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# Characterising the transcriptomic response of bovine peripheral blood mononuclear cells to a mycobacterial cell wall fraction

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ABSTRACT

*Background:* Innate immune stimulants, including mycobacterium cell wall fractions (MCWF), offer an alternative control option to prevent and treat disease in livestock, by appropriately augmenting the innate immune response. However, the functional response to mycobacterium cell wall fractions in cattle is not well defined. In this study we report the transcriptomic response of bovine peripheral blood mononuclear cells to MCWF in the product Amplimune®.

*Methods:* Amplimune-induced transcriptomic changes in bovine peripheral blood mononuclear cells were determined following an initial pilot study and a later time course experiment. These cells were cultured *in vitro* for 24 h. In the pilot experiment, cells were stimulated with 0, 2, 5, 12.5 or 31.25 μg/mL Amplimune. In the time course experiment, cells were stimulated with 0 or 31.25 μg/mL Amplimune. In both experiments the total RNA was extracted at 0 h, 6 h and 24 h following stimulation. Ribosomal RNA depleted samples were sequenced, and data analysed to determine differential gene expression profiles. Differential gene expression was further analysed to determine enriched biological processes and pathways and a co-expression network.

*Results and conclusion:* Amplimune induced dose- and time-dependent gene expression profile changes in bovine peripheral blood mononuclear cells, which were enriched into GO-BP regulation of signalling receptor activity, response to cytokine and inflammatory response. Enriched pathways from KEGG analysis were cytokine-cytokine receptor interaction, IL17 signalling and TNF signalling pathways. Selected genes involved in these processes and pathways included *IFNG, IL17A, TNF, IL22* and *IL23A*. *PDE1B, CSF2* and *IL36G* were identified as the most connected genes in a co-expression network, while the connection between *SAA2* and *SIGLEC5* was the most important for flow of information within the network. Genes encoding for pro-inflammatory cytokines *TNF, IL1B, IL6, IL2,* and *IL12B*, and chemokines *CCL3, CCL4* and *CCL20* were also upregulated at 6 and 24 h post stimulation, as was the β-defensin gene *TAP*. These results assist in understanding how mycobacterial cell wall fractions alter immune function and may contribute to our understanding of the immune stimulant response attributed to Amplimune.

## 1. Introduction

Bovine respiratory disease (BRD) is a significant threat to the health of cattle managed in feedlot systems (Blakebrough-Hall et al., 2020). Currently, vaccines are used to aid in prevention of BRD and antibiotics are used to treat the disease (Blakebrough-Hall et al., 2020), however, both approaches have limitations. Immune stimulants such as mycobacterial cell wall fragments (Solano-Suárez et al., 2021) and liposome-TLR complexes (Wheat et al., 2020) have the potential to be used in disease control strategies, due to a reported ability to enhance immune system function (Wu et al., 2021). Innate immune stimulants enhance innate and adaptive immune responses by training or shaping

*Abbreviations:* AGRF, Australian Genome Research Facility; AMP, Amplimune; BCG, Bacille Calmette-Guerin; BoHV-1, Bovine alphaherpesvirus type 1; BRD, Bovine respiratory disease; BVDV, bovine viral diarrhoea virus; ConA, Conconavalin A; DE, differentially expressed; EDTA, ethylenediaminetetraacetic acid; MCWF, mycobacterium cell wall fractions; MRNA, messenger ribonucleic acid; NT, no stimulation; PBMC, peripheral blood mononuclear cells.

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innate immune cells via epigenetic, transcriptomic and metabolic alterations, modifying the state of alertness and allowing either an enhanced or tolerised reaction to secondary challenge (Netea et al., 2020). *In vitro* human leukocyte culture experiments and *in vivo* mouse trials have demonstrated that innate immune stimulants often induce a pro-inflammatory response (Arts et al., 2018; Quintin et al., 2012; Saeed et al., 2014). As such, pro-inflammatory markers have primarily been used to monitor induction of immune responses following stimulant administration. However, only rudimentary knowledge exists regarding the induction of gene expression in cattle leukocytes by immune stimulants, which are known to establish trained immunity responses and have potential to ameliorate disease in livestock.

Immune stimulants such as cell wall fractions of non-pathogenic mycobacteria (MCWF), yeast and fungi, are being investigated as potential disease control agents for animal production systems. The immune stimulant Amplimune® (AMP), contains MCWFs of *Mycobacterium phlei,* a non-pathogenic mycobacterium found naturally in the environment (Filion et al., 1999), and has been investigated for this application. Previously, AMP has been shown to increase the number of major histocompatibility complex Class $II^{+}CD4^{+}$ T cells in peripheral blood of neonatal dairy calves following subcutaneous injection (Griebel, 1999). A study that measured both pro and anti-inflammatory responses, following administration of MCWF from *M. phlei* to pregnant mares, found increased expression of the anti-inflammatory cytokine *IL10*, but no differential expression of *IL8* (Fumuso et al., 2007). Other studies have found treatment with AMP reduced the incidence of metritis and mastitis in dairy cows (Solano-Suárez et al., 2021), reduced the need to treat calves for respiratory disease following transportation (Omontese et al., 2020), and improved weight gain, reduced morbidity and mortality in calves entering a feedlot (Nosky et al., 2017). Similarly, Alexander et al. (2022) determined that AMP is safe to use in weaned beef cattle and reported moderate immune activation via increased concentrations of TNFα in serum, without excessive inflammation.

It is thought that the trained immune response begins with the induction of the innate response, triggering specific pathways of activation via receptors such as toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene 1-like receptors (RLRs) and c-type lectins (Alberts et al., 2015; Brueggeman et al., 2022; Butcher et al., 2018; Cheng et al., 2014; Domínguez-Andrés, Joosten, et al., 2019). Adaptor proteins or receptor chains recruit kinases and proteins in multiple pathways (Brueggeman et al., 2022; Butcher et al., 2018; Domínguez-Andrés, Novakovic, et al., 2019; Morgan et al., 2022; Owen et al., 2021; Quintin, 2019; Saeed et al., 2014) that activate complexes, initiate phosphorylation or glycolysis, interact with the TCA cycle (influencing energy production pathways) or lead to signalling in the nucleus via transcription factors such as BATF3, IRF8 (Arnold et al., 2019), ATF7, AP1 and NFκβ (Butcher et al., 2018; Owen et al., 2021; Quintin, 2019). Within the nucleus, production of inflammatory mediators, inhibitory type I interferons (IFNs) (Butcher et al., 2018), or downregulation of succinate-dehydrogenase (SDH) (Domínguez-Andrés, Novakovic, et al., 2019) result in transcriptional changes in histone marks, enhancers and promotors, leading to the trained or tolerized response to secondary challenge (Saeed et al., 2014). It has also been proposed that trained immunity can last for up to a year through the effect of stimulants polarizing cytokines and inducing specific antigen presenting cell marker expression (such as $CD14^{+}$ and $CD11b^{+}$), affecting T-cell responses (Kleinnijenhuis et al., 2014). Influences on T cells could impact the balance of Th1, Th2 or Th17 responses (Kleinnijenhuis et al., 2014; Murphy et al., 2021), to shape the adaptive immune response.

Pro-inflammatory immune responses are typical of the response to immune stimulants although the exact nature of the response has been shown to be variable. A study investigating the transcriptional response of bovine macrophages to *in vitro* challenge with *Mycobacterium avium* spp *paratuberculosis* (MAP) demonstrated the stimulant induced a strong inflammatory response but a concomitant pro-inflammatory, anti-inflammatory and regulatory expression profile was also observed via TLR, NFκβ and IL17 pathways (Ariel et al., 2020). Specifically, expression of pro-inflammatory genes such as *TNF, CCL3, CXCL2, PTGS2, NOS2* and *IL6*, regulatory genes such as *LIF* and *IL1RA* and anti-inflammatory genes such as *IL10* and *ATF3* were all significantly upregulated (Ariel et al., 2020). In contrast, a study investigating the transcriptional response of bovine PBMC to *in vitro* challenge with live *Mycobacterium bovis,* found that expression of genes encoding inflammatory cytokines *TNF, IL12B, IL1B* and *IFNG* were downregulated, as was expression of genes encoding anti-inflammatory markers *IL10* and *MAP3K8* (Cheng et al., 2015), suggesting a possible tolerised state. In one study investigating trained immunity, training primary porcine monocytes with live Bacille Calmette-Guerin (BCG, an attenuated form of *M. bovis*) or β-glucan followed by secondary stimulation with LPS did not induce significant changes in expression of *TNFA* or *IL1B* but did increase expression of *IL18* and *CASP1*, genes associated with the NLRP3 inflammasome (Byrne et al., 2020). In contrast, *in vivo* vaccination of humans with BCG and then stimulation of *ex vivo* PBMC with *Candida albicans* resulted in increased production of pro-inflammatory cytokines IFNα, IFNγ, IL1β, IL6 and TNF and anti-inflammatory cytokines IL10 and IL1Rα in supernatant (Cirovic et al., 2020). Further, peripheral blood mononuclear cells (PBMC) from calves vaccinated with BCG showed increased expression of genes encoding cytokines *TNFA, IL1B* and *IL6* following *ex vivo* stimulation with LPS from *Escherichia coli* or tripalmitoylated lipopeptide (Guerra-Maupome et al., 2019).

Despite the studies reported above, much remains unknown about the mechanistic response of livestock to different innate immune stimulants. Characterisation of the immediate early global transcriptional response following MCWF stimulation induced by such stimulants has not been reported and as such, characterisation of the response has been based on data inferred from human or mouse studies. Furthermore, there are concerns that these stimulants may be detrimental to production animals if they induce strong pro-inflammatory response pathways that promote fever and loss of appetite (Amadori and Spelta, 2021), and repartition energy resources away from other important biological functions, thereby increasing the vulnerability of the production animal to ill-thrift. To address these concerns this study was designed to investigate the transcriptomic responses of bovine peripheral blood mononuclear cells (PBMC) following stimulation with AMP (containing MCWF) *in vitro*.

## 2. Materials and methods

### 2.1. Overview

A pilot experiment and a time course experiment were conducted at the CSIRO, F. D. McMaster research station located near Armidale in New South Wales, Australia.

### 2.2. Animals and experimental design

Six Angus cross steers aged 18–20 months were enrolled in the two experiments. None of the steers showed any clinical symptoms of disease. The pilot experiment was conducted on a single steer (liveweight of 414 kg) to gauge an optimal dose of Amplimune (AMP) for *in vitro* cell stimulation. Following results of the pilot experiment, an *in vitro* PBMC stimulation time course experiment was conducted using PBMC isolated from the blood of five steers (liveweight of 543 ± 44 kg). For both trials during the time of blood collection, cattle were housed in a paddock and had *ad libitum* access to water and good quality hay.

### 2.3. Sample collection, cell culture, stimulation and extraction

Blood samples were collected via jugular venepuncture into sterile EDTA coated vacutainers (Becton Dickson, USA) and processed as previously described (Novak et al., 2018). Briefly, blood was chilled on ice

for one hour, then mixed gently and diluted 1:2 with Hank's balanced salt solution. Diluted blood was layered onto Ficoll-paque (GE Healthcare Life Sciences, USA) and then spun at 400 x *g* without brake for 45 min. The isolated PBMC were counted on an automated haematology analyser (Cell-Dyn 3500 R, Abbot Diagnostics, Australia) and resuspended in complete culture media (High-glucose DMEM, 10 % foetal calf serum, 1 % Penicillin/Streptomycin, 1 % glutamax; ThermoFisher Scientific, USA) at a concentration of $1 \times 10^6$ cells per mL. A total of $1 \times 10^6$ cells in 1 mL of medium, per well were seeded in 24 well plates (Sarstedt, Germany) and incubated for 24 h at 37 ℃ in a humidified 5 % $CO_2$:95 % air atmosphere (InCU Safe Sanyo $CO_2$ Incubator, USA) before stimulation. For both experiments, treatments were replicated in seven wells on the plate.

For the pilot experiment, following the initial 24 h of culture without AMP, PBMC were treated with either media only (NT), or 2 μg, 5 μg, 12.5 μg, or 31.25 μg AMP per mL medium. AMP is an emulsion of 0.5 mg/mL fragmented sections of *M. phlei* cell wall with nucleic acids conserved onto it, adjuvanted in 2 % squalane, final solution delivered in phosphate buffered solution (NovaVive Inc., Napanee, Ontario, Canada). Previous *in vitro* studies had used mycobacterium cell wall extract of *M. phlei* at 12.5 μg/mL, hence the decision for a concentration range with that concentration in the middle (Archambault et al., 1989; Vézina and Archambault, 1997). At 0 h (T0), 6 h (T6) or 24 h (T24) post-stimulation, supernatants were discarded, cells were washed twice with 2 mL sterile PBS. Cells were lysed as per RNA mini kit instructions as described below.

For the time course experiment, after the initial 24 h of culture without AMP, PBMC were treated with either media only (NT), or 31.25 μg AMP per mL medium (NovaVive Inc., Napanee, Ontario, Canada). At 0 h (T0), 6 h (T6) or 24 h (T24) post-stimulation, supernatants were discarded, cells were washed twice with 2 mL sterile PBS and total RNA was then extracted using the method described below.

The four most homogenous wells were selected from the seven technical replicates (identified by visual assessment of the number and spread of cells under a microscope) for total RNA extraction. Following washing with 2 mL sterile PBS, two wells were combined by lysing cells in one well, then transferring the lysed cells and lysis buffer into a second cell. Lysed cells and lysis buffer were then transferred to the shredder column supplied in the Isolate II RNA Mini Kit (BioLine, Meridian LifeScience Inc., USA) and the protocol used to extract RNA was followed as per manufacturer's instructions. The kit incorporates the use of genomic DNA eliminator spin columns. The yield of total RNA was determined using a Nanodrop 8000 Spectrophotometer (v2.3.2, Thermo Fisher Scientific, USA). The 260:280 nm absorbance ratios for prepared samples were between 2.0 and 2.22. The samples were stored in DNAse/RNAse free 2 mL microtubes at –80 °C until required for RNA sequencing.

For the pilot experiment only one RNA sample per AMP treatment at each time point was extracted for analysis (11 samples in total). For the time course experiment RNA was extracted from five NT samples at T0, four NT samples and four AMP samples at T6 and five NT samples and five AMP samples at T24 (see supplementary material Additional File 1: Table S1 In Vitro Treatments).

### 2.4. RNA sequencing and mapping

Thirty-five total RNA samples were sent to the Australian Genome Research Facility (AGRF, Melbourne Australia) for RNA Illumina Sequencing. NovaSeq Control Software v1.7.5 and Real Time Analysis v3.4.4 were used to perform image analysis. Ribosomal RNA depletion was conducted using the Ribo-Zero Gold rRNA depletion method (AGRF, Melbourne Australia). In total, thirty-five 100 bp paired-end libraries for sequencing were prepared from rRNA depleted RNA. Illumina bcl2fastq 2.20.0.422 pipeline was used to generate sequence data (AGRF, Melbourne, Australia). A minimum of 100 million paired-end reads were obtained for each sample. Eleven libraries from the pilot experiment were sequenced with > 120 M reads. Each sample had > 95 % reads mapped to the bovine reference genome (*Bos taurus* Hereford UMD3.1). Twenty-four libraries from the time course experiment were sequenced with > 200 M reads. Each sample had > 92 % reads mapped to the bovine reference genome (*Bos taurus* Hereford UMD3.1). Sample reads were mapped using the RNASeq analysis function in CLC Genomics Workbench v21.0.4 (digitalinsights.giagen.com). The filtering strategy included removal of contaminated paired-end reads (allowing up to 2 mismatches, 3 insertions and 3 deletions), removal of poor quality paired-end reads, removal of paired-end reads that did not meet the required minimum fraction length of 0.8 and similarity of 80 % with a maximum of 10 hits for a read.

### 2.5. Analysis

#### 2.5.1. Pilot experiment

The raw counts for unique genes across all 11 libraries in the pilot experiment were normalised by trimmed mean of M-values (TMM) using the EdgeR package (Robinson et al., 2010) in R (R-Core-Team, 2018) and only genes presenting more than 1 count per million mapped reads (CPM) in at least 5 samples were kept for downstream analysis. Because there were no replicates per treatment x time, differential expression was performed by grouping samples into treated (all AMP concentrations) and untreated (NT) and genes were considered differentially expressed (DE) if FDR < 0.05. Principal Components Analysis (PCA) and heatmaps were generated using log2CPM values.

#### 2.5.2. Time course experiment

As for the previous analysis, the raw counts for unique genes across all 24 libraries in the time course experiment were normalised by the TMM method and only genes presenting more than 1 CPM in at least 3 samples were kept for the downstream analysis. Raw counts were log2CPM transformed to be used in PCA analysis. To investigate the effect of the treatment after 6 h and 24 h AMP-stimulation (T6 and T24), we used a generalised linear model and a quasi-likelihood F-test with the EdgeR package considering a multilevel design, as per example 3.5 in the EdgeR User's Guide (Robinson et al., 2010). To achieve this, we first initialised the design matrix with the animal effect to account for baseline differences between individuals, and then appended the time-specific treatment effect (i.e., AMP-T6 and AMP-T24). After estimating the dispersions, we fitted a linear model using glmQLFit function to find genes responding to treatment at T6 (AMPT-T6 vs all NT) and genes responding to treatment at T24 (AMP-T24 vs all NT). Genes responding to treatment regardless of time were also identified (all AMP vs all NT), representing the effect of treatment alone (hereafter called T6+T24), as well as genes responding differently to treatment from T6 to T24 (AMP-T6 vs AMP-T24), hereafter called T6≠T24. This approach allowed the identification of genes that may not have been DE at either T6 or T24 individually, but that were DE between those time points. Genes were considered significant for each comparison if FDR < 0.05 and log Fold Change > 2 or < −2. Three outlying samples were removed from analysis in the time course experiment.

### 2.6. Gene ontology enrichment, normalised expression matrix and co-expression analysis

Gene ontology enrichment analyses of the DE genes in the time course experiment were conducted using the GeneOntology Over-representation Test, Biological Process NonRedundant, via WebGestalt (Liao et al., 2019) to generate biological processes (GO-BP) associated with those genes. The 13,459 expressed genes in PBMC were used as background for the enrichment analysis. KEGG pathway enrichment analysis was also conducted on the DE genes in the time course experiment using the KEGG functional database via WebGestalt (Liao et al., 2019), to generate enriched pathways associated with those genes. Again, the 13,459 expressed genes in PBMC were used as the reference

set for the enrichment analysis.

A normalised expression matrix of the 442 DE genes at T6 and T24 was used to generate a heatmap where genes and samples were clustered based on Euclidean distances after centring and scaling data by using the pheatmap package (Kolde, 2019) in R (R-Core-Team, 2018). Figures were generated in R (R-Core-Team, 2018), using packages pheatmap (Kolde, 2019) and ggplot2 (Wickham, 2016).

DE genes from T6 and T24 were assessed in a co-expression network conducted using the Partial Correlation and Information Theory algorithm (PCIT (Reverter and Chan, 2008)). PCIT uses a gene expression matrix to test all possible 3-way combinations in the dataset and only keeps correlations between genes if they are significant and independent of the association of another gene. The first network was created using all 442 DE genes from T6 and T24 with FDR < 0.05 and partial correlations above 0.50. Filtering for partial correlations above 0.95 only reduced the number of genes in the network to 301. Then, a subnetwork was created by selecting genes directly connected (first neighbours) of two genes of interest (*IFNG* and *IL17A*). A fourth network was created filtering the subnetwork for partial correlations above 0.90. Networks were visualised in Cytoscape v3.8.2 (Shannon et al., 2003) and searchable PDF images of the networks are provided in supplementary material Additional Files 8–11.

## 3. Results

### 3.1. Proportions of leukocyte cell types in PBMC

Data from the cell count of cells in the PBMC from steers in the pilot experiment and time course experiment confirmed that more than 98 % of isolated cells were mononuclear cells as expected. The predominant cell type was lymphocytes, followed by monocytes.

### 3.2. Pilot experiment to test AMP dosage

Unsupervised two component PCA of gene expression data from AMP treated PBMC isolated from a single steer accounted for 79 % of experimental variation. The PCA demonstrated both time- and concentration-dependent clustering of PBMC compared to T0 and T6-NT, T24-NT (Fig. 1A). Both the AMP-T6 and AMP-T24 gene expression profiles clustered independently of each other, highlighting an effect of time following stimulation. Within T6 and T24, the gene expression profiles from the 2 μg/mL to 31.25 μg/mL AMP treatments clustered in a dose-dependent pattern on the PCA plot (Fig. 1A), suggesting that AMP induced a significant increase in gene expression in cattle PBMC. Overall, 642 genes were differentially expressed in PBMC treated with AMP compared to media only (NT) PBMC with an FDR < 0.05

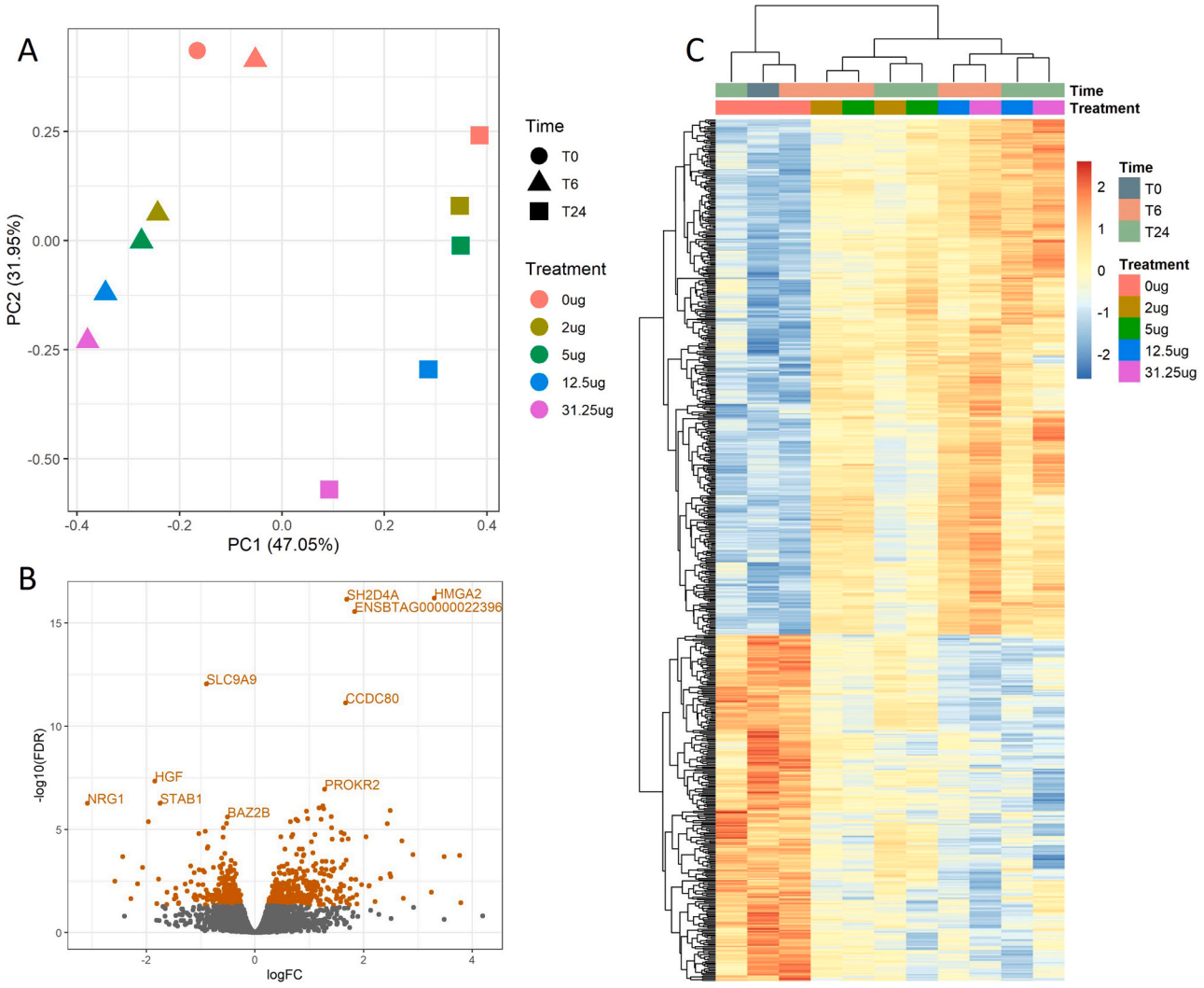


**Fig. 1.** Results from Pilot Amplimune Dosage Experiment. (A) Unsupervised PCA of genes in bovine PBMC samples treated with different concentrations of Amplimune (AMP) in the pilot experiment. (B) Volcano plot of all genes expressed by bovine PBMC treated with AMP in the pilot experiment. (C) Heatmap of DE genes from bovine PBMC treated with different concentrations of AMP in the pilot experiment.

(supplementary material Additional File 2: Table S2 Pilot Experiment Differentially Expressed Genes). The top five candidate genes that were upregulated by AMP, were *HMGA2, SH2D4A, ENSBTAG00000022396, CCDC80 and PROKR2*, whereas the top five most downregulated genes were: *SLC9A9, HGF, STAB1, NRG1 and BAZ2B* (Fig. 1B). The heatmap analysis of the DE genes indicated a cluster of up-regulated genes and a cluster of downregulated genes. The intensity of gene expression in the up- and downregulated clusters increased in an AMP concentration and time-dependent manner (Fig. 1C). Therefore, based on the AMP induced PBMC gene expression profile of the Pilot trial (Fig. 1C), the AMP dose for use in a 24 h time course *in vitro* PBMC stimulation experiment was identified as 31.25 μg/mL.

### 3.3. Differentially expressed genes in the time course experiment

The results from the Pilot experiment indicated that the expression of genes was greatest when cells were stimulated with 31.25 μg/mL AMP, therefore, this concentration was used in the AMP stimulated PBMC gene expression time course experiment. An unsupervised two component PCA of all samples in the time course experiment accounted for approximately 63 % of the total experimental variation and revealed distinct clusters of NT samples compared to AMP (31.25 μg/mL) samples (Fig. 2A). However, NT samples from T0 and T6 clustered together, suggesting that the 6 h cultured PBMC had a similar expression profile to freshly isolated PBMC. In contrast, NT samples from T24 were not superimposed with the NT samples from T0 or T6, suggesting that after 24 h, culture conditions significantly changed the PBMC expression profile when no treatment had been applied.

In the contrast T6-AMP vs all NT, (hereafter called T6) there were 122 DE genes (FDR $< 0.05$, LogFC $>2$ or $< -2$), and in the contrast T24-AMP vs all NT, this number increased to 419 (hereafter called T24) (FDR $< 0.05$, logFC $>2$ or $< -2$). These genes (122 DE at T6 and 419 DE at T24) are represented in a Venn diagram Fig. 2B. There were 99 genes commonly DE at T6 and T24 which corresponded to the contrast of all AMP (treated) vs all NT (non-treated), hereafter called T6+T24 (FDR $< 0.05$, logFC $> 2$ or $< -2$). These 99 genes (in the intersection of the Venn diagram, Fig. 2B) responded to treatment with AMP independent of time. The 23 genes DE at T6 but not at T24 suggested a time dependent response to AMP. The 320 DE genes at T24 but not T6 also suggested a time dependent response to AMP.

In the contrast AMP-T6 vs AMP-T24, we identified 218 genes responding differently to treatment between T6 and T24, hereafter called T6≠T24 (FDR $< 0.05$, logFC $> 2$ or $< -2$). These genes either increased (139 genes) or decreased (79 genes) expression over time. These genes are presented in a graph showing the number of genes and direction of change in expression (Fig. 3). The full list of DE genes in each group are presented in supplementary material Additional File 3: Table S3 Time Course Experiment Differentially Expressed Genes.

The top 5 upregulated and top 5 downregulated DE genes at T6 and at T24 in terms of log fold change are presented in Table 1. Similarly, the top 5 upregulated and top 5 downregulated genes DE at T6+T24 and between T6≠T24 in terms of log fold change, are presented in Table 2.

### 3.4. Functional enrichment and gene clustering in the time course experiment

GO enrichment analysis was conducted on genes that were upregulated at T6 or T24 and genes that were downregulated at T6 or T24, (relative to NT at each of those time points) (supplementary material Additional File 3, Table S3: Time Course Experiment Differentially Expressed Genes), revealing biological processes (GO-BP) that were more functionally present in PBMC stimulated with AMP. The top 5 GO-BPs associated with upregulated genes at T6 and T24, along with the four GO-BPs associated with downregulated genes at T24 are displayed in Table 3 and a full list of GO-BP associated with upregulated or downregulated genes at T6 and T24 and the genes contributing to those processes can be viewed in supplementary material Additional File 4: Table S4 Gene Ontology Biological Processes.

Common GO-BP associated with upregulated genes at both T6 and T24 included regulation of signalling receptor activity, response to cytokine and inflammatory response. The upregulated genes associated with these GO-BP included some that were consistent at both times; *IL12B, CXCL2* and *PTGS2*, and some that were upregulated only at the specific time points; *IL6* and *IL2* at T6, and *TNF* and *IL17A* at T24. The analysis revealed that no GO-BP were associated with downregulated genes at T6, but 21 GO-BP were associated with upregulated genes at T6 including positive regulation of cell death and positive regulation of intracellular signal transduction. The analysis also revealed 80 GO-BP associated with upregulated genes at T24 including leukocyte migration and response to biotic stimulus, along with four GO-BP associated

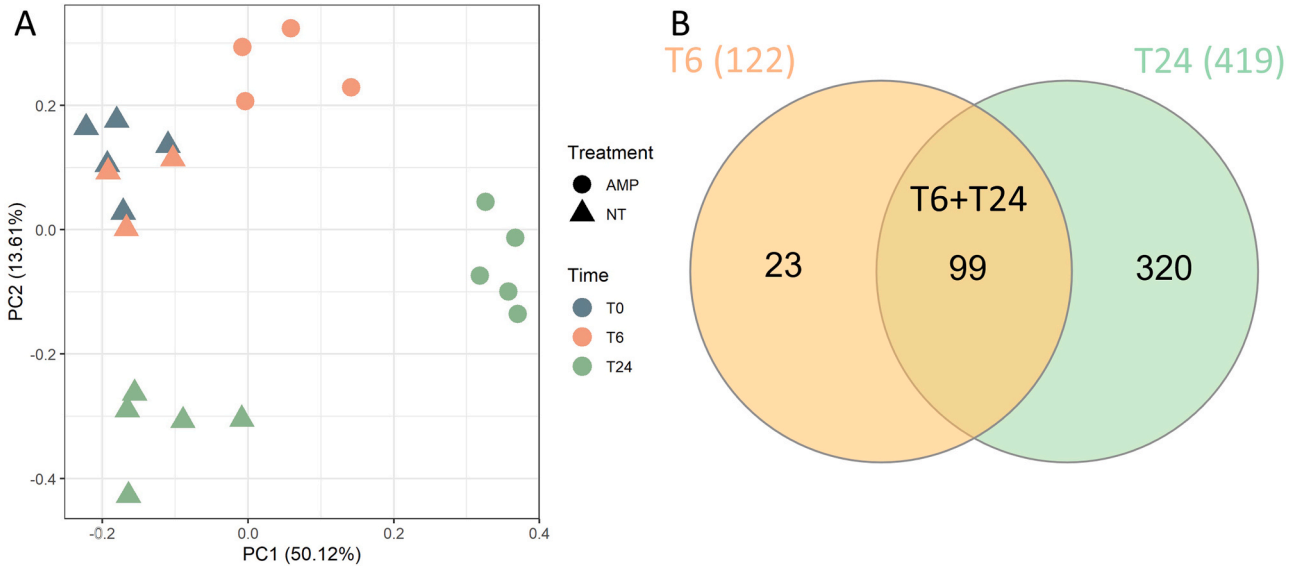


**Fig. 2.** Results from time course experiment. (A) Unsupervised PCA of all genes in bovine PBMC in untreated (NT) or Amplimune (AMP) treated samples and extracted at different times post-stimulation in time course experiment. (B) Venn diagram of differentially expressed (DE) genes at T6 and T24 post-AMP stimulation (compared to all NT). Of the 122 gene DE at T6, 23 were only DE at T6 not T24, 99 genes were commonly DE at both T6+T24 (centre of the Venn) and of the 419 genes DE at T24, 320 were only DE at T24 not T6.

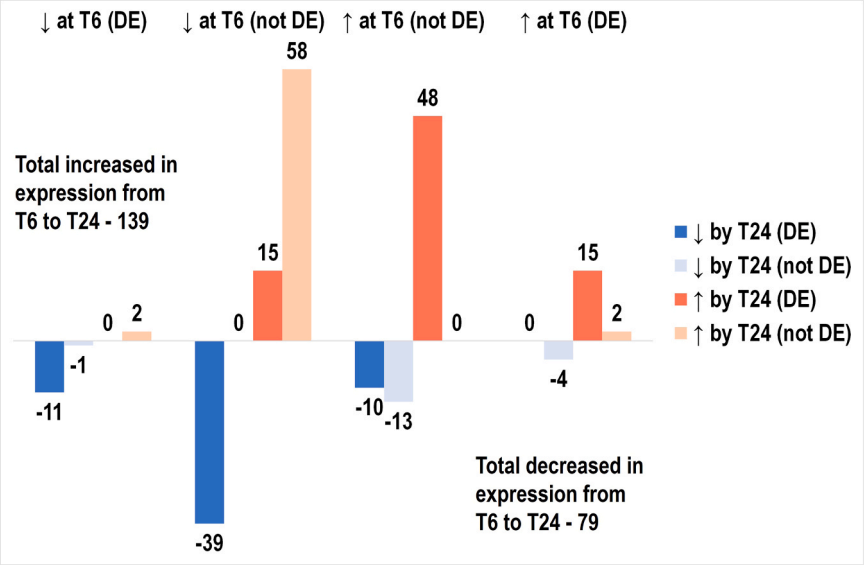


**Fig. 3.** Genes expressed differently between T6 and T24 (T6≠T24). The number of genes that responded differently to treatment from T6 to T24, either increasing or decreasing in expression. These are genes that may not have been DE at either T6 or T24 individually but that were DE between those time periods.

**Table 1**
Top 5 upregulated genes and top 5 downregulated DE genes at T6 or T24.

| T6[a] | | | T24[a] | | |
|---|---|---|---|---|---|
| **Gene** | **logFC** | **FDR** | **Gene** | **logFC** | **FDR** |
| TGM3 | 4.1 | 3.0E−04 | EDN1 | 6.8 | 5.5E−10 |
| CCL4 | 3.9 | 9.1E−09 | CCL3 | 6.7 | 1.9E−10 |
| CCL3 | 3.8 | 1.3E−06 | IL22 | 6.6 | 7.0E−12 |
| CCL20 | 3.8 | 1.6E−07 | IL36G | 6.4 | 6.8E−11 |
| F3 | 3.8 | 2.5E−08 | CSF2 | 6.4 | 4.7E−13 |
| CD300LF | −4.1 | 5.0E−03 | FOLR2 | −6.2 | 3.9E−05 |
| FGL2 | −4.1 | 6.5E−05 | TGFBI | −6.3 | 2.5E−11 |
| Regakine−1 | −5.1 | 0.01 | NRG1 | −7.2 | 1.4E−10 |
| ENSBTAG00000047363 | −5.1 | 3.0E−04 | CD163 | −8.2 | 2.9E−11 |
| CLEC7A | −6.7 | 1.7E−06 | ENSBTAG00000047363 | −8.6 | 2.1E−06 |

FDR, p-value adjusted for false discovery rate; logFC, log fold change in bovine PBMC gene expression, treated versus not treated; T6, 6 h post-AMP stimulation; T24, 24 h post-AMP stimulation.

[a] top 5 upregulated and top 5 downregulated genes out of 122 at T6 and 419 at T24.

with downregulated genes at T24 including protein activation cascade and taxis. The downregulated genes associated with GO-BP included *C1QA*, *CLEC7A, IL1R2* and *CD163*.

Further KEGG database pathway enrichment analysis revealed a range of pathways enriched by the DE genes at T6, T24 T6+T24 and T6≠T24. Top pathways enriched by DE genes at T6, T24 and T6+T24 were cytokine-cytokine receptor interaction, IL17 signalling pathway and TNF signalling pathway (FDR < 0.05 for all pathways). Additional pathways enriched at T24 included signalling pathways for Toll-like receptor, JAK-STAT, NF-kappa B, PI3K-Akt and MAPK (FDR < 0.05 for all five pathways). As an example, DE genes enriching the IL17 pathway at T6 included *CSF2, TAP, GRO1, CXCL2, IFNG, IL6, CSF3, PTGS2* and *CCL20*. The pathway was further enriched by the additional DE of *IL1B, CXCL10, IL17A, FOSL1, MMP13, IL13, IL17F, MAPK12, CXCL8, TNF* and *MAPK11* by T24 (the gene *IL6* was no longer contributing to enrichment). Pathways enriched by DE genes at T6≠T24 included complement and coagulation cascades and neutrophil extracellular trap formation (FDR < 0.05 for both pathways). The pathways and genes enriching them can be found in supplementary material Additional File 5: S5 KEGG Enrichment Analysis, Enriched Pathways.

The heat map (Fig. 4) shows the clustering of the 442 DE genes according to a similar expression pattern at T0, at T6 and T24 with and without treatment (AMP, NT). Genes are displayed in two clusters (on the y-axis) and four blocks (on the x-axis). In the y-axis, the top cluster indicates genes that were upregulated prior to treatment (block 1, NT T0 and NT T6) and which over time became more upregulated (block 2, NT T24), but when treatment was added, became downregulated over time (blocks 3 and 4, AMP T6 and AMP T24). The bottom cluster on the y-axis indicates genes that were negatively expressed or downregulated prior to treatment (blocks 1 and 2, NT T0, NT T6 and NT T24) and when treatment was added, became positively expressed or upregulated over time (blocks 3 and 4, AMP T6 and AMP T24) (supplementary material Additional File 6: Table S6, Heatmap Clusters). Two genes (*IFNG* and *IL17A*) strongly associated with Th1 and Th17 type immune responses (Damsker et al., 2010; Hurley et al., 2019; Infante-Duarte et al., 2000) have been highlighted in the bottom y-axis clusters. Previous research with mycobacterial and yeast cell wall fractions has resulted in changes in pro-inflammatory immune cells and cytokines including T-lymphocytes, IFNγ, IL17, TNFα and many others (Brandão et al., 2016; Filion and Phillips, 1997; Griebel, 1999; Hurley et al., 2019; Korf et al., 2005; Paik et al., 2010). The genes IFNG and IL17A became a focus of interest due to their well-known roles in Th1 and Th17 immune responses (Damsker et al., 2010; Hurley et al., 2019; Infante-Duarte et al., 2000), which have also been highlighted as important in trained immunity focused studies (Kleinnijenhuis et al., 2014), that are of interest to the authors. This decision was further strengthened following the KEGG

**Table 2**
Top 5 upregulated and top 5 downregulated DE genes at T6+T24 or T6≠T24.

| T6+T24[a] | | | |
|---|---|---|---|
| **Gene** | **logFC@T6** | **logFC@T24** | **FDR** |
| CSF2 | 2.1 | 6.4 | 1.8E−12 |
| CCL4 | 3.9 | 5.7 | 1.5E−11 |
| PTGS2 | 3.4 | 5.6 | 2.5E−12 |
| HMGA2 | 3.1 | 5.6 | 2.2E−11 |
| LOC527744 | 3.0 | 5.0 | 2.9E−11 |
| IL1R2 | −2.1 | −5.0 | 1.6E−10 |
| CLEC12A | −2.6 | −5.8 | 1.1E−11 |
| TGFBI | −3.5 | −7.2 | 3.1E−11 |
| CD163 | −2.7 | −8.4 | 6.3E−11 |
| ENSBTAG00000047363 | −5.1 | −8.6 | 9.2E−07 |

| T6≠T24[a] | | | | |
|---|---|---|---|---|
| **Gene** | **logFC between T6≠T24** | **FDR** | **logFC@T6** | **logFC@T24** |
| SIGLEC15 | 7.8 | 6.9E−07 | −0.6 | 4.2 |
| SPP1 | 7.6 | 1.2E−05 | −1.6 | 6.0 |
| MT3 | 5.9 | 5.9E−05 | −0.9 | 5.0 |
| MT1E | 5.3 | 1.6E−05 | 0.9 | 4.2 |
| LOC407163 | 5.0 | 6.5E−08 | 0.5 | 5.5 |
| AQP9 | −5.0 | 1.4E−06 | −0.3 | −5.3 |
| BST1 | −5.2 | 9.7E−06 | −0.04 | −5.2 |
| F13A1 | −5.6 | 2.4E−06 | −0.2 | −5.7 |
| FLT4 | −5.8 | 6.4E−06 | −0.1 | −5.9 |
| CD163 | −5.8 | 5.7E−08 | −2.7 | −8.5 |

FDR, p-value adjusted for false discovery rate; logFC, log fold change in bovine PBMC gene expression, treated versus not treated (T6+T24) and treated versus treated (T6≠T24); T6+T24, DE at both T6 and T24 post-AMP stimulation; T6≠T24, differently expressed between T6 and T24 post-AMP stimulation.
[a] top 5 upregulated and top 5 downregulated genes out of 99 at T6+T24 and 218 between T6≠T24.

database pathway analysis which revealed the enrichment of the IL17 signalling and cytokine-cytokine receptor interaction pathways in which IL17 and IFNG contributed to enrichment. Twenty genes grouped around *IL17A,* and 89 genes grouped around *IFNG* including *TNF*. The 10 genes grouped closest to those two genes are displayed in Table 4. Gene ontology (GO) enrichment analysis of the genes grouped with *IL17A* revealed two enriched GO-BP which were regulation of signalling receptor activity and ion homeostasis. GO enrichment analysis of the genes grouped with *IFNG* revealed 65 GO-BP including regulation of signalling receptor activity, response to cytokine and inflammatory response (supplementary material Additional File 6: Table S6 Heatmap Clusters).

As per the PCA in Fig. 2A, clustering of samples in blocks on the x-axis of the heatmap (Fig. 4) demonstrates the similarities and differences between samples stimulated with AMP, or not (NT) over time. The NT samples from T24 clustered further away from NT samples from T0 and T6 in block 1. The NT samples from T0 and T6 cluster together first based on similarities due to the animal replicate samples rather than due to the different time of sampling.

The enhanced cytokine profile, specifically including *IFNG, TNF, TAP* and *IL17A*, and these associated biological processes and pathways suggest that PBMC stimulated with AMP were expressing genes indicative of a response to cytokines and an inflammatory response and were possibly primed to initiate a Th1/Th17 type response.

### *3.5. Time course experiment co-expression network analysis of AMP induced DE genes in PBMC*

To investigate the relationships between DE genes induced by AMP stimulation of PBMC, all 442 DE genes at T6 and T24 were analysed in a co-expression network (Fig. 5A and supplementary material Additional File 8: Figure S5A, gene co-expression network, searchable image). This Main network was further filtered to include only connections with

**Table 3**
Top 5 Gene ontology biological processes associated with DE genes.

| **T/D** | **Biological Processes** | **ER** | **FDR** | **Genes DE from PBMC stimulated with AMP associated with biological processes** |
|---|---|---|---|---|
| **6/↑** | Regulation of signalling receptor activity | 17.0 | 0 | CSF2, IL36G, RETN, IL12B, EDN1, IFNG, IL6, TNFSF15, IL1RN, RAMP3, IL2, CCL20, CSF3, CCL3, CCL4, CXCL2, GRO1 |
| | Inflammatory response | 7.53 | 4.5E−06 | IL36G, IL12B, F3, PTGS2, IL6, IL1RN, CCL20, SAA2, CCL3, CCL4, CXCL2, GRO1 |
| | Response to cytokine | 6.73 | 3.7E−07 | CSF2, IL36G, IL12B, F3, SPRY4, IFNG, PTGS2, IL6, IL1RN, CCL20, CSF3, CCL3, CCL4, CXCL2, GRO1 |
| | Positive regulation of cell death | 6.25 | 2.6E−04 | IL12B, F3, ATF3, IFNG, IL6, TNFSF15, RAMP3, CCL3, HMGA2, G0S2 |
| | Positive regulation of intracellular signal transduction | 5.76 | 5.5E−06 | CSF2, IL36G, IL12B, F3, EDN1, IFNG, IL6, TNFSF15, IL1RN, RAMP3, CCL20, CSF3, CCL3, CCL4 |
| **24/↑** | Regulation of signalling receptor activity | 9.24 | 0 | CSF1, IL1B, CSF2, CXCL10, IL36G, IL17A, INHBA, IL12B, GDNF, SPP1, CCL5, LIF, EDN1, GAL, IL1A, OSGIN1, IFNG, IL13, IL17F, TNFSF15, IL1RN, CXCL8, RAMP3, ADM, CCL20, CSF3, CCL3, CCL4, TNF, CXCL2, CDC42EP2, GRO1, CXCL9, TNFSF9 |
| | Leukocyte migration | 5.48 | 1.1E−06 | CSF1, IL1B, CXCL10, IL36G, IL17A, HSD3B7, CCL5, ITGB3, IL1RN, CXCL8, CCL20, CCL3, CCL4, TNF, CXCL2, GRO1, CXCL9 |
| | Inflammatory response | 4.78 | 4.0E−10 | CSF1, IL1B, CXCL10, IL36G, IL17A, IL12B, IL1RL2, CCRL2, NOS2, F3, CCL5, IL1A, LDLR, PTGS2, PTGIR, VNN1, IL13, IL17F, IL1RN, CXCL8, CCL20, SAA2, CCL3, CCL4, TNF, CXCL2, GRO1, CXCL9 |
| | Response to cytokine | 4.52 | 6.0E−13 | CSF1, IL1B, CSF2, CXCL10, IL36G, IL17A, GFPT2, IL12B, IL1RL2, CCRL2, F3, CCL5, SPRY4, MX2, IFIT3, IL1A, IFNG, EBI3, PTGS2, OAS2, ISG15, CNTFR, IL13, MT3, CYP27B1, IL1RN, CXCL8, IL2RA, CCL20, CSF3, CCL3, CCL4, TNF, CXCL2, CDC42EP2, GRO1, CXCL9 |
| | Response to biotic stimulus | 3.33 | 2.2E−06 | ADAMTS5, IL1B, CXCL10, IL36G, IL17A, IL12B, NOS2, CCL5, MX2, IFIT3, CCDC80, IFNG, PTGIR, OAS2, ISG15, RSAD2, CYP27B1, IL1RN, CXCL8, RGS1, CCL3, TNF, CXCL2, SERPINB9, GRO1, CXCL9, TAP |
| **24/↓** | Protein activation cascade | 19.4 | 1.0E−03 | C1QA, F13A1, C1QB, CFD, FCN1 |
| | Humoral immune response | 8.05 | 4.2E−02 | C1QA, RNASE6, C1QB, CFD, FCN1 |
| | Taxis | 5.26 | 3.9E−04 | LOC504773, CH25H, CSF1R, ENPP2, HGF, SEMA6A, C5AR1, CMKLR1, CXCR1, ARRB2, C5AR2, CXCR2, DEFB6 |

(*continued on next page*)

**Table 3** (*continued*)

| T/D | Biological Processes | ER | FDR | Genes DE from PBMC stimulated with AMP associated with biological processes |
|---|---|---|---|---|
| | Inflammatory response | 3.71 | 2.5E−02 | THBS1, OLR1, IL1R2, LOC504773, ALOX5AP, CLEC7A, HGF, CD163, C5AR1, CMKLR1, C5AR2 |

ER, enrichment ratio; FDR, p-value adjusted for false discovery rate; T/D, time and direction of regulation; ↑, biological processes associated with upregulated genes; ↓, biological processes associated with downregulated genes; 6, 6 h post-AMP stimulation (treated versus not treated); 24, 24 h post-AMP stimulation (treated versus not treated).

partial correlations over 0.95, a strict cut-off parameter, which only reduced the genes included in the network to 301 (supplementary material Additional File 9: Network with partial correlations over 0.95, searchable image). The strong connections between all the genes in the Main network of Fig. 5A may be interpreted as a highly coordinated response of PBMC to stimulation with AMP over time. A small group of genes (in pale and dark green ellipses) were involved in initial leukocyte responses after 6 h AMP stimulation, and a larger group of genes (in pale and dark pink, pale and dark blue) were involved in the response from 6 to 24 h of AMP stimulation. A Sub network was created to more closely explore the connections surrounding two genes, *IFNG* and *IL17A* (Fig. 5B and supplementary material Additional File 10: Figure S5B, IL17A IFNG network, searchable image). Again, this network was filtered to include only partial correlations over 0.90 (supplementary material Additional File 11: IL17A IFNG network with partial correlations over 0.90, searchable image).

Within a network, different genes had different capacities to influence the expression of other genes which is represented by the strength and number of direct connections. Analysis for topological measures within the Main network in Fig. 5A revealed the average number of connections in the network was 261. The top three connected genes in the Main network were *PDE1B, IL36G* and *CSF2* with 365, 362 and 362 connections respectively. Other genes including *IL12B, TNF, MAPK11, IFNG, IL23A, IL22, MAPK12, CXCL8, IL1B, IL17A* and *TAP* had 357, 355, 352, 343, 336, 324, 309, 307, 298, 216 and 97 connections respectively. The edge connection (termed Edge Betweenness Centrality) between SIGLEC5 and SAA2 had the strongest value (530), indicating their importance of these two genes for communication between many parts

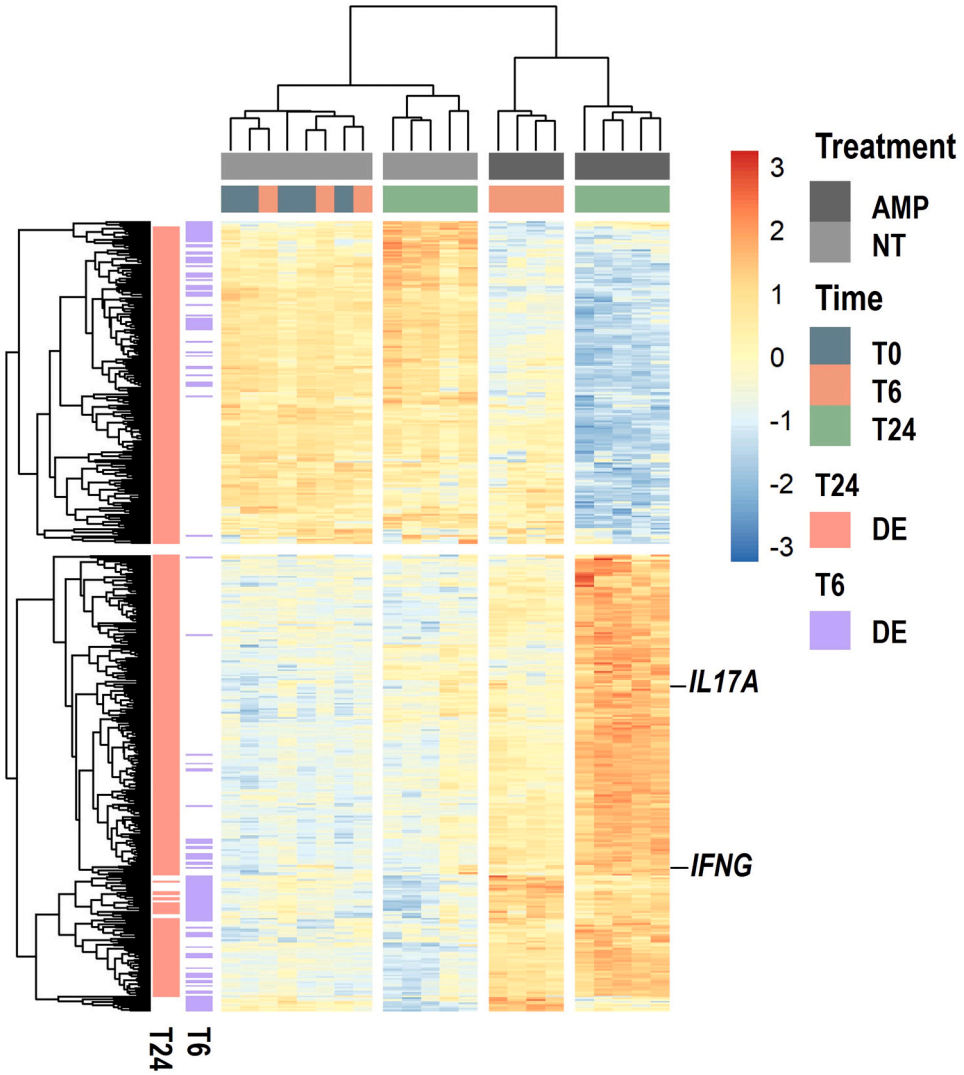


**Fig. 4.** Heatmap of differentially expressed genes. Genes differentially expressed (DE) between samples stimulated with Amplimune (AMP) or not (NT) at 6 h (T6) and 24 h (T24) post-stimulation in the time course experiment. Gene expression levels were centred and scaled by gene and are represented by colours varying from red to blue. Red representing increased expression, blue representing decreased expression. DE genes at T6 and T24 are indicated on the left side of the plot. Two genes of interest, IL17A and IFNG, are indicated on the right side of the plot.

**Table 4**
10 DE genes in heatmap clusters closest to IL17A or IFNG from Fig. 4.

| IL17A | | | IFNG | | |
|---|---|---|---|---|---|
| **Gene** | **logFC T24** | **FDR** | **Gene** | **logFC T24** | **FDR** |
| MT1E | 6.1 | 1.6E−09 | HMGA2 | 5.6 | 4.9E−12 |
| ENSBTAG00000038067 | 4.1 | 9.9E−06 | ENSBTAG00000037775 | 3.3 | 7.0E−12 |
| IL17F | 3.9 | 4.3E−09 | ANKRD33 | 3.2 | 1.2E−08 |
| ADM | 3.8 | 1.8E−04 | IL23A | 3.1 | 2.4E−13 |
| GZMA | 3.2 | 1.0E−07 | CYP27B1 | 3.1 | 4.6E−11 |
| EID2 | 2.4 | 2.5E−08 | CD9 | 2.6 | 9.4E−13 |
| RAI14 | 2.2 | 1.3E−06 | TUBA1C | 2.5 | 7.1E−10 |
| PCDH18 | 2.1 | 4.5E−08 | PDE1B | 2.3 | 3.0E−12 |
| SCNN1G | 2.0 | 2.7E−05 | SLC2A3 | 2.0 | 1.9E−08 |
| IDO1 | 2.0 | 9.6E−09 | IGFBP3 | 2.0 | 2.2E−04 |

FDR, p-value adjusted for false discovery rate; logFC, log fold change in bovine PBMC gene expression, treated versus not treated at T24 (24 h post-AMP stimulation).

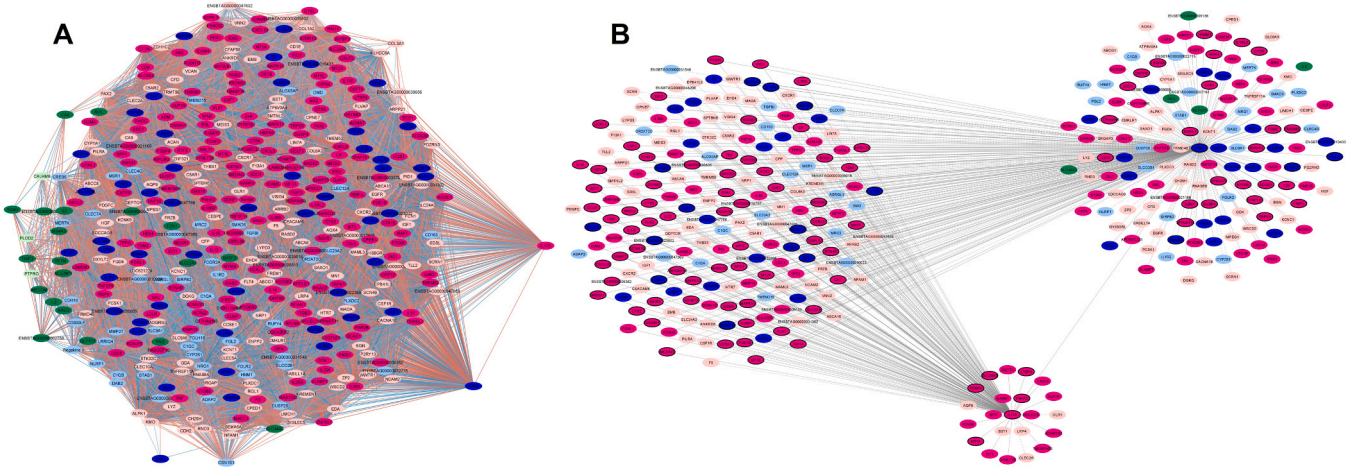


**Fig. 5.** Co-expression gene networks of DE genes induced in PBMC stimulated with AMP. (A) All DE genes at T6 and T24 (as per the heatmap in Fig. 4). IL17A is located in the middle far right of network A (dark pink ellipses). IFNG is located in the bottom far right of network A (dark blue ellipses). (B) First neighbours of IL17A and IFNG considering the network in A. Ellipses colours for Fig. 5A and B: Pale green: genes downregulated only at T6, dark green: genes upregulated only at T6. Pale pink: genes downregulated only at T24, dark pink: genes upregulated only at T24. Dark blue: genes upregulated at both T6 and T24, pale blue: genes downregulated at both T6 and T24. Genes in Fig. 5B with black borders around ellipses are those in the clusters with IL17A and IFNG from the heatmap in Fig. 4.

of the network. These genes are displayed in Table 5 and in supplementary material Additional File 7: Table S7 Topological Analysis of Differentially Expressed Gene Network.

## 4. Discussion

This study has demonstrated that AMP enhanced the transcriptional response of genes associated with innate immune activation functions in PBMC in a time-dependent manner. After 24 h of PBMC stimulation with AMP, biological processes such as leukocyte migration, inflammatory response and response to biotic stimulus were upregulated along with genes such as *IFNG, IL17A, TNF, CCLs, CXCLs* and *TAP*. Many of these genes contribute to immunity to different diseases, notably the β-defensin protein tracheal antimicrobial peptide (TAP) which is known for its bactericidal activity against bacteria associated with bovine respiratory disease (Taha-Abdelaziz et al., 2013). Alternatively, biological processes such as taxis, protein activation cascade and humoral response were downregulated in PBMC after 24 h post-treatment with AMP, along with the downregulation of genes such as *CXCR2, IL1R2, CLEC7A* and *CFD*.

The bovine PBMC response to stimulation with AMP by 6 h was defined by initiation of the innate immune system through DE of genes involved in cytokine-cytokine receptor interaction, complement and coagulation cascades and IL17 and TNF signalling pathways and the expression of genes such as *CSF2, IL12B, CCL3* and *IL6*. The chemokine CCL3 in particular, is involved in the functional capacity of cytotoxic T-lymphocytes (Jones et al., 2003) and in humans is reported to be a strong attractant for CD4+ T-lymphocytes and B cells (Schall et al., 1993). Of note is decreased expression of *IL1R2*, the receptor for IL1β, which might indicate cells have already been activated, leaving the remaining *IL1B* to act on other leukocytes migrating to the site of 'infection'.

Some genes were expressed by PBMC after 6 h AMP stimulation and in the following period until 24 h, either increased or decreased that expression, making the expression between times significantly different. Examples of these genes are *SIGLEC15, MT1E* and *CCNB1* which increased in expression between 6 and 24 h, and *CD163, FLT4* and *F13A1* which decreased in expression significantly from 6 to 24 h. This approach allowed the identification of genes that may not have been DE at either T6 or T24 individually, but that were DE between those time points and as such may show their involvement in response specialization or amplification. CD163 has a major role in the response to viral infection of the respiratory system in pigs but is downregulated by pro-inflammatory cytokines (Zhu et al., 2020), which in the current study were upregulated. The metallothioneins (MTs) are proteins involved in cellular processes including cell to cell communication and intracellular signalling (Vignesh and Deepe, 2017). MTs, particularly MT1 and MT2, are directly involved in regulating zinc availability for metabolism in T and NK cells and if that regulation is faulty, can result in host vulnerability to pathogenic infections (Vignesh and Deepe, 2017). The significantly increased or decreased expression of these genes (MT1E and MT2A) between 6 and 24 h AMP stimulation indicate the importance of

**Table 5**
Topological measures of selected genes in the Main networks from Fig. 5A.

| Genes selected for Degree of Connectedness in the network | | | | | |
|---|---|---|---|---|---|
| **Gene** | **Degree** | **LogFC T6** | **FDR** | **LogFC T24** | **FDR** |
| PDE1B | 365 | 0.93 | 5.8E−06 | 2.29 | 3.0E−12 |
| IL36G | 362 | 2.44 | 2.2E−03 | 6.42 | 6.8E−11 |
| CSF2 | 362 | 2.13 | 2.9E−06 | 6.38 | 4.7E−13 |
| IL12B | 357 | 2.37 | 1.3E−05 | 4.62 | 6.6E−12 |
| TNF | 355 | 1.45 | 1.4E−05 | 2.75 | 2.5E−10 |
| MAPK11 | 352 | NA | NA | 2.93 | 8.7E−10 |
| IFNG | 343 | 2.44 | 2.9E−06 | 4.25 | 1.1E−10 |
| IL23A | 336 | 0.67 | 5.1E−04 | 3.08 | 2.4E−13 |
| IL22 | 324 | 1.82 | 1.7E−03 | 6.65 | 7.0E−12 |
| MAPK12 | 309 | NA | NA | 3.26 | 1.4E−09 |
| CXCL8 | 307 | NA | NA | 2.85 | 4.3E−13 |
| IL1B | 298 | 1.81 | 1.5E−06 | 2.46 | 1.2E−09 |
| IL17A | 216 | NA | NA | 3.81 | 3.4E−08 |
| TAP | 97 | 2.15 | 5.5E−06 | 3.92 | 3.6E−11 |

| **Genes selected for Edge Betweenness Centrality in the network** | | | | | | |
|---|---|---|---|---|---|---|
| **Gene** | **EBC value** | **Degree** | **LogFC T6** | **FDR** | **LogFC T24** | **FDR** |
| SAA2 | 530 | 4 | 2.43 | 1.1E−03 | 2.55 | 2.0E−04 |
| SIGLEC5 | | 195 | −1.32 | 2.1E−05 | −2.07 | 6.5E−09 |

Degree, degree of connection (number of connections in network); EBC, Edge Betweenness Centrality (importance of the connection between the pair of genes for the whole network); FDR, p-value adjusted for false discovery rate; LogFC, log fold change in bovine PBMC gene expression; NA, gene not DE at that timepoint; T6, 6 h post-AMP stimulation (treated versus not treated; T24, 24 h post-AMP stimulation (treated versus not treated).

their role in communication and energy regulation of the PBMC immune response.

Trained immunity has been linked to both Th1 and Th17 T cell responses along with the production of pro-inflammatory mediators such as IL6, TNF, IFNγ and IL1β and the inhibition of anti-inflammatory mediators such as IL10 (Kleinnijenhuis et al., 2014; Murphy et al., 2021). Similarly, Th17 T lymphocytes are reported to be involved in the immune defence against extracellular bacterial and fungal pathogens (Jankovic and Feng, 2015). While a direct link cannot be inferred between trained immunity and the results of the current study, it was interesting to note the upregulation of genes including *IL6, TNF, IFNG, IL17A* and *IL1B* in response to AMP, while *IL10* was not DE. It was similarly interesting to note the significant enrichment of TNF and IL17 signalling pathways both at T6 and T24. If training the bovine immune system using innate immune stimulants to respond with a more Th1/Th17 type bias is possible, it may be beneficial to cattle in environments where exposure to mycobacterial and viral infections is more likely, such as in a feedlot environment.

Heatmap clustering data further supported the finding that *ex vivo* PBMC stimulated with AMP may have initiated an early polarization towards a Th1/Th17 response. Genes, which showed similar expression profiles, in the cluster containing *IL17A* included *IL22, RAI14, GZMA, ICAM1, IDO1* and *IL17F. IL17A, IL17F* and *IL22* are Th17 associated transcripts (Corbishley et al., 2014), and the expression of them in the current study, alongside the enrichment of the TNF and IL17 signalling pathways may suggest the early steps of a Th17 type response. The co-expression of *IDO1* and *RAI14* with the *IL17A* cluster may suggest the beginnings of an immune response with the ability to control excessive inflammation (Moura et al., 2012; Xiao et al., 2020), which aligns with the low level of inflammation in cattle treated with AMP reported previously (Alexander et al., 2022).

Genes which showed similar expression profiles with *IFNG* included *IL23A, HMGA2, CD9, SCL2A3* and *ANKRD33*, along with *TNF* and 83 other cytokines, chemokines, colony stimulating factors, cell markers, receptors and even genes in the p38/MAPK signalling pathway *MAPK11* and *MAPK12* (Morgan et al., 2022). The enrichment of the MAPK signalling pathway in the current study emphasizes the importance of these upregulated genes. In porcine lymphocytes, the expression of *CD9* on effector T cells was associated with the ability of those cells to retain long-lived cytokine production specificity against influenza A virus (Milburn et al., 2021). Results from the current study suggest *CD9* may play a role in shaping the adaptive PBMC T cell immune responses, leading to the establishment of memory responses to mycobacterial products.

Gene co-expression networks of the AMP induced DE genes expressed by PBMC revealed strong gene to gene associations between all DE genes. Based on the topological analysis and degree of connection, the importance of genes such as *IL12B, IFNG, IL17A, IL23A, IL22, IL36G, IL1B, TAP* and *TNF,* was confirmed. Three of the most connected genes were *PDE1B, CSF2* and *IL36G* being the 1st, 2nd and 3rd most connected in the network, suggesting they may have driven the response of PBMC to AMP stimulation. This idea is strengthened by the fact that *CSF2* and *IL36G* were DE at T6 and were among the top 5 genes upregulated at T24, while *PDE1B* was DE at T24 and was among the gene cluster with *IFNG*. Several genes were also found to have important edge relationships in connecting sides of the network that may otherwise not have been connected, including *SAA2* with *SIGLEC5*. This topological measure 'edge betweenness centrality', reveals relationships between genes that are important for connection between different parts of the network (Lu and Zhang, 2013). The relationship is so important that if the genes were absent, their absence may impact communication between other genes of the network (Lu and Zhang, 2013). In other words, the connection between *SAA2* and *SIGLEC5* was the most important in the network in terms of maintaining the flow of information between the majority of other genes. The combination of DE, edge betweenness centrality and enrichment in biological processes and pathways are all additional evidence supporting the importance of those genes to the PBMC response to AMP.

Furthermore, in considering the first neighbours of *IFNG* and *IL17A*, genes such as *CXCL9, CXCL2, IL23A, PTGIR, CCL5, IL22, TNF* and *IL12B* were revealed as highly co-expressed with a strong relationship to those two genes of interest. These genes were found commonly in many of the enriched biological processes and pathways enriched following AMP stimulation of PBMC, including response to cytokine GO-BP, cytokine-cytokine receptor interaction pathway, IL17 signalling pathway, TNF signalling pathway inflammatory response and leukocyte migration. We were also able to confirm the importance of genes revealed in the heatmap cluster as being closely connected with *IFNG* and *IL17A* via their network connections, including genes just mentioned; *IL22, CCL5, PTGIR, TNF, IL12B, IL23A* and *CD9,* as well as the most connected gene in the network, *PDE1B* which was also one of the genes clustered closest to *IFNG*. *PDE1B* is a gene encoding for the 1B variant of cyclic nucleotide phosphodiesterase enzymes, which can regulate cAMP and cGMP signalling in the brain, and as the PDE1B2 variant, is involved through an interaction with CSF2, in the differentiation of monocytes to macrophages (Ahmad et al., 2022; Bender et al., 2005). The range of results in which *PDE1B* appears as significant, suggest that it was very important for the immune response of the PBMC to AMP stimulation.

Results from the current study indicated the activation of genes involved in an inflammatory response during the immediate early response of PBMC to offer protection against both viral and mycobacterial infection. Likewise, the elevated expression of the antimicrobial defensin *TAP* also hints at the identity of pathogen elimination processes. Further work should involve investigation of transcription factor binding sites at the bioinformatic level, and ChIP analysis at the experimental level to confirm potential histone modifications induced by stimulation of PBMC and epithelial barrier cells with AMP.

## 5. Conclusions

In this study the transcriptional response of *ex vivo* bovine PBMC to AMP was assessed. Results showed that the response to treatment with

AMP was characterised by inflammatory cytokine production, innate immune response processes and pathways, cellular processes influenced by pathogen infection, through differential expression of genes including *IFNG, IL23A, IL17A, TNF* and *IL22*. The results are consistent with results from previous studies examining the response to other mycobacterial products. The exact pattern of gene expression associated with trained immunity is dependent on the stimulant used, however, many studies concur that a pro-inflammatory gene expression profile and a Th1/Th17 type response is induced by most stimulants.

## Ethics approval

All experimental procedures for the Pilot and Time course experiments were pre-approved by the CSIRO, Chiswick Animal Ethics Committee (Animal Research Authority 20/24).

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## CRediT authorship contribution statement

**Annika Alexander:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **E. Doyle:** Writing – review & editing. **I. Colditz:** Writing – review & editing, Methodology. **A. Ingham:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **A. Reverter:** Writing – review & editing, Methodology. **P. Alexandre:** Writing – review & editing, Methodology, Formal analysis. **B. Hine:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **T. Vuocolo:** Writing – review & editing, Formal analysis. **N. Andronicos:** Writing – review & editing, Methodology.

## Declaration of Competing Interest

The authors declare no competing interests.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2024.11.005.

## Data availability

The datasets supporting the conclusions of this article are available in the BioStudies Array Express Annotare Repository, [RNA-seq of bovine peripheral blood mononuclear cells stimulated with an innate immune stimulant containing cell wall fractions of Mycobacterium phlei compared with untreated cells. Accession E-MTAB-13418, https://www.ebi.ac.uk/biostudies/ArrayExpress/studies/E-MTAB-13418], and as Supplementary files 1 to 8.

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