves are prospective contender genes for the classification of biomarkers for immune-competence development and will contribute towards a greater understanding of the development of an immune response in neonatal calves.

Collaborating Institutions: AFBI

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1. Project background:

New-born calves are immunologically naïve at birth, offering the ideal scenario to observe the development of immune-competence through time. The protection of the womb environment during the pre-partum period, coupled with syndesmochorial placentation, results in a lack of exposure to pathogens, meaning that calves are born with an essentially non-functional immune system. Failure of passive transfer of colostrum derived IgGs (FPT, serum IgG <10 mg/mL) markedly increases morbidity and mortality in calves. There is tremendous variation in the passive immune status of dairy calves and, generally passive immunity of dairy calves is much lower than that of beef calves. This difference is primarily attributed to variation in colostrum Ig concentrations, whereby dairy cows produce relatively large volumes of colostrum with relatively low concentrations of Ig while beef cows produce the opposite. In the suckled beef calf, there are also large differences in passive immunity between cow breed types.
The use of a systems approach such as RNA sequencing offers advantages over other molecular based techniques such as microarray, enabling unbiased opportunities towards the profiling of developing immune-competence using a global unbiased view of relative transcriptomic alterations.

2. Questions addressed by the project:

In this project we aimed to elucidate the molecular mechanisms involved in the development of immune-competence, from birth through the first 7 days of life of dairy calves in addition to two beef breeds.
Understanding such mechanisms would be a step towards integrating optimum husbandry practices, and to identify possible biomarkers associated with development of immune-competence for breeding of resilient calves.

3. The experimental studies:

3.1 Material and methods

Jugular whole blood samples were collected into RNA tempus tubes from Holstein dairy calves (n= 8) artificially fed 5% B.W. colostrum, and from naturally suckled Charolais-Limousin (CL; n = 7) and Limousin-Friesian beef calves (LF; n = 7), for subsequent RNA isolation. Blood samples were harvested at 0, 48, 72 and 168 hours (h) post-birth. mRNA was isolated from the whole blood, cDNA libraries prepared from mRNA and subsequently sequenced with single-end reads. RNAseq processing was carried out as described by Johnston *et al.,* 2016. Quality assessment of the filtered data was performed by FastQC and reads were UMD3.1 Bos taurus genome. Read alignment was performed using STAR. Analysis of the count data was performed using DESeq2, and functional analysis of DEG was carried out using ingenuity pathway analysis (IPA).

4. Main results:

4.1 IgG concentration

Serum IgG concentrations at 0 h, 48 h, 72 h and 168 h post birth in dairy and beef calves are shown in Table 1. There was a significant effect of breed ($p<0.05$) sampling time ($p<0.0001$) and breed × sampling time interaction ($p<0.0001$) for serum IgG concentrations. As expected, at 0 h, prior to the first feed of colostrum, baseline serum IgG concentrations were lower ($P<0.0001$) compared with all other sampling times. In LF calves, serum IgG concentrations were greater ($P<0.001$) compared to dairy calves at 48, 72 and 168 h post-birth and were not different from CL, except at 168 h when concentrations were lower in CL compared with LF. Colostrum IgG concentrations (mean (SD)) were not different across the three cow breed types (H; 63.1 (14.4), CL; 63.0 (25.2) LF (71.0 (14.5) mg/mL).

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Table 1. Least square means (SEM) for serum IgG concentrations (mg/mL) from birth (d 0) to 168 h hours (h) for the calf progenies of H, CL and LF cows.

	0 h	12 h	24 h	48 h	72 h	168 h	SE	T	S	T × S
								0.04	P<.0001	P<0.0001
H	0.64 ^{a}	14.34 ^{b,x}	13.59 ^{b,x}	14.47 ^{b,x}	13.47 ^{b,x}	9.01 ^{b,x}	1.51			
CL	0.01 ^{a}	14.34 ^{b}	18.34 ^{b,x}	17.19 ^{b}	15.93 ^{b}	14.35 ^{b,z}	1.61			
LF	0.01 ^{a}	19.34 ^{b,y}	24.91 ^{b,y}	22.31 ^{b,y}	19.57 ^{b,y}	17.75 ^{b,y}	1.61			

T; Treatment, S (sampling time);
Cow genotype; H = Holstein; CL = Charolais × Limousin (n=7), LF = Limousin × Friesian (n=7); ^{a,b} = Within rows, LSmeans differ from d 0 by P < 0.05. ^{x,y,z} = Within columns Lsmeans differ across breed by P < 0.05.

4.2 *Differentially expressed genes and Pathway analysis across all comparisons*

4.2.1 Over enriched networks in dairy calves over the first 168 h post birth

Comparisons of dairy calves at 0 h, 48 h, 72 h and 168 h, were examined for the presence of enriched molecular and cellular functional networks, physiological system developmental networks, and for the presence of biologically interesting canonical pathways. The top enriched pathway in the comparison of 0 h calves to those sampled at 48 h was the role of pattern recognition receptor in recognition of bacteria and viruses, in which enriched genes were present at higher levels of abundance in the 0 h samples. The complement system pathway was also enriched in the comparison, with the majority of the genes present at higher levels in the 48 h calves than in the 0 h.

Comparison of the transcriptomic profile of calves aged 168 h to those sampled at 72 h highlighted *Il-8* signalling as the most enriched canonical pathway, with the majority of genes present at lower levels in the 168 h calves than in the 72 h. Networks of interest included 'Cell signalling, Molecular transport, Vitamin and mineral transport' (Network 3), and 'Cellular development, Cellular growth and Proliferation' (Network 4). On investigation of any upstream effects of DEG enriched in the comparison of 168 h and 72 h dairy calves, we identified inhibition of a growth factor at 168 h, *TGFβ1.* Additionally, inhibition of a transmembrane receptor, *EPOR,* was identified as -5.487 times lower in the 168 h calves, with 22 out of the proposed 366 genes involved in its upstream regulation present in the DEG list.

4.2.2 Over enriched networks in beef calves over the first 168 h post birth

(i) Comparisons over time within crossbreeds

Comparisons were carried out within each beef breed across the four time points, 0 h, 48 h, 72 h and 168 h, to investigate the transition of the developing immune response over time. The top over-represented networks are described. Firstly, biologically interesting pathways were investigated within the CL crossbred calves. Comparison of 48 h to 0 h CL calves resulted in 2095 DEG, 1766 of which were successfully mapped in IPA. Biologically interesting enriched molecular, cellular and physiological networks included cellular movement, immune cell trafficking, and lymphoid tissue structure and development. Top enriched canonical pathways included cholesterol biosynthesis, which was upregulated in the 48 h old calves, the complements system, also upregulated at 48 h, and two interleukin signalling pathways, *Il-9* and *Il-12* in which the majority of genes were present at higher levels after 48 h. Investigating upstream analysis effector genes identified a number of significantly altered upstream regulators including a number of growth factors (*Areg, Hgf, VEGFA),* cytokines *(c5f2, PRL, IFNa2),* and also the Fc gamma receptor upstream pathway activated in the CL calves at 48 h.

The calves were then compared at the later time point of 168 h to 72 h, to investigate the transition of the immune response evident through the blood transcriptome from day. Only 383 DEG were identified between the two time points in the CL calves. Top molecular cellular and physiological networks again included cellular movement, immune cell trafficking and also cellular function and maintenance. There were no canonical pathways identified as significantly enriched in the comparison. Overall a major stabilization of the immune response was evident through the inhibition of a number of immune related regulators.

The LF calves were also compared across the four time points (0 h, 48 h, 72 h and 168 h) to investigate the development of immune-competence during the first week of life. Similarly high levels of DEG were evident at the early time point comparison of 48 h versus 0 h, with 2021 DEG identified in total, with 1640 of these being successfully mapped in IPA. Top enriched molecular and cellular networks included cellular movement, cellular function and maintenance; immune cell trafficking, and lymphoid tissue structure and development. A number of upstream regulators including a large number of cytokines (*TNFα, Il1β, IFN)*,

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transmembrane receptors (*TLR3, TLR4, TLR7, TLR9)* exhibited activation in the 48 h calves compared to 0 h.

LF calves were then compared at 168 h versus 72 h to identify observable alterations to key genes and pathways involved in the development of immune-competence. Overall there were 1897 DEG identified, with 1562 being successfully mapped using IPA for functional analysis. The vast majority of total DEG, 1362 out of 1897, were present at lower levels in the 168 h calves, highlighting the stabilization on the initial surge in immune gene expression. A number of biologically interesting networks were enriched in pathway analysis of the 168 h and 72 h calves, including network 14 mapping enrichment of immune cell trafficking and the inflammatory response, of which the majority of genes are present at lower levels of expression by 168 h. Network 23 however, humoral immune response, showed upregulation of a number of genes in the 168 h calves. A significant number of upstream gene regulators including cytokines (*Il1α, IFNγ, Il1P, OSM),* and immune regulatory complexes (*MAP2K1, NFκB)* were inhibited by 168 h.

(ii) Comparison of CL calves to LF calves across time

In order to investigate any differences in the development of a competent immune response during the first week of life in the two beef crossbreeds, the breeds were compared at each time point. Only the 48 h time point comparison generated a significant number of DEG, 716 in total, 594 of which were mapped for functional analysis using IPA. A number of interesting networks were highlighted as being enriched in the comparison of 48 h, including network 5, antimicrobial response, in which the majority of the genes were present at lower levels in the CL calves. Biologically important upstream regulators were also identified as being significantly differentially expressed in the comparison of beef CL to LF, including *NFκB* and *Il12* complexes, both inhibited in CL calves compared to LF at 48 h. A number of immunologically important upstream regulators were also inhibited in the beef CL calves, including *CD40LG,* the cytokine that binds to CD40.

5. Opportunity/Benefit:

Colostrum management on both beef suckler and dairy farms is of critical importance to the prevention of calf morbidity and mortality. In this study, we observed for the first time, the development of an immune response in calves of different breeds over the first seven days of life, using peripheral blood and RNA sequencing technologies. The molecular variations in key genes such as cytokines and B cell receptors across all breeds gives a tremendous insight into possible reasons as to why certain cattle breeds are more susceptible than others to illness during the neonatal period. It is clear form these data, that dairy calves undergo a systemic stress response following separation from the dam at birth and following artificial feeding of colostrum. Key genes increased in LF calves at 168 h including the immunoglobulin receptors *FCER1A* and *FCRLA,* and *IL-12r,* an immune regulator consistently down regulated in beef CL and in dairy calves, offer possible targets for biomarker discovery and a greater understanding into the development of immune-competence.

6. Dissemination:

In-service training, Animal Health Ireland (CalfCare events), Teagasc Open Days, Teagasc Fact sheets

Scientific publications:

1. Dunn, A., Duffy, C., Gordon, A., Morrison, S.J., Argűello, A., Welsh, M., **Earley**, B. **2018.** Comparison of single radial immunodiffusion and ELISA for the quantification of immunoglobulin G in bovine colostrum, milk and calf sera. Journal of Applied Animal Research, 46:1, 758-765.
2. **Earley**, B., Tiernan, K., Duffy, C., Dunn, A., Waters, S.M., Morrison, S., McGee, M. **2018.** Effect of suckler cow vaccination against glycoprotein E (gE)-negative bovine herpesvirus type 1 (BoHV-1) on passive immunity and physiological response to subsequent bovine respiratory disease vaccination of their progeny. Research in Veterinary Science, 118, Pages 43-51.
3. Dunn, A., Duffy, C., Gordon, A., Morrison, S.J., Argűello, A., Welsh, M., **Earley**, B. **2018.** Effect of passive transfer status on response to a glycoprotein E (gE)-negative bovine herpesvirus type 1 (BHV-1) and bovine respiratory syncytial virus (BRSV) vaccine and weaning stress in pre-weaned dairy calves. Journal of Applied Animal Research, 46 (1) 907-914.

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