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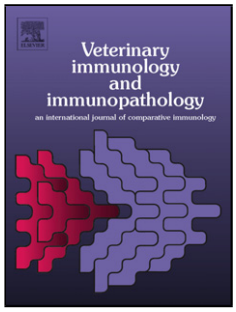

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Application of the TruCulture® whole blood stimulation system for immune response profiling in cattle

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HIGHLIGHTS

- The commercially available TruCulture® assay facilitates the standardised profiling of immune responses from human peripheral blood cells at both the transcript and protein level and here, we optimise this system for use in cattle.
- Standardised concentrations of LPS and zymosan pathogen-associated molecular patterns (PAMPs) stimulate an immune response in bovine whole blood, detected via significantly enhanced IL-1β and IL-8 protein expression. This assay induces less cell death than whole blood culture assays and does not negatively affect cell viability.

ABSTRACT

Capturing the phenotypic variation in immune responses holds enormous promise for the development of targeted treatments for disease as well as tailored vaccination schedules. However, accurate detection of true biological variation can be obscured by the lack of standardised immune assays. The TruCulture® whole blood stimulation system has now been extensively used to detect basal and induced immune responses to a range of pathogen-associated molecular patterns (PAMPs) in human peripheral blood. This study demonstrates the optimisation of this commercially available assay for systemic immune phenotyping in cattle. The early immune response in Holstein-Friesian bull calves (n=10) was assessed by haematology, flow cytometry and cytokine expression profiling after 24 hour *ex-vivo* PAMP (LPS, poly (I:C) and Zymosan) stimulation in TruCulture® tubes. A comparative analysis was also performed with a traditional whole blood stimulation assay and cell viability using both systems was also evaluated. *Results:* Supernatant collected from TruCulture® tubes showed a significant increase in IL-1β and IL-8 expression compared to null stimulated tubes in response to both LPS and zymosan. In contrast, a detectable immune response was not apparent at the standard concentration of poly (I:C). Conventional whole blood cultures yielded similar response profiles, although the magnitude of the response was higher to both LPS and zymosan, which may be attributed to prokaryotic strain-specificity or batch of the stimulant used. Despite being a closed system, *HIF1A* expression – used as a measure of hypoxia was not increased, suggesting the TruCulture® assay did not negatively affect cell viability. This represents the first reported use of this novel standardised assay in cattle, and indicates that the concentration of poly (I:C) immunogenic in humans is insufficient to induce cytokine responses in cattle. We conclude that the low blood volume and minimally invasive TruCulture® assay system offers a practical and informative technique to assess basal and induced systemic immune responses in cattle.

Keywords: TruCulture®, PAMPs, bovine peripheral blood, innate immune response.

1. Introduction

Significant inter-individual heterogeneity in the immune response has been documented across multiple studies in humans and other animal species, and such differences are likely to significantly affect both disease susceptibility and responses to vaccination. As an illustration, studies employing LPS as a PAMP to mimic the immune response to Gram negative bacteria in cattle, found statistically significant inter-individual variation in multiple clinical, haematological and biochemical responses [1]. In fact, subsequent work by the same authors attributed between 21% and 29% of the variation in expression of acute phase proteins such as serum amyloid A and haptoglobin after *in vivo* LPS administration to the individual cow [2]. This may not be surprising given that cattle are an outbred species [3], but such individual variation may not only impede the detection of significant differences for any experimental treatment under investigation but is also biologically informative, and yet is often ignored. Current approaches to livestock immunity are usually disease-based and thereby assess the treatment and control animal groups as collective entities rather than individual subunits, and this can potentially obscure relevant insights into the complexity of immune responses at both the individual and population level. The magnitude of IFN-γ responses has been shown to vary considerably between individual calves in response to antigens from *Mycobacterium avium* subsp. *paratuberculosis* - the causative agent of Johne's disease for example [4], and this could affect interpretation of the test to detect positive animals.

Additionally, a multitude of methodologies used in previous work to profile the immune response has precluded the repeated and comparative analysis of the healthy immune response in livestock. As a result, what constitutes a healthy, basal immune response remains unclear. Whole blood stimulation assays have been particularly informative and have been previously utilised in cattle [5, 6], but accurate comparisons between studies is complicated by variation in cell preparation procedures, stimulation times, incubation conditions and PAMP concentrations. Furthermore, the type of anticoagulant used for whole blood assays as well as the time between the initial bleed and isolation of plasma has also been shown to affect the expression levels of a number of cytokines and chemokines including IL-1β and IL-8 [7, 8]. As a result, separating true biological from technical variation remains a challenge and there is an urgent unmet need for standardised immune phenotyping approaches.

The successful capture of such inter-individual variation as well as the establishment of a 'healthy' immune response baseline has been significantly advanced in human immunological studies through the advent of TruCulture® whole blood stimulation system from Myriad RBM [9]. TruCulture® is a syringe-based *in vitro* model system which combines whole blood collection and culture with various natural and synthetic immunostimulants in a closed system, allowing for retention of all immune cells including mononuclear cells, granulocytes, and platelets along with circulating soluble factors for characterizing the *in vivo* stimulation and response of circulating immune cells. In contrast to the various methods employed previously in livestock cell culture systems, the TruCulture® system does not require extensive sample manipulation, specialized equipment or technical expertise in cell culture. Furthermore, because the TruCulture® syringe retains the cell pellet and culture supernatant, both gene expression profiling and protein secretion analysis can be easily performed. The first study using this stimulation system in humans developed and tested 27 stimulation systems (including bacteria, fungi, and viruses; agonists specific for defined innate immunity sensors; cytokines; and activators of T cell immunity) built into whole-blood syringes [10]. Since this initial study, these assays have been extensively used in human analyses to deconvolute the complexity of the immune response and increased our understanding of human health and disease pathogenesis including defining immune function after haematopoietic stem cell transplantation [11]. Importantly, the use of these assays has been reported to reduce the coefficient of variation to between 5 and 14%, facilitating meaningful comparisons on immune response results between laboratories [12]. With this study, we aim assess the use of the TruCulture® as an immunoprofiling tool in cattle and compare it to a traditional whole blood culture assay.

2. Materials and Methods

2.1 Animal Selection

Ten male Holstein-Friesian calves were purchased from a commercial dairy farm and moved the Teagasc Animal and Biosciences Research Centre, Dunsany 3 weeks after birth. Calves were immunised on arrival with Bovipast® RSP. They were kept outdoors on pasture throughout the experimental period which began one week after arrival. The calves were bucket fed 2L of milk replacer twice daily for one month after arrival. After this period it was reduced to 2L once a day for a further two weeks before weaning. They were also provided with calf pellets and fresh water *ad libitum.* Full ethical approval for sampling was obtained from the Irish Health Products Regulatory Authority (HPRA).

2.2 Haematology and flow cytometry

1 mL of blood collected in a 9 mL heparin coated vacutainer was lysed in 10 mL of high yield lysis buffer (Thermo Scientific) for 10 min. The solution was then centrifuged at 300 xg for 10 min. The supernatant was discarded and the pellet was resuspended in 2mL of PBS with 1% BSA. 200 µL per well was transferred to a 96 well plate. Primary antibodies WC1, γδTCR, CD3 and CD8 (Monoclonal Antibody Centre, Washington State Univeristy) were conjugated to the Pacific Blue and AlexaFlur488 fluorophores using the APEX Antibody Labelling Kit (Thermo Scientific) according to manufacturer's instructions. The CD4 antibody was conjugated to FITC (Bio-Rad). 0.5 µL of each of these was added per well for 30 min at 4°C in the dark. Afterwards, the plate was centrifuged at 300 xg for 5 min. The supernatant was discarded and 200 µL of PBS 1% BSA was added to each well to resuspend the cells. The sIgM antibody (Monoclonal Antibody Centre, WSU) was unconjugated; therefore it had a secondary anti-mouse FITC antibody (Dako) added after one wash step. This plate was incubated at 4°C in the dark for 30 min. Live/Dead cell staining was performed using propidium iodide (PI) (Thermo Scientific). 30,000 events were acquired using the Attune Flow Cytometer (Applied Biosystems) from a leukocyte gate based on a FSC SSC profile. The cells were then gated based on single cells and viability. The results were analysed using the Attune software by looking at positive staining for each cell marker. Isotype controls were also included. Haematology profiles were established from blood collected in 6 mL EDTA coated vacutainers processed using the ADVIA 2120 haematology system to acquired total lymphocyte, monocyte, neutrophil, basophil and eosinophil cell numbers.

2.3 TruCulture® and whole blood culture

Standardised TruCulture® tubes (Myriad RBM) were purchased pre-filled with a proprietary medium along with either LPS (*E. coli,* O55:B5) (100 ng/mL), poly (I:C) (30 µg/mL), zymosan (50 µg/mL) and also a control (null). They were left to thaw at room temperature for 1 hour before use. Blood was drawn directly into each tube via jugular venepuncture. The tubes were transferred within 30 min to a heat block and incubated at 37°C for 24 hours. After 24 hours, the tubes were centrifuged at 600 x g for 10 min. The tubes were opened and the Seraplas valve separator was inserted via a plunger to separate the cells and supernatant. The supernatant was transferred to microfuge tubes and stored at -20°C and the cells were transferred to Trizol for RNA extraction.

For whole blood culture, blood was collected in 9 mL heparin coated vacutainers. Six well plates containing 2 mL of RPMI 1640 with 1% penicillin/streptomycin. Each well also contained one of the following LPS (*E. coli,* O55:B5) (100 ng/mL) (Sigma), poly (I:C) (30 µg/mL) (InVivogen), zymosan (*S. cerevisiae*) (50 µg/mL) (InVivogen) or a negative control (no stimulant). 1 mL of blood per well was transferred to the six well plate within 30 min of being collected. The plates were then incubated at 37°C with 5% CO_2 for 24 hours. After 24 hours, the well contents were transferred to microfuge tubes and centrifuged at 600 xg for 10 min. The supernatant was transferred to microfuge tubes and stored at -20°C and cells were transferred to Trizol for RNA extraction.

2.4 ELISA

The bovine IL-8 ELISA used to measure concentration in cell supernatants and serum was carried out as instructed by Cronin et al [13]. The bovine IL-1β ELISA (Thermo Scientific) were performed as per the manufacturer's instructions.

2.5 RNA extraction, cDNA synthesis and qPCR

A combination method of Trizol and the RNeasy Plus Mini Kit (Qiagen) was used to extract RNA. Chloroform was added to the cell pellet in Trizol and shaken vigorously. The solution was then centrifuged for 15 min, 12000 xg at 4°C. The RNA containing aqueous layer was transferred to a clean microfuge tube. An equal amount of 70% ethanol was then added and mixed vigorously. This solution was then transferred to an RNeasy kit column. The manufacturer's instructions were then followed from this point. RNA quantity was measured using the Nano-Drop ND-1000 and quality was assessed using the RNA 6000 Nano Kit on

the 2100 Bioanalyser (Agilent Technologies) according to manufactures instructions. The RNA samples were diluted down to the sample with the lowest concentration. The RNA was then converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) according to manufacturers' instructions. cDNA samples were prepared on a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems) with primers using Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. The following primers were designed using NCBI Primer-BLAST:

HIF1A forward–CAGAAGAACTTTTGGGCCGC, *reverse*-TACAATGCACTGGGGCTGAG; *GUSB forward*-ACCATCGCCATCAACAACAC, *reverse*-TCCCGCGTAGTTGAAGAAGT; *HSP90AB1 forward*-GCATGAAGGAGACGCAGAAG, *reverse*- TCCTTGAGCTGCTGTACACA; *RPS15 forward*-GCGACATGATCATTCTACCCG, *reverse*-GGTAGTGGCCGATCATCTCA; *ACTB forward*-AGATGACCCAGATCATGTTCGA, *reverse*-TGACCCCGTCACCGGAGTCCATCACGAT; *GAPDH forward*-CTCCCAACGTGTCTGTTGTG, *reverse*-TGAGCTTGACAAAGTGGTCG.

No template and no reverse transcriptase controls were also included. The samples were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). The following parameters were used: 95°C for 20 sec, 40 cycles of 95°C for 3 sec and 60°C for 30 sec. From a panel of five reference genes, *RPS15* and *HSP90AB1* were selected to use for normalisation based on low M-values calculated by the geNorm tool in the GenEx software v.5.2.7.44. Cq values were adjusted for primer efficiency and replicates averaged. The values were then normalised to the reference genes and relative gene expression was calculated to the lowest expressed sample which received a value of 1 and higher expressed samples received a value of >1.

2.6 Statistical Analysis

Statistical analysis (including Pearson correlations) was carried out using GraphPad Prism 7. TruCulture® stimulation data was assessed for statistical significance using an ordinary one-way ANOVA for each individual time point. All other data was assessed using a student's t-test. The percentage coefficient of variation was calculated using the following formula: (SD/Mean) *100.

3. Results and Discussion

3.1 TruCulture® induces an IL-1β and IL-8 response across multiple timepoints

The bovine neonatal period is a time of immense change in terms of immune system development, and the stage of immune cell maturity can have a direct influence on the functional capacity of immune cells, including cytokine expression levels [14]. Hitherto, temporal assessment of changes in immune competence was hampered by the availability of standardised immune assays.

In this this study, for the first time, the TruCulture® system was used to profile the immune response in whole bovine peripheral blood stimulated with a PAMPs representing bacterial, viral and yeast agents which are responsible for significant neonatal mortality in cattle. Repeated immunoprofiling showed a clear significant, consistent and reproducible increase in both IL-1β and IL-8 expression at all time points in response to both lipopolysaccharide (LPS) and the fungal PAMP, zymosan. Maximal responses for both the cytokine and the chemokine were evident in blood from calves at 1 month of age (Fig 1A&B), compared to unstimulated control tubes. Multiple studies have evaluated the immune response to LPS in either bovine PBMCs or whole blood *in vitro* [15] and *in vivo* [16-18] and levels shown here are comparable. Although fungal infections like ringworm represent important issues in cattle, studies evaluating the immune response to the fungal PAMP zymosan are less common. Zymosan has been previously reported to induce an IL-1β response via toll like receptor 2 (TLR2) in mice [19]. It has also been studied as a counteractant against immunosuppression in the heat stressed dairy cow as well as activating the immune response in the udder during mastitis [20, 21]. Here we show that zymosan is a potent activator of both IL-1β and IL-8.

Very clear induced responses to both PAMPs are sustained in blood from the same calves at months 3 and 5 for IL-1β. In contrast, the expression levels of IL-8 are considerably muted at months 3 and 5 compared to initial expression levels (Fig. 2B). It is generally understood that TLR mediated cytokine responses are muted in human neonates but this appears to be selective depending on the cytokine or chemokine as IL-8, IL-6 and IL-1β showed higher induced secretion when compared with adult blood [22]. A similar phenomenon was also reported in horses where IL-8 again was expressed at similar or greater levels in newborn foals when compared with older foals [23].

In contrast, no significant induction of either the cytokine or chemokine was evident in response to poly (I:C). This result contrasts sharply with results in human studies at the same dose of poly (I:C) [24], showing a species-specific difference in the innate immune response. Previous studies have shown that poly (I:C) can activate the immune response in cattle and therefore, the results detected here are likely a consequence of sub-optimal PAMP concentration [22].

3.2 Immune cell population profiling

In order to identify biological correlates which may explain the age-specific differences in PAMP sensitivity in calves, analysis of immune cells was performed. At a macro-level, no significant changes were observed between calves in terms of lymphocyte and other leukocyte cell populations in blood over the first 5 months of life (Fig. 2A). Interestingly however, whereas a relative decrease in circulating levels of T cells ($CD4^+$, $CD8^+$ and γδ TCR^+) were detected from 1 month of age by flow cytometry (Fig. 2B), in contrast the number of B-cell marker (surface IgM) positive cells increased until 5 months of age. This data is in agreement with previous reports showing that γδ T-cells become less abundant in calves with age [25]. Less data is available on B-cell markers but the steady increase detected here corresponds with results of a previous study which reported that the mean proportion of B-cells was shown to gradually increase across the first six months of life [26]. The absolute numbers do not change but the shift within the whole lymphocyte population from T-cells to B-cells may explain the change in IL-8 levels. It is possible that the significant decrease in all T-cell subtypes analysed explain the muted response in IL8 expression profile after month 1. T-cells have been documented to secrete IL-8 in humans [27, 28] and it is possible that neonatal T cells have an important role in this regard.

3.3 Comparison of TruCulture® with conventional whole blood stimulation

As the TruCulture® system represents an unconventional method of culturing cells – i.e. in a closed system without the usual 5% CO_2, we sought to compare our immune read-outs with those produced using a conventional whole blood assay from the same calves. Using a consistent volume of blood and media as with the TruCulture® system, stimulations were performed using same concentration of PAMPs. Results showed a consistent induction of both cytokine and chemokine expression over null-stimulated samples. Interestingly however, a significant difference in IL-1β expression was detected between assays in response to zymosan (Fig. 3A) with elevated expression detected using the conventional

whole blood stimulation assay. Similarly, the expression of IL-8 was significantly higher in response to both LPS and zymosan using the conventional whole blood cell stimulation (Fig. 3B). Coefficients of variation for biological replicates for both assay designs (n=10 calves) were calculated for both IL-1 and IL-8 expression levels. Despite generally elevated cytokine and chemokine expression levels detected using the whole blood cell stimulation assay, results revealed generally higher CV values for this assay compared to TruCulture tubes. IL-1β coefficients of variation ranged from 66.82-117.84 and 51.57-124.38 for the Truculture and whole blood culture assay respectively. Similarly for IL-8 expression levels, coefficients varied between 6.61-47.13 and 4.63-186.90 for both assays, depending on stimulus used (Supplementary tables 1 and 2 for IL-1 and IL-8, respectively). Pearson correlation coefficients were also calculated between assays and a more robust relationship between expression levels were detected for the IL-1 expression levels, but not for the IL-8 expression levels (Supplementary Figure 1). Although previously, LPS serotypes have been shown to elicit variable responses, most notably in the case of IL-8 expression [29], in this study the same strain of *E. coli* (O55:B5) was used in both assays. Therefore, the most likely explanation for the differences in cytokine and chemokine expression levels between assays may lie in the origin of the zymosan used in the commercially produced and laboratory-based assays or with the proprietary TruCulture® cell medium and foetal serum sources. Although information is not available on the specific type of zymosan (or other PAMPs) used in the TruCulture® tubes, the zymosan A from *S. cerevisiae* used in this system seemed to induce high pro-inflammatory response as evidenced by IL-1β levels. This finding highlights the importance of choice of strain from which the PAMP of choice is isolated for profiling immune responses. Future work on larger panels of calves will include Bland-Altman plots to assess the relationship between assay measurements across additional immune read-outs. Interestingly, cell death was significantly higher in the conventional whole blood culture system (Fig 3C), and in order to rule out hypoxia accounting for the difference in results between the conventional and TruCulture® system, the expression of the hypoxia-inducible factor 1-alpha (*HIF1A)* gene was investigated in cells from both assays. No significant difference in *HIF1A* gene expression was detected relative to non-stimulated cells (or between assays) (Fig 3D) and therefore we conclude that hypoxia is not a factor in any differences detected. Poly (I:C) did not induce an adequate response in either assay to be deemed as a reliable viral stimulant at this concentration in cattle.

Conclusion

Assay standardisation is the key to facilitating temporal and multi-stimulant (or disease) comparisons [30], and progress has been made in human studies. Along with the successful use of TruCulture for immune function monitoring [10], similar immune system assays have been designed to measure lymphocyte proliferation [31]. An equivalent standardised immunophenotyping assay is not yet available for use in cattle. One exception is the Quantiferon™ assay is available commercially for the detection of specific adaptive immune responses to *Mycobacterium bovis* antigens [32]. For other pathogens however, or for the assessment of innate immune responses, similar high-throughput assays have not been developed. Standardised assays are critical too to mitigating the introduction of technical variation inherent in previous generations of *in vitro* assays. Here, we report that the TruCulture® system is a useful immunoprofiling tool to examine the *ex vivo* bovine innate immune response to bacterial and fungal stimulants and we predict will be widely used to overcome the limitations associated with conventional immune phenotyping in cattle. Applying simple-to-use technologies, such as the TruCulture® system offers enormous potential to provide in-depth understanding of the phenotypic variance of immune responses in cattle and offers relevance across livestock species. Capturing this variation offers potentially valuable insights which can be used to improve disease diagnosis, improve adjuvant formulation and guide targeted therapeutic interventions including vaccination regimens.

Declarations

All authors declare that they have no competing interests

Authors Contributions

Conceived the study: KGM. Performed experiments and interpreted data: MOB, KGM and RMcL. Wrote the manuscript: MOB, KGM and RMcL. All authors read and approved the final manuscript.

Funding

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Ethics Statement

All procedures described were conducted under ethical approval and experimental license from the Irish Health Products Regulatory Authority in accordance with the Cruelty to Animals Act 1876 and in agreement with the European Union (Protection of Animals Used for Scientific Purposes) regulations 2012 (S.I. No. 543 of 2012).

Acknowledgements

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Consent for Publication

Not applicable.

Availability of data and material

The datasets analysed during the current study are available from the corresponding author on reasonable request.

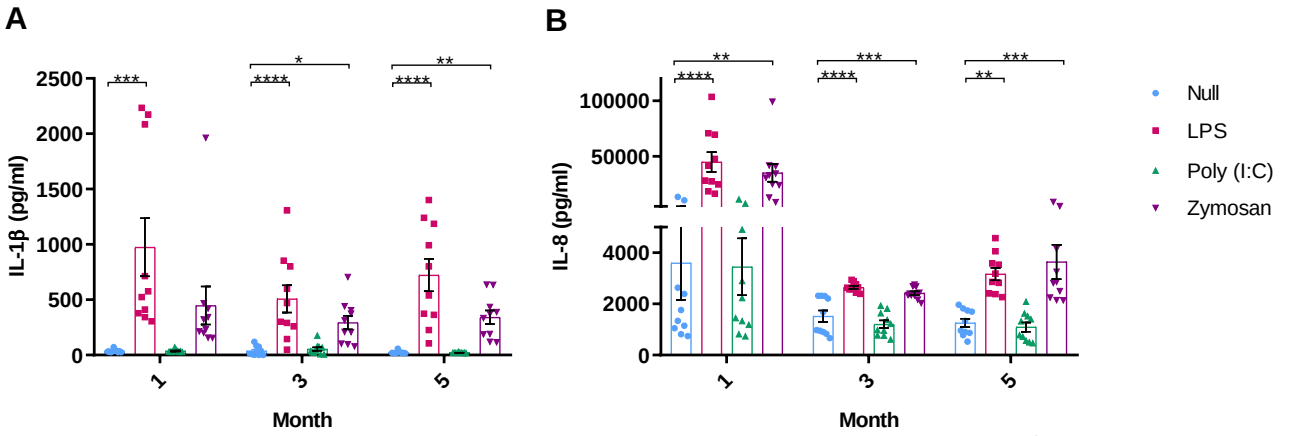
Figure legends:

Figure 1. IL-1β and IL-8 protein expression of Holstein-Friesian calves in response to LPS, poly (I:C) and zymosan using TruCulture®. Blood was taken via jugular venepuncture across one to five months of age and stimulated for 24 hours at 37°C with LPS (100 ng/mL), poly (I:C) (20 µg/mL) and zymosan (50 µg/mL) using the TruCulture® system. (A) IL-1β and (B) IL-8 concentration was measured from the supernatant by ELISA and represented in pg/mL. Error bars represent the SEM of n=10. Significance is between haplotypes where *P<0.05 , ***P<0.001 and ****P<0.0001.

Figure 2. Haematological and lymphocyte profiles of Holstein-Friesian calves across one to five months of age. Blood from calves was taken via neck venepuncture monthly and processed by the (A) ADVIA 2120 system and (B) flow cytometry analysis for lymphocyte populations. Error bars represent the SEM of n=11.

Figure 3. Comparison of TruCulture® and a conventional whole blood culture system by IL-1β and IL-8 expression. Blood was taken via jugular venepuncture from three month old calves and stimulated for 24 hours at 37°C with LPS (100 ng/mL), poly (I:C) (20 µg/mL) and zymosan (50 µg/mL) using each system. An unstimulated negative control (null) was also included. (A) IL-1β and (B) IL-8 concentration was measured from the supernatant by ELISA and represented in pg/mL n= 10. Error bars represent SEM. Significance is between systems where ***P<0.001 and ***P<0.0001.

Figure 4. Assessment of cell viability and HIF1A expression in the TruCulture® and a conventional whole blood culture system. Blood was taken via jugular venepuncture from six month old calves and stimulated for 24 hours at 37°C with LPS (100 ng/mL), poly (I:C) (20 µg/mL) and zymosan (50 µg/mL) using each system. A pre-incubation sample (-24hr) and unstimulated negative control (null) were also included. (A) Cell viability was quantified by PI staining for flow cytometry and (B) *HIF1A* expression was assessed via qPCR. Error bars represent SEM of n=3. Significance is between systems where *P<0.05.



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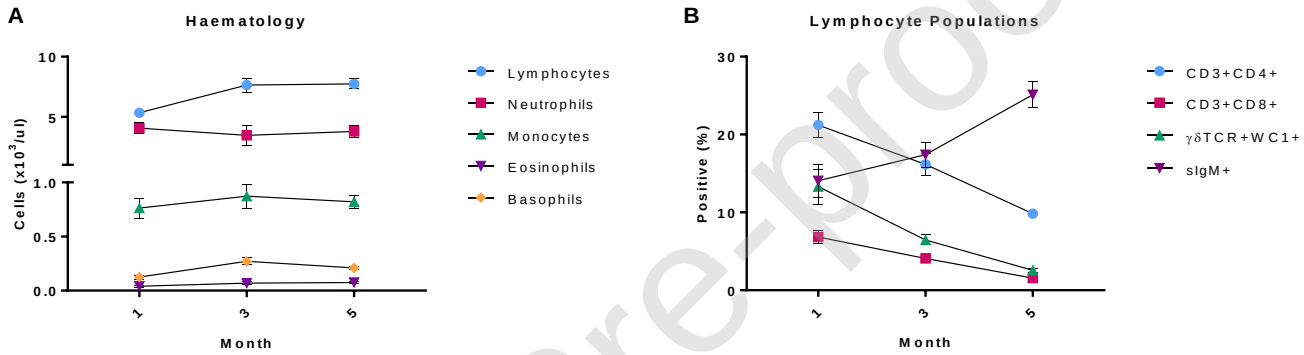

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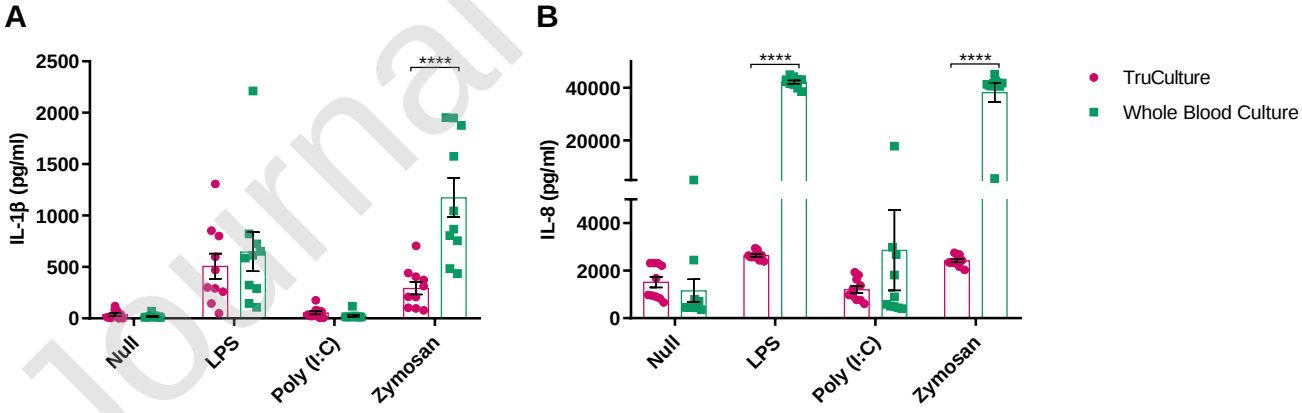

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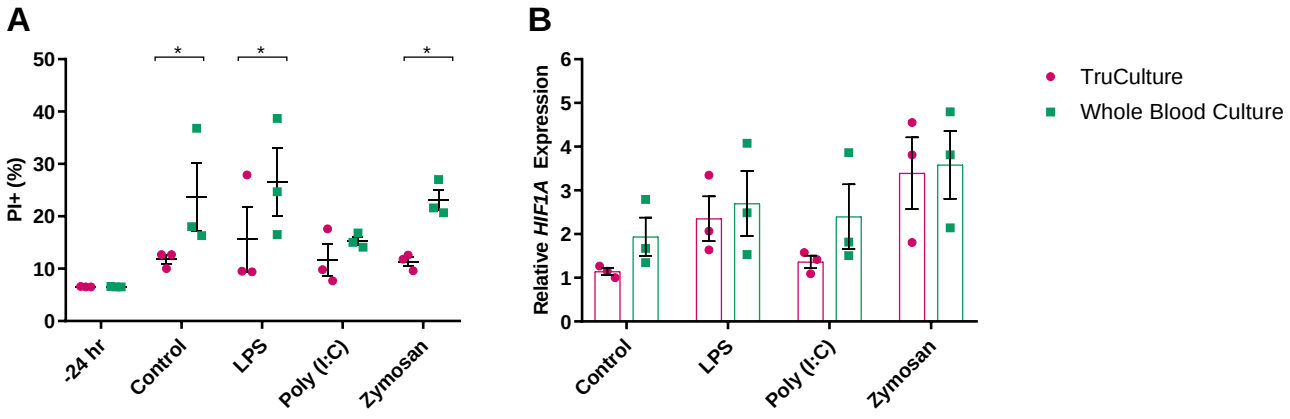

Figure 4.

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