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Negative energy balance alters global gene expression and immune responses in the uterus of postpartum dairy cows

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ABSTRACT

Most dairy cows suffer uterine microbial contamination postpartum. Persistent endometritis often develops, associated with reduced fertility. We used a model of differential feeding and milking regimes to produce cows in differing negative energy balance status in early lactation (mild or severe, MNEB or SNEB). Blood hematology was assessed pre-slaughter at 2 weeks postpartum. RNA expression in endometrial samples was compared using bovine Affymetrix arrays. Data were mapped using Ingenuity Pathway Analysis. Circulating concentrations of IGF1 remained lower in the SNEB group, whereas blood non-esterified fatty acid and β-hydroxybutyrate concentrations were raised. White blood cell count and lymphocyte number were reduced in SNEB cows. Array analysis of endometrial samples identified 274 differentially expressed probes representing 197 recognized genes between the EB groups. The main canonical pathways affected related to immunological and inflammatory disease and connective tissue disorders. Inflammatory response genes with major up-regulation in SNEB cows included matrix metalloproteinases, chemokines, cytokines and calgranulins. Expression of several interferon inducible genes including *ISG20, IFIH1, MX1* and *MX2* were also significantly increased in the SNEB cows. These results provide evidence that cows in SNEB were still undergoing an active uterine inflammatory response 2 weeks postpartum, whereas MNEB cows had more fully recovered from their energy deficit with their endometrium reaching a more advanced stage of repair. Severe NEB may therefore prevent cows from mounting an effective immune response to the microbial challenge experienced after calving, prolonging the time required for uterine recovery and compromising subsequent fertility.

Key words: microarray, bovine, endometrium, innate immune system, anti-microbial peptides

INTRODUCTION

High yielding dairy cows are under considerable metabolic stress in early lactation, as they cannot meet the energetic demands for milk production entirely from feed intake (1). Gestational changes cause insulin resistance in late pregnancy (2). Immediately after calving high rates of body condition score loss are associated with a severe negative energy balance (NEB) status, indicated by alterations in blood metabolite and hormone profiles. Both non-esterified fatty acids (NEFAs) and β-hydroxybutyrate (BHB) concentrations are elevated, indicative of lipid mobilization and fatty acid oxidation (73). The liver coordinates the extensive biochemical and morphological modifications required via up-regulation of genes involved in fatty acid oxidation and gluconeogenesis, and down-regulation of triacylglycerol synthesis (42). However, excessive fat accumulation postpartum impairs liver function, compromising glucose production and increasing inflammatory responses. Production of insulin and IGF-I are also reduced at this time (14,17).

Cows are expected to re-breed within 2-3 months of calving for optimum economic return. Poor fertility is a serious economic consideration in the dairy industry and is strongly linked to the animal's health around calving. Excessive lipid mobilization is associated with metabolic and reproductive disorders (57). Cows with a low nadir in IGF1 in the first 2 weeks postpartum take longer to resume estrous cyclicity and are less likely to conceive when the breeding period is reached (6,70). Immune function is also suppressed over the periparturient period (7,32,43), and poor EB status and fatty liver can impair peripheral blood neutrophil function (24,39,77).

Following calving, the uterus must undergo extensive remodelling to reduce in size, remove cellular debris, and restore normal architecture (21,39,65). Microbial contamination of the uterus is almost universal during the first week postpartum and continues for about two weeks in around 90% of animals (18,40,66). The most common recognised pathogens are *Arcobacterium pyogenes, Escherichia coli, Fusobacterium necrophorum, Prevotella melaninogenicus* and *Proteus* spp. Uterine defences rely initially on classical innate immunity and mucosal defence systems rather than adaptive immunity (33,66). Failure in this defence system results in uterine disease. Metritis is present in 40% of cows within 2 weeks of calving and 15% have a persistent endometritis in the 3-6 week postpartum period (40,66). Subclinical endometritis, from 6 weeks postpartum onwards, is characterised by an extensive leucocytic

infiltration of the endometrium and chronic inflammation (18,66) and is associated with longer intervals to conception and a greater likelihood of culling (19,22,40).

The experiment described here was designed to test the hypothesis that metabolic changes in postpartum cows can delay uterine repair mechanisms and promote a state of chronic inflammation, resulting in an unfavourable uterine environment likely to contribute to reduced fertility. To this end we developed a model of differential feeding and milking regimes to produce cows in differing negative EB status in early lactation (mild or severe, MNEB or SNEB) as confirmed by markedly divergent metabolic and endocrine profiles (17). Uterine tissue samples were collected at 2 weeks postpartum. This time point was chosen as infection would have had sufficient time both to manifest and to be influenced by the differences in energy demand between groups (77). It was, however, before cows had ovulated, to avoid differences between cows associated with exposure to luteal progesterone which can influence uterine resistance to infection (60).

MATERIALS AND METHODS

Animals and management. All procedures were carried out under license in accordance with the European Community Directive, 86-609-EC. Multiparous Holstein-Friesian cows with a mean parity of 4.7 and an average previous lactation yield of 6,477 ± 354 kg were used. Cows were blocked two weeks prior to expected calving according to parity, body condition score and previous yield and were randomly allocated to 2 treatments (each n = 6 cows) designed to produce mild or severe NEB. From day 2 after calving, mild (MNEB) cows were fed *ad lib* grass silage with 8 kg/day of a 21% crude protein dairy concentrate and milked once daily; severe (SNEB) cows were fed 25 kg/day silage with 4 kg/day concentrate and milked three times daily. The chemical composition of silage and concentrate offered (as previously described (53)) was the same across treatment groups. Daily measurements of milk yield, milk composition, dry matter intake (DMI), body weight, and dietary energy intake were used to calculate EB, based on the French net energy for lactation ($NE_{L)}$ system. Net EB was calculated as UFL/day in which 1 unité fourragère lait (UFL) is the NE_L equivalent of 1 kg of standard air-dry barley as described previously (29). Samples of endometrium were collected from all cows following slaughter at 14 ± 0.4 days postpartum as described below. Array data from one MNEB cow failed the inter-array

quality control analysis (see below) so this animal was excluded from all analyses, leaving 5 cows in the MNEB group.

Blood sampling, hormone and metabolite assays. Blood samples were collected after morning milking (08:00 h) by jugular venipuncture twice weekly throughout the 2 week treatment period up to and including the day of slaughter. Samples were collected into lithium-heparin primed vials and were immediately placed on ice before centrifugation at 2,000 x g for 10 min. Plasma was decanted and stored at -20°C for subsequent analysis of hormones and metabolites.

IGF1 was measured using human OCTEIA IGF1 kits (IDS, Tyne and Wear, UK). The inter-assay coefficient of variation (CV) was 8.7% while the intra-assay CVs were 8.7% and 16.8% for samples with mean values of 81.5 and 4.7 ng/ml respectively. The sensitivity of the assay was 1.9 ng/ml. Insulin was assayed using a solid-phase radioimmunoassay (Coat-a-Count, Diagnostics Products Corp, CA). The inter- and intra-assay CV were 14.2% and 9.8%, and 9.8% and 3.9%, for control samples with a mean insulin concentration of 6.4 and 13.1μIU/ml respectively. The minimum detectable concentration of the assay was 1.6 ± 0.03 μIU/ml.

Concentrations of the $PGF_{2\alpha}$ metabolite PGFM were quantified using a charcoal-dextran RIA method as described previously (56). In brief, the tritiated tracer of PGFM (13,14-Dihydro-15-keto-[5, 6, 8, 9, 11, 12, 14(n)-^{3}H]-prostaglandin $F_{2\alpha}$) was from Amersham International plc (Amersham, Bucks, UK). The standards were supplied by Sigma. The PGFM antiserum was a kind gift from Dr R Kelly (University of Edinburgh, Edinburgh, UK). The limit of detection was 1 pg/tube and the intra-assay and inter-assay CVs were 7.6 % and 14.3 %. The cortisol radiommunoassay was based on a similar methodology (56) using plant cortisol (H4001; Sigma-Aldrich) as standard, sheep antiserum to cortisol (Diagnostics Scotland) and ([1, 2, 6, 7-^{3}H] Cortisol (Amersham Biosciences Biosciences) as tracer. Plasma samples were first extracted with diethyl ether and the extracts were reconstituted in assay buffer. Separation was by charcoal-dextran. The sensitivity was 0.7 nmol/L plasma, the recovery was 103 %, and the intra-assay CVs for samples with a mean of 3.6 and 51.4 cortisol nmol/L of plasma were 5.6 % and 6.1 % respectively. The inter-assay coefficient of variation was 18.5 %. Measurement of plasma estradiol concentrations used the Estradiol MAIA assay kit (BioStat Diagnostic Systems, Stockport, Cheshire, UK). The intra-assay CVs for samples with a mean of 1.1 and 4.6 estradiol pg ml^{-1} of plasma were 15.8 and 7.3 % respectively. The inter-assay CV for the same samples was 0.6 and 5.6 %, respectively.

Serum amyloid A (SAA) concentrations were measured in serum using the Tridelta Phase™ range SAA solid phase ELISA kit according to the manufacturer's guidelines (Tridelta Co., Kildare, Ireland). The intra- and inter-assay coefficients of variation for low, medium and high concentrations within the effective range were all <12 and <18%, respectively.

Samples of blood plasma obtained on the day of slaughter were also analyzed for glucose, NEFAs, BHB and urea using appropriate kits and an ABX Mira auto-analyzer (ABX Mira, Cedex 4, France).

Hematology. Blood samples were taken the morning before slaughter from each cow. Blood parameters were determined in unclotted (EDTA treated) whole blood samples using an electronic particle Nihon Kohden hematology analyser (Celltac MEK-610K, Nikon-Kohdon, Tokyo, Japan).

Uterine tissue collection and RNA isolation. The uterus was opened and samples of inter-caruncular endometrial tissue weighing approximately 1 g were dissected from the mid portion of the previously gravid horn approximately 1 cm anterior to the bifurcation of the uterus. These were rinsed in RNase free phosphate buffer, snap-frozen in liquid nitrogen and stored at –80°C. Total RNA was prepared from 200-300 mg of fragmented frozen endometrial tissue and homogenized in TRI reagent (Molecular Research Centre Inc, Cincinnati, OH, USA). RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). RNA integrity was confirmed for all samples using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips according to manufacturers' instructions (Agilent, Waldbronn, Germany).

Microarray analysis. Microarray hybridization and data acquisition were carried out in ARK-Genomics (Roslin Institute, Edinburgh, UK) using 24 K Affymetrix GeneChip Bovine Genome Arrays based on the established ARK-Genomics protocols (http://www.ark-genomics.org/protocols). The acquired data were analyzed using S+ ArrayAnalyzer 2.1 built in S-Plus Enterprise Developer 7.0 software package (Insightful Corp, Seattle, Washington, USA). The probe level expression data generated by the scanner (.CEL files) were imported into the ArrayAnalyzer. They were filtered out if the detection was absent or if the pairs used were less than 7 (11 pairs in total). The probe pairs were summarized into a single value per gene using Robust Multichip Analysis (RMA) with a primary Quantiles normalization. After this filtration and summarization, about 20,000 probes/genes were available. The inter-array quality control analysis using MvA and box plots showed that the sample from one cow did not meet the

requirements so this animal's data were excluded from all the analyses. The summarized data were further normalized with Median Inter-quartile Range (IQR). The differentially expressed genes were identified using a Local Pooled Error (LPE) test at P=0.05 with Bonferroni (BON) false discovery rate (FDR) adjustment. The significantly expressed genes were loaded into the Affymetrix website for annotation (http://www.affymetrix.com). The GEO-deposited data can be accessed at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15544.

Pathway analysis. The annotated genes were organized using Entrez Gene combined with gene symbols as identifiers and fold changes and adjusted P-values as observations. They were loaded into Ingenuity Pathway Analysis (IPA) V7.5 software server (Ingenuity Corp, Redwood City, CA, USA) for mapping into relevant functional groups and pathway analysis. Among 274 significantly expressed probes, 197 were found to be previously reported genes with recognized gene symbols. About half of the identified genes (n=103) had known involvement with immune and inflammatory functions. These were selected and reloaded into IPA for further analysis of immune and inflammatory pathways using gene symbols as identifiers and fold changes and adjusted P-values as observations.

Quantitative real-time PCR (qPCR). Total RNA from each sample was treated for potential genomic DNA carryover in a single reaction with DNase based on the guidelines supplied by Promega (Promega Corporation, Madison, WI). From this reaction, precisely 1 μg of DNase-treated RNA was reverse transcribed into cDNA using random hexamer primers and processed accordingly (Reverse Transcription System Kit; Promega). A mastermix of reagents was prepared for the above reaction to minimize potential variation from pipetting. Selected negative control samples were also prepared by including all reagents as above, minus the reverse transcriptase. Assays were designed for 22 genes of interest (see Supplementary Table 1). Three housekeeping genes were also analyzed: 18S rRNA, ribosomal protein L19 (*RPL19*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences were designed online using the Primer3 web software (http://frodo.wi.mit.edu/primer3/input.htm) based on the target sequences of Affymetrix GeneChip Bovine Genome Array probes (http://www.affymetrix.com). Primer alignment specificity and compatibility were checked using the BLAST search tool (NCBI) and Amplify software (v3.1) respectively. Gene symbols, sequence information, accession numbers and expected product lengths are provided in Supplementary Table 1.

Gene transcripts were quantified as described in detail previously (17). Briefly, for each assay a mastermix was prepared that contained a final concentration of 1x absolute qPCR SYBR

Green Mix (ABgene, Epsom, Surrey, UK), 500 nM forward and reverse primers and nuclease-free water. Primer annealing and amplicon-specific melting temperatures were determined using the gradient function of the DNA Engine Opticon 2 thermal cycler (MJ Research Inc, Waltham, MA, USA). Equivalent amounts of sample cDNA were added to each reaction in duplicate. To minimize variation, all samples included in each analysis were derived from the same RT batch, prepared under the same conditions and were analyzed on a single plate. Thermal cycling conditions applied to each assay consisted of an initial *Taq* activation step at 95°C for 15 min followed by 38 cycles of denaturation (95°C), annealing (range 50.0-64.2°C), extension (72°C) and an amplicon-specific fluorescence acquisition reading (range 74-84°C). A melting curve analysis was performed for each amplicon between 50-95° and as such any smaller non-specific products such as dimers were melted (if present) prior to fluorescence acquisition. All qPCR results were recorded with the Opticon Monitor Analysis Software (V2.02; MJ Research). Values for r^2 and amplification efficiency (E) were derived from the linear regression analysis of Log (input cDNA) versus cycle number at threshold (C_t) plot. The slope created for each set of standards was used to determine E according to $E=10^{-slope}-1$ where an E value of 1.0 would correspond to 100% cDNA replication at each cycle. Variation within each assay was determined from the average standard deviation across the whole quantification range and converted to percentage [ng input cDNA] using the equation ±% [ng input cDNA] = $[(E+1)^{SD}-1]\times100\%$ (62). For comparison of expression data, absolute values were derived from standard curves generated from purified cDNAs identical to amplified products and expressed as fg/µg reverse-transcribed RNA.

Data analysis. Gene expression data from qPCR and values for energy balance, endocrine, metabolic, and hematological parameters were compared between the two energy balance groups using independent samples t-test in SPSS for Windows, v16.0. Data were log transformed if necessary to achieve homogeneity of variance. Differences were deemed significant where $P<0.05$. Array data were analysed as described above.

RESULTS

Metabolic, hormonal and energy balance status of cows in mild and severe NEB. All cows achieved a state of NEB between calving and the time of tissue collection 2 weeks later (17). Cows exposed to a restricted dietary intake and increased milking frequency (SNEB) were in a

lower EB status compared with MNEB cows at tissue collection ($P<0.02$; Table 1). The divergence in EB status at tissue collection was confirmed by changes in measured metabolites: systemic NEFA and BHB were elevated ($P<0.001$) while glucose was reduced ($P<0.001$) in the SNEB compared with the MNEB group (Table 1). Concentrations of IGF1 were low following calving in both groups, then started to increase again after one week in the MNEB cows but remained basal in the SNEB group, resulting in a significant difference at tissue collection ($P<0.05$, Fig. 1). Although insulin and estradiol levels were also numerically lower in SNEB cows this did not achieve statistical significance. Concentrations of both SAA and PGFM were elevated at calving in all cows and then fell by the time of tissue collection, but values did not differ significantly between groups (Fig. 1). No cows had ovulated again postpartum at the time of tissue collection, so progesterone levels remained low in all animals.

Hematology. Blood samples taken on the day before slaughter showed several significant differences in hematological parameters between groups (Table 2). White blood cell (WBC) count and lymphocyte number were both reduced in SNEB cows. The red blood cell distribution width was higher and the platelet crit and mean platelet volume were lower in SNEB cows. Other parameters were not significantly altered.

Arrays and pathways. Array analysis of endometrial samples identified 274 differentially expressed probes between the energy balance groups representing 240 genes of which 197 had recognized gene symbols. Of this total, 176 genes (73%) were significantly up-regulated and 64 (27%) were down-regulated. Initial analysis of the top 20 differentially expressed genes increased in the SNEB cows revealed up-regulation of many key genes known to be involved in inflammatory responses including *MMP1, MMP3* and *MMP13, CXCL5, HLA-DQB* (MHC class II antigen), *S100A8, S100A9* and *S100A12* (calgranulin A, B and C), *AHSG, IL1R, IL8* and *IL8RB* (Table 3). These were increased between 28.8 to 6.4 fold in the SNEB group. The most highly down-regulated genes (real fold change down -5.9 to -2.6) represented a much more diverse group with no clear theme (Table 4). Seven of the 20 most highly down regulated genes were unidentified. Of particular interest were *PLA2G10* (a phospholipase A_2 involved in lipid accumulation and modification of lipoproteins), *NTRK2* and *CCNB1* (involved in cell differentiation and mitosis respectively), *PTHLH* (important in epithelial-mesencymal interactions), *SLC2A5* (also known as *GLUT5*, a facilitated glucose/fructose transporter), *NOV* (influential in cell adhesion) and *MYB* (involved in cell proliferation and a target for estrogen

receptor signaling). *IL2* (a cytokine produced by $CD4^+$ T-helper Th1 cells) was also down-regulated, contrasting with *IL1B* which was increased.

IPA was used to place all the differentially expressed genes into different function and disease categories. This confirmed that the main canonical pathways and biofunctions affected related to immunological and inflammatory disease, connective tissue disorders and cellular growth, proliferation and interaction (Table 5).

All genes identified as belonging to immune or inflammatory pathways (n = 103, listed in supplementary Table 1) were further classified into immune sub-pathways (Table 6). Pathways with the greatest number of molecules represented included acute phase response, complement system, pattern recognition receptors and leukocyte extravasation signaling. These are all consistent with the processes known to be required to remodel the postpartum uterus and to clear any microbial infections likely to be present (21,40,65). In addition the subpathway "hepatic fibrosis" was highly significant. This pathway includes several MMPs, as the condition is associated with increased deposition and reduced degradation of collagen in diseased liver.

The four top networks identified by IPA are illustrated in Figs. 2-5. These were: (1) antigen presentation, cell-mediated immune response, humoral immune response (score 49, 24 focus molecules); (2) cellular movement, hematological system development and function, immune cell trafficking (score 40, 20 focus molecules); (3) hematological system development and function, humoral immune response, tissue morphology (score 29, 17 focus molecules and (4) post-translational modification, cell death, connective tissue development and function (score 23, 13 focus molecules).

Network 1 featured a number of genes associated with activation of the complement system (*C1QA, C1QB, C1QC, C3, C8, CFB, CFH*), which were all up-regulated in SNEB cows. CD79A is a B lymphocyte antigen receptor whose activation precedes rearrangement of heavy chain immunoglobulins during B cell ontogeny. This network also included *IL1R* and the antagonist *IL1RN*. IL1 receptor signaling can activate the NFκB signaling pathway. This is important in macrophages during the respiratory burst, and is thought to be a key link between this and other inflammatory responses (28). PDCD1LG2 is a programmed cell death ligand involved in antigen receptor signaling. *CXCL5* was one of the most highly up-regulated genes in SNEB cows. This is produced with IL-8 in response to stimulation by IL-1 or TNFα and is a potent chemokine. Expression of another chemokine *CXCL2* was also increased, which is important for neutrophil recruitment.

297 Network 2 showed strong associations with the EB model used as the basis of the
298 experiment, in which plasma concentrations of IGF1 were reduced while NEFA was greatly
299 elevated. Local endometrial expression of *IGF1* was also reduced, whereas *IGFBP1* was
300 increased in SNEB cows. Associations between these genes are shown with plasma GH and
301 insulin and STAT5, a transcription factor that is phosphorylated in response to cytokine
302 signaling. Network 2 also featured signaling of the pro-inflammatory cytokine IL1 and linked this
303 to prostaglandin production, as *PTGS1* and *PTGS2* were both higher in SNEB cows. *CD36*
304 encodes a protein found on platelets which acts as a receptor for thrombospondins, which are
305 involved in cell adhesion processes. CD36 also binds to collagen, anionic phospholipids and
306 oxidized LDL and may play a role in fatty acid transport. The fatty acid binding protein *FABP4*
307 was also up-regulated whereas *GSTA1* (glutathione S-transferase) was decreased. GSTA1 can
308 detoxify products of oxidataive stress, providing protection from reactive oxygen species and the
309 products of peroxidation. These changes may be related to the raised NEFA concentrations.
310 *CXCL13* and *CXCL14* are attractants for B cells and monocytes respectively. This network also
311 included the antimicrobial calgranulin genes *S100A8* and *S100A9* which were both highly
312 increased in SNEB.
313 Network 3 was also identified as having a role in hematological system development and
314 function. It featured a number of genes involved in T cell development and activation (*CD69,*
315 *CCR5, CCR7, PTPN22, ZAP70*). T cells play a key role in inflammation and tissue remodeling
316 through activation of MMPs which break down extracellular matrix. *MMP3* encodes an enzyme
317 which degrades fibronectin, laminin and several types of collagen and is thought to be of
318 particular importance in wound repair. *MMP3* is linked to *PLAUR*, the receptor for urokinase
319 plasminogen activator. PLAU converts plasminogen to plasmin, the active form of a proteolytic
320 enzyme which plays an important initial step in MMP activation. This pathway is activated
321 through a MAP kinase signaling cascade and *MAK3K8* and *MAPK13* were both up-regulated in
322 SNEB.
323 Network 4 also relates to connective tissue remodeling. *MMP1, MMP9* and *MMP13* were
324 all highly up-regulated in SNEB endometrium. The uterus must undergo extensive remodeling
325 during the postpartum period. IL8 is a major mediator of inflammatory responses and is also a
326 potent angiogenic factor. Other genes associated with inflammation were *VCAM1* and *SELL*
327 which are both involved with leucocyte-endothelial cell adhesion. *MYB* encodes a transcription
328 factor which regulates progression through the cell cycle and both this and the cyclin *CCNB1*

were down regulated in SNEB. Network 4 also featured the interferon responsive genes *MX2* and *ISG20,* in addition to *DEFB1* which can also respond to viral infections. Differential expression of IFN genes themselves between groups was not detected, but three other IFN-inducible were differentially increased in endometrium of SNEB cows (*MX1, IFIH1* and *Loc512486,* see Supplementary Table 2).

Antimicrobial genes. A separate search of the entire bovine Affymetrix array was made to identify genes representing antimicrobial peptides. From this 16 genes were identified (5 different β-defensins, 6 different cathelicidins, *LAP, LEAP-2, TAP, CAMP* and *SLP1*). Of these two were differentially expressed using the array analysis: *DEFB1* and *TAP* were increased 2.29 fold and 3.56 fold respectively in endometrium of SNEB cows (see Supplementary Table 2). There were trends for several other of these genes to be more highly expressed in SNEB cows (*LAP, DEFB5, DEFB7, BBD(C7), SLP1*), but these did not achieve statistical significance. On the other hand, none of the cathelicidins (*CATHL1, CATHL2, CATHl4, CATHL5, CATHL6* or *CATHL7*) showed any change in expression between the two groups (data not shown).

Other differentially expressed genes. Those 137 genes identified from the array analysis as being differentially expressed between groups but which were not placed into the immune and inflammatory categories are listed in Supplementary Table 3. Of these genes 86 were up-regulated and 51 were down-regulated. They are not discussed any further here in order to maintain the focus of this paper.

Quantitative PCR analysis. In order to validate the array data, uterine samples were analyzed by qPCR for 22 genes. The selected list included genes which were identified as being either up- or down-regulated or unchanged between groups in the array analysis. Expression data for these genes were compared between the two EB groups (Table 7), and concentrations obtained by qPCR were also compared directly to the array data. Correlation between the two techniques was consistently high, with most genes having r^2 values >0.8. Thirteen genes tested showed significantly different values between EB groups by both techniques and 4 were non-significant by both. Four genes identified as significant using the array data also had fold changes of 1.6- to 2-fold by qPCR, but these were not significant largely due to some within-group variation. One gene (*LAP*) was shown to be increased 1.9 fold by qPCR ($P<0.04$) but was not significant on the array. Expression of the housekeeping genes *RPL19, GAPDH* and 18SrRNA was not altered by EB status.

DISCUSSION

All cows deal with postpartum uterine infection initially using the innate immune system, but with time the adaptive response gains importance. The results of this study provided evidence for an ongoing inflammatory response in the uteri of cows in SNEB at 2 weeks postpartum. Accompanying this, cows in SNEB had higher expression levels of several antimicrobial genes, notably *S100A8, S100A9* and *S100A12*. In comparison, cows in the MNEB group had nearly recovered from their energy deficit by this stage as evidenced by increased plasma IGF1 and glucose concentrations together with lower NEFA, BHB and WBC. Several of the genes which were highly down–regulated in SNEB are involved in cell proliferation and in interactions between cells (eg *NTRK2, CCNB1, MYB, NOV*). *PTHLH* is of particular interest as parathyroid hormone-like hormone is known to have a role in epithelial-mesenchymal interactions, for example during development of the mammary gland (16). The postpartum uterus must replace the epithelium which has been lost following placental separation, and this step will be important in re-establishing the innate defence system. Histological analysis confirmed a greater degree of tissue repair in the uterus of MNEB cows (unpublished observations). A poor energy balance status may therefore inhibit the ability of the cow to mount an effective immune response to the bacterial challenge experienced after calving and also delay the general repair process within the endometrium, thus prolonging the time required for the recovery phase. The array data were analysed and have been presented according to the original experimental design, with 6 cows in the SNEB group and 5 in the MNEB group. A cluster analysis based on the 274 differentially expressed genes/probes for all cows indicated that two of the SNEB cows occupied an intermediate position between the two main treatment groups, suggesting that these two animals may have reached a different stage of uterine recovery. They could not, however, be distinguished from the rest of the SNEB group in terms of the blood parameters measured (data not shown). Further study of a larger number of cows will be therefore be required to identify in more detail additional factors which may affect recovery rates in individual animals.

Effects of treatment on metabolism, acute phase response and oxidative stress. The SNEB cows had clear evidence of liver damage, associated with lipid infiltration, high circulating NEFAs and BHB and reduced glucose and IGF1 (17). Although the differential feeding and milking treatments did not begin until after calving, adaptive changes are initiated pre-partum. In late pregnancy falling insulin and elevated placental lactogen stimulate adipose mobilisation,

providing nutrients for fetal growth (2). The main control of the insulin signaling pathway occurs downstream of the insulin receptor. Insulin receptor activation results in tyrosine phosphorylation of IRS proteins-1 and -2 (74). This step is inhibited by lipid accumulation in muscle, contributing to the pregnant mother developing peripheral insulin resistance (34). Insulin signaling was restored within a few days of giving birth in normal weight women but this took up to 15 weeks in obese women (68). Acute infections are also accompanied by tissue insulin resistance (15). AHSG, a natural inhibitor of insulin receptor signaling (46), is a plasma protein, produced primarily by the liver, whose circulating concentration is positively associated with insulin resistance and hepatic fat accumulation (69). In our study uterine expression of *AHSG* was significantly increased in cows in SNEB, implying that local insulin receptor signaling was likely to be impaired.

Acute-phase proteins are primarily produced by hepatocytes and can directly neutralise inflammatory agents, minimising the extent of tissue damage. Our results confirmed previous studies in showing a peak in circulating SAA between 1 and 3 days postpartum (64). SAA can affect a variety of cellular functions including adhesion, migration and proliferation (71). Cows are also under oxidative stress around calving, as indicated by an increase in reactive oxygen metabolites, decreased CuZn-superoxide dismuatse (SOD), and raised plasma Se-glutathione peroxidase (GSH-Px) (3). Analysis of livers from the cows in the present study using Affymetrix arrays showed elevated stress response genes (eg *GPX* and *HSP70*) (47). In the SNEB cows there was altered expression in uterine endometrium of a number of key genes involved in both the acute phase response and Nrf-2 mediated oxidative stress. For example, *C3* and *SOD2* were increased, whereas expression of *PIK3R1, GSTA1* and *CRABP1* were decreased. PI3-kinase controls Nrf2 in response to oxidative stress and *GSTA1* contains functional anti-oxidant response elements (31). *CRABP1* alters cellular responses to retinol. Overall the evidence suggests that there is a complex inter-relationship in postpartum cows between acute infection and tissue insulin resistance. Together with the elevated circulating NEFA concentration this may predispose the animals to peroxidative damage of lipids and other macromolecules, chronic inflammation and a reduced capacity for tissue repair. In addition, previous studies have shown that NEFAs can directly impair the functional capacity of mononuclear cells from sheep (36).

Hematology. Previous work in cattle has indicated that immune function is suppressed over the periparturient period (7,32,43). Blood leucocyte counts decrease in the first 2 weeks postpartum then recover over the following 3 weeks (67). Increased liver triacylglycerol content in the first

two weeks post calving is also associated with decreased functional capacities of polymorphonuclear leucocytes derived from both blood and uterus (77,24). We showed here that the circulating WBC, in particular lymphocyte number, was reduced in SNEB cows. In addition the red blood cell distribution width (RDW) was higher and the platelet crit and mean platelet volume were lower. In humans low platelet counts and greater RDW are both associated with liver disease (26,44). In our study the platelet crit was positively associated with circulating IGF1 ($r = 0.696$, $P<0.02$) and negatively with circulating SAA ($r = -0.690$, $P<0.04$), supporting an involvement with liver function in early postpartum cows.

Effects of NEB on the innate and mucosal immune system. In both the gastrointestinal and urogenital tracts the epithelium normally forms a critical physical and chemical defense barrier that separates the underlying mucosa from the luminal contents and transmits signals generated in response to microbial infection to cells of the innate and acquired immune systems (8,30,33,76). Epithelia prevent adhesion of bacteria and subsequent colonization in part by the release of antimicrobial substances (eg lactoferrin, lipocalin) into the overlying mucosal fluid. This system is now known to include antimicrobial peptides such as alpha- and beta-defensins and cathelicidins (8,76). Beta-defensins are found in both macrophages and epithelial cells, have a broad range of anti-microbial activity and are rapidly inducible as an initial part of the innate immune system (12,58).

Following parturition in the cow there is substantial damage to the surface epithelium (21,39). In the ewe regeneration of luminal epithelium did not commence until after day 8 and was completed by days 28-31 postpartum (23). There is thus a lengthy period after calving when the normal mucosal defence system is jeopardised. In a previous study *BBD119, BBD123, BBD124, LAP, DEFB7, BNBD4* and *BNBD5* were all identified in bovine uterus using qPCR (10). We have here confirmed these findings in the postpartum uterus. Furthermore, we show that expression of *DEFB1, TAP* and *LAP* (significant by qPCR only) was increased in the SNEB cows. Although expression of several members of the cathelicidin family were also readily detectable by the array analysis, none of these were altered by EB although in ewes the cathelicid bactenecin-1 was up-regulated eight-fold in cervico-vaginal fluid in association with labor (75).

S100A8, S100A9 and S100A12 are all members of the S100 family of Ca^{2+} binding proteins. S100A8 and S100A9 together form a heterodimer called calprotectin which has antimicrobial properties and plays an important role in innate immunity. S100A8 and S100A9 can be produced by a variety of cell types including neutrophils, activated macrophages,

keratinocytes and fibroblasts and their presence acts as a marker of inflammation in oral mucosa, skin disorders and wounds (49,54,59). Expression in vitro can be up-regulated by a variety of pro-inflammatory cytokines including IFNG, TNFA, IL1 or FGF2 but is suppressed by TGFB or retinoic acid (27,49,54). Proteomic analysis of human amniotic fluid has identified S100A8 and S100A9 as markers of inflammation associated with intra-uterine infection, a major cause of preterm labour in human pregnancies (52,61). Our data indicate that the members of this family are significantly elevated in postpartum cows in association with uterine infection, and they may thus represent a useful marker of inflammation in this situation.

Effects of NEB on inflammatory responses in uterus. Bacteria that overwhelm the early mucosal defenses activate an immune response by signaling through receptors such as TLR4, which recognize bacterial LPS (11). TLR4 signaling can increase synthesis of β-defensins, whose role is discussed above (20). Some previous studies have also suggested a link between TLR4 signaling and metabolism. For example, LPS can inhibit *GHR* expression (11,72). The TLR4 signaling cascade involves MyD88, CD14, IRAK-1, IRAK-4, IRAK-M and TRAF-6, activating NFκB and MAPK, leading to cytokine production (20). LPS binds to circulating LBP and this complex binds to cell membrane bound CD14. With persistence of increasing numbers of microbes, chemokines such as IL8 and pro-inflammatory cytokines such as IL1 and TNFA up-regulate expression of vascular adhesion proteins which attract and activate large numbers of neutrophils, macrophages and T-lymphocytes (25). Both *IL1* and *IL8* were up-regulated in SNEB cows. IL1 is a key player in both Network 1 and Network 2. CCL2 and CXCL5 are strong chemoattractants for neutrophils and monocytes and *CXCL5* was the second most highly up-regulated gene detected (see Table 3) (25). A neutrophilic influx into the superficial endometrium characterises the early response of the uterus to surface infection and macrophages, lymphocytes, eosinophils and mast cells may subsequently be mobilised (4). This is associated with vascular congestion and stromal oedema. In this study expression of many chemokines, pro-inflammatory cytokines and their receptors and vascular adhesion molecules were all higher in the SNEB cows at 14 days postpartum (see Table 6 and Network 4).

Several genes associated with interferons were up-regulated in SNEB cows: *MX1, MX2, IFIH1, ISG20, GBP1* and *Loc512486*. The type 1 interferons IFNA and IFNB increase in response to many viral infections, have potent anti-viral activities and can also promote expression of IFNG in T cells. They increase cellular responsiveness to other stimuli including LPS, but can also produce excessive responses leading to tissue damage (27). MX1 and GBP1

both have antiviral activity and GBP1 also has potent anti-angiogenic activity in endothelial cells (50). *Loc512486* on the array is identified as being similar to *GBP1*.Viral causes of uterine disease are rarely considered in cattle, but there is some evidence to associate bovine herpesvirus 4 with bovine metritis (13). Antimicrobial polypeptides such as defensins provide intial mucosal protection against viruses. In humans, impairment of this system can increase susceptibility to HIV-1 infection in the cervix and vagina (9).

Tissue repair. Many of the other genes showing greatest differential expression between the two groups of cows have previously been associated with tissue remodelling and inflammatory responses (see Networks 3 and 4). *MMP1, MMP3, MMP9* and *MMP13* were all highly up-regulated in the SNEB group. In human uterus *MMP1, MMP3, MMP7* and *MMP9* mRNAs all increase at menstruation when progesterone withdrawal is an important stimulus (63). In this situation MMP7 is produced by epithelial cells, MMP1 and MMP3 by stromal cells and MMP9 by migratory immune cells, attracted by chemokines. Mast cell activation releases the pro-inflammatory cytokines IL1 and TNFA (which increase stromal cell MMP1 and MMP3 production) and proteases (which releases active MMPs from pro-MMPs), leading to breakdown of the extracellular matrix (63). Plasmin is one such proteolytic enzyme and the increased expression of *PLAUR* is indicative of activation of this system.

MMP expression is also related to that of *AHSG*, another highly up-regulated gene in SNEB cows. In addition to its' role in insulin signaling discussed above, AHSG is found on the cell surface where it functions to anchor other molecules to the plasma membrane (38). It interacts with a variety of MMPs, both activating them and protecting them from autolytic cleavage (35,55). In bone MMP2 is an important activator of IGFBPs and its production is stimulated by IGF2 and TGFB (48). *IGFBP1* expression was increased in SNEB cows and we have previously shown that IGF2 is highly expressed in endometrial stroma (41), although expression was not affected by EB status. Together these data suggest that IL1 signaling in the postpartum uterus induces MMP expression and activation. The much higher levels of MMP expression in the SNEB cows suggests that the necessary remodelling of the uterus following calving, which requires extensive tissue breakdown, was proceeding more slowly in SNEB than in MNEB cows.

Conclusions. In conclusion, available evidence suggests that there is a complex inter-relationship in the postpartum uterus between acute infection, a predisposition to chronic inflammation and a reduced capacity for tissue repair. The ability to clear the infection is impaired in cows in SNEB,

thus also delaying the repair processes. Such effects may be mediated directly by altered concentrations of metabolic hormones and metabolites acting on the uterine cellular mechanisms. Indirect effects are also likely, as cows in SNEB are predisposed to liver damage and increased peripheral insulin resistance, both of which can have a negative impact on the immune system. Our work thus supports previous suggestions that uterine involution and elimination of contaminant bacteria will be delayed in animals in a negative energy state post calving (40). An increased rate of uterine involution is associated with earlier resumption of ovarian activity (45). Conversely, endometrial damage associated with sub-clinical endometritis delays cervical involution, disrupts the pre-ovulatory LH surge and perturbs embryo survival leading to prolonged intervals to conception with many cows failing to conceive at all (5,22,51,65).

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769 **Figure legends**

770

771 Fig. 1. Changes in circulating concentrations of: (a) IGF1, (b) PGFM and (c) serum amyloid A
772 (SAA) following calving. IGF1 concentrations increased to significantly higher values in MNEB
773 cows $P<0.05$, but PGFM and SAA values did not differ between groups.

774

775 Fig. 2. IPA Network 1. Differentially regulated genes in endometrium involved in antigen
776 presentation, cell mediated immune responses and humoral immune response, with 24 focus
777 molecules and a score of 49. The network is displayed graphically as nodes (gene/gene products)
778 and edges (the biological relationship between nodes). The node colour intensity indicates the
779 expression of genes; red up-regulated; green, down-regulated in SNEB versus MNEB
780 endometrium. The fold value and P values are indicated under each node. The shapes of nodes
781 indicate the functional class of the gene product as shown in the key.

782

783 Fig. 3. IPA Network 2. Differentially regulated genes in endometrium involved in cellular
784 movement, haematological system development and function and immune cell trafficking with
785 20 focus molecules and a score of 40. The network is displayed graphically as nodes (gene/gene
786 products) and edges (the biological relationship between nodes). The node colour intensity
787 indicates the expression of genes; red up-regulated; green, down-regulated in SNEB versus
788 MNEB endometrium. The fold value and P values are indicated under each node. The shapes of
789 nodes indicate the functional class of the gene product as shown in the key given in Fig. 2. Solid
790 lines indicate a direct interaction and dotted lines an indirect interaction.

791

792 Fig. 4. IPA Network 3. Differentially regulated genes in endometrium involved in haematological
793 system development and function, humoral immune response and tissue morphology with 17
794 focus molecules and a score of 29. The network is displayed graphically as nodes (gene/gene
795 products) and edges (the biological relationship between nodes). The node colour intensity
796 indicates the expression of genes; red up-regulated; green, down-regulated in SNEB versus
797 MNEB endometrium. The fold value and P values are indicated under each node. The shapes of
798 nodes indicate the functional class of the gene product as shown in the key given in Fig. 2. Solid
799 lines indicate a direct interaction and dotted lines an indirect interaction.

800

Fig. 5. IPA Network 4. Differentially regulated genes in endometrium involved in post-translational modification, cell death, connective tissue development and function with 13 focus molecules and a score of 23. The network is displayed graphically as nodes (gene/gene products) and edges (the biological relationship between nodes). The node colour intensity indicates the expression of genes; red up-regulated; green, down-regulated in SNEB versus MNEB endometrium. The fold value and P values are indicated under each node. The shapes of nodes indicate the functional class of the gene product as shown in the key given in Fig. 2. Solid lines indicate a direct interaction and dotted lines an indirect interaction.

Table 1. Mean detectable concentrations of circulating hormones and metabolites at the time of tissue collection in cows maintained under mild or severe NEB

		MNEB	SNEB	*P*
	No. cows	5	6	
	Days postpartum	14 ± 0.73	14 ± 0.68	
Energy	Net EB (UFL/ day)	-1.7 ± 1.17	-6.1 ± 1.03	0.018
Endocrine	IGF1 (ng/ml)	51.4 ± 10.33	10.6 ± 1.09	0.002
	Insulin (μIU/ml)	0.2 ± 0.07	0.1 ± 0.02	0.12
	PGFM	34.0 ± 8.72	55.0 ± 17.65	0.34
	Cortisol (ng/ml)	13.2 ± 5.80	5.47 ± 1.61	0.24
	Estradiol (pg/ml)	2.2 ± 0.33	1.6 ± 0.24	0.14
Metabolism	Glucose (mmol/L)	4.1 ± 0.15	2.7 ± 0.15	<0.001
	NEFA (mmol/L)	0.3 ± 0.05	1.4 ± 0.14	<0.001
	BHB (mmol/L)	0.5 ± 0.09	3.7 ± 0.20	<0.001
	SAA mg/ml	62 ± 37.1	133 ± 29.2	0.16

Values are mean ± SEM. EB, energy balance: UFL, unité fourragère lait: IGF1, insulin like-growth factor-I: NEFA, non-esterified fatty acid: BHB, β-hydroxybutyrate: SAA, circulating serum amyloid A.

Table 2. Comparison of hematological parameters between groups. Significant differences are shown in **bold.**

	MNEB	SNEB	P
n	5	6	
White blood cell count (WBC)	**9.4 ± 0.62**	**6.0 ± 0.69**	**0.006**
Granulocyte No.	0.6 ± 0.30	0.1 ± 0.04	0.12
Granulocyte %	6.9 ± 3.92	2.2 ± 0.55	0.23
Lymphocyte No.	**8.9 ± 0.84**	**5.8 ± 0.70**	**0.021**
Lymphocyte %	92 ± 4.2	95 ± 1.5	0.59
Monocyte No.	0.06 ± 0.02	0.15 ± 0.06	0.26
Monocyte %	0.7 ± 0.34	3.0 ± 1.27	0.14
Red blood cell count	6.3 ± 0.43	7.4 ± 0.17	0.23
Haemoglobin concentration	9.8 ± 0.36	10.4 ± 0.19	0.12
Haematocrit	26 ± 1.5	29 ± 0.6	0.11
Mean corpuscular volume	42 ± 1.1	43 ± 0.5	0.73
Mean corpuscular hemoglobin	15.8 ± 0.64	15.4 ± 0.33	0.53
Red blood cell distribution width	**16.0 ± 0.58**	**17.9 ± 0.52**	**0.035**
Platelet crit	**0.23 ± 0.02**	**0.15 ± 0.02**	**0.028**
Mean platelet volume	**5.3 ± 0.21**	**4.2 ± 0.36**	**0.033**

Table 3. Top 20 genes ranked by real fold change up in endometrium of cows in SNEB compared with MNEB (all P < 0.0001)

Fold Change Up	**Entrez Gene ID**	**Unigene ID**	**Gene symbol**	**Entrez gene name**
28.8	281308	Bt.3417	*MMP1*	Matrix metallopeptidase 1
14.8	281735	Bt.7165	*CXCL5*	Chemokine (C-X-C motif) ligand 5
14.0/12.9#	281309	Bt.18504	*MMP3*	Matrix metallopeptidase 3 (stromelysin 1)
11.3	539241	Bt.350	*HLA-DQB1*	MHC class II, DQ beta 1
9.4	616818	Bt.9360	*S100A8*	S100 calcium binding protein A8
9.4	281914	Bt.39	*MMP13*	Matrix metallopeptidase 13 (collagenase 3)
8.3	532569	Bt.87249	*S100A9*	S100 calcium binding protein A9
8.3	515640	Bt.9175	*IL1R*	Interleukin 1 receptor
8.2	282467	Bt.357	*S100A12*	S100 calcium binding protein A12
8.2	505080	Bt.13628	*TGM3*	Transglutaminase 3
8.1	280828	Bt.49740	*IL8*	Interleukin 8
8.0/7.7/6.9#	280988	Bt.23250	*AHSG*	Alpha-2-HS-glycoprotein
8.0	522269	Bt.13633	*ACTN4*	Actinin, alpha 4
7.6	505518	Bt.6410	*C15H11ORF34*	Chromosome 11 open reading frame 34 ortholog
7.4	505317	Bt.19959	*TRPA1*	Transient receptor potential cation channel, subfamily A, member 1
7.4	504598	Bt.5878	*MATN4*	Matrilin 4
7.4/6.1#	514346	Bt.56517	*SDS*	Serine dehydratase
7.3	515200	Bt.23199	*CTSL1*	Cathepsin L1
7.2	---	Bt.65714	---	---
6.4	281863	Bt.4208	*IL8RB*	Interleukin 8 receptor, beta

represented by more than 1 probe on the array
--- unidentified gene

Table 4. Top 20 genes ranked by real fold change down in endometrium of cows in SNEB compared with MNEB (all P < 0.0001)

Fold Change Down	**Entrez Gene ID**	**Unigene ID**	**Gene symbol**	**Entrez gene name**
-5.9	---	Bt.1296	---	---
-4.6	---	Bt.24179	---	---
-4.5	613966	Bt.22381	*PLA2G10*	Phospholipase A2, group X
-4.0	505824	Bt.64757	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2
-3.9	617336	Bt.22389	*SH1SA2*	Shisa homolog 2 (Xenopus laevis)
-3.8	---	Bt.69297	---	---
-3.8	---	Bt.26467	---	---
-3.7	327679	Bt.15980	*CCNB1*	Cyclin B1
-3.6	---	Bt.92107	---	---
-3.4	286767	Bt.12848	*PTHLH*	Parathyroid hormone-like hormone
-3.4	528174	Bt.37396	*GPR133*	G protein-coupled receptor 133
-3.3	507243	Bt.22741	*CLIC6*	Chloride intracellular channel 6
-3.1	---	Bt.31265	---	---
-3.1	511596	Bt.41310	*IL2*	Interleukin 2
-3.1	---	Bt. 17034	---	---
-2.9/-2.6/ -2.6#	533044	Bt.13588	*PSAT1*	Phosphoserine aminotransferase 1
-2.9	282868	Bt.19805	*SLC2A5*	Solute carrier family 2 (facilitated glucose/fructose transporter) member 5
-2.8	505727	Bt.27716	*NOV*	Nephroblastoma over-expressed gene
-2.8	317776	Bt.12781	*MYB*	v-myb myeloblastosis viral oncogene homolog (avian)
-2.7	---	Bt.84625	---	
-2.6	504879	Bt.15901	*MGC127236*	Amyloid P component like

represented by more than 1 probe on the array
--- unidentified gene

Table 5. Main functions identified using Ingenuity Pathway Analysis (all with P-value <0.0001)

Top canonical pathways	**Ratio**
Hepatic fibrosis/hepatic stellate cell activation	10/135 (0.074)
Complement system	6/36 (0.167)
Acute phase response signaling	11/178 (0.062)
HIF1α signaling	8/105 (0.076)
Leucocyte extravasation signaling	10/195 (0.051)

Top Bio functions:	**Molecules**
Diseases and disorders	
Cancer	93
Inflammatory response	58
Reproductive system disease	48
Renal and urological disease	26
Hematological disease	49
Molecular and cellular functions	
Cellular movement	52
Antigen presentation	53
Cell-to-cell signaling and interaction	51
Cellular growth and proliferation	75
Cell death	70
Physiological system development and function	
Tissue morphology	57
Hematological system development and function	64
Immune cell trafficking	40
Cell-mediated immune response	70
Humoral immune response	55

.

Table 6. Top 20 canonical sub-pathways from IPA analysis (all P<0.001) associated with immune response which were differentially influenced by energy balance status in bovine endometrium#. Genes in **bold** decreased in SNEB, remainder increased.

Sub-pathway	**Gene symbol**
Hepatic fibrosis	*IL8, VCAM1, CCR5, MMP13, IL1B, IL1R, CCR7, MMP1, MMP9,* ***IGF1***
Acute phase response	*C3, AHSG, IL1R, MAPK13, HP, SOD2, IL1RN, CFB, IL1B,* ***PIK3R1, CRABP1***
Complement system	*C3, CFB, C1QA, C1QB, C1QC, CFH*
Pattern recognition receptors	*IFIH1, C3, IL1B, C1QA, C1QB, CIQC,* ***PIK3R1***
IL17 signaling	*IL8, MMP3, MAPK13, CXCL5, PTGS2* ***PIK3R1***
Airway pathology in chronic obstructive pulmonary disease	*IL8, MMP1, MMP9*
Leucocyte extravasation signaling	*VCAM1, MMP3, MMP13, MAPK13, ACTN4, MMP1, MMP9,* ***PIK3R***
Primary immunodeficiency signaling	*ZAP70, IGHM, CD79A, IGLL1,* ***IGHG1****,*
HIF1α signaling	*MMP3, MMP13, MAPK13, MMP9, MMP1,* ***PIK3R***
IL-10 signaling	*CCR5, IL1RN, IL1B, MAPK13, IL1R*
LXR/RXR activation	*IL1RN, CD36, IL1B, IL1R, MMP9*
Oncostatin M signaling	*MMP3, MMP13, CH13L1, MMP1*
Glucocorticoid receptor signaling	*VCAM1, IL8, IL1RN, IL1B, MAPK13, MMP1,* ***FKBP4, PIK3R1***
Bladder cancer signaling	*IL8, MMP3, MMP13, MMP9, MMP1*
Il-6 signaling	*IL8, IL1RN, IL1B, MAPK13, IL1R*
NF-κB signaling	*IL1RN, ZAP70, IL1B, IL1R, MAP3K8,* ***PIK3R1***
HMGB1 signaling	*IL8, VCAM1, IL1R, MAPK13,* ***PIK3R1***
P38 MAPK signaling	*IL1RN, IL1B, MAPK13, IL1R,* ***PLA2G10***
Dendritic cell maturation	*IL1RN, IL1B, MAPK13, CCR7,* ***PIK3R1, IGHG1***
IL-8 signaling	*IL8, VCAM1, IL8RA, PTGS2, MMP9,* ***PIK3R1***

#Based on significant differential expression of 103 immune-related genes using Bovine Affymetrix arrays.

Table 7. Comparison of some genes analysed by both qPCR and Affymetix arrays.

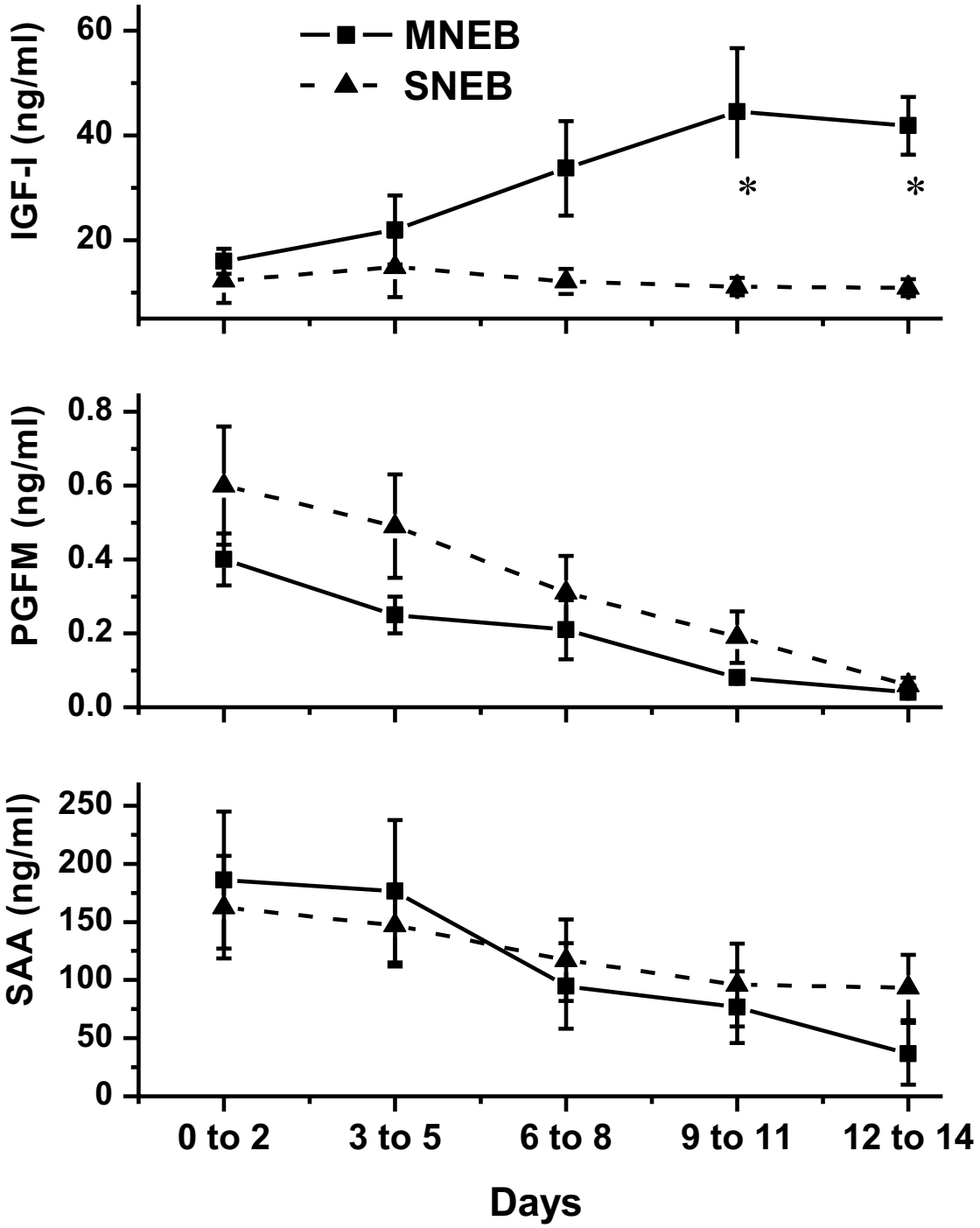
Gene	MNEB (n=5 cows)	SNEB (n=6 cows)	Fold change in SNEB by qPCR	P for qPCR data*	Fold change in SNEB by array	P for array data
MMP1	0.2 ± 0.10	11.1 ± 6.09	55.5 up	0.003#	28.8 up	$<10^{-10}$
MMP3	0.1 ± 0.04	1.9 ± 0.97	19.0 up	0.009#	14.0/12.9 up+	$<10^{-10}$
IL8	2.5 ± 1.3	44.3 ± 27.8	17.7 up	0.10#	8.1 up	$<10^{-10}$
IL1R	15 ± 5.9	217 ± 115	14.5 up	0.016#	8.3 up	$<10^{-10}$
AHSG	0.1 ± 0.02	1.2 ± 0.52	12.0 up	0.009#	6.9 up	$<10^{-10}$
IL1B	0.6 ± 0.13	6.5 ± 4.34	10.8 up	0.026#	2.7 up	$<10^{-5}$
S100A8	0.05 ± 0.02	0.36 ± 0.13	7.2 up	0.004	9.4 up	$<10^{-10}$
MMP9	0.3 ± 0.11	2.0 ± 0.55	6.7 up	0.02	5.3 up	$<10^{-10}$
IL8RB	1226 ± 681	7486 ± 1847	6.1 up	0.017	6.4 up	$<10^{-10}$
MMP13	0.1 ± 0.03	0.5 ± 0.25	5.0 up	0.032#	9.4/5.3 up+	$<10^{-10}$
IGFBP1	0.5 ± 0.16	2.1 ± 0.76	4.2 up	0.062#	2.3 up	$<10^{-5}$
CXCL5	1.1 ± 0.41	3.7 ± 1.34	3.4 up	0.068#	14.8 up	$<10^{-10}$
AKR1C4	0.8 ± 0.13	2.1 ± 0.55	2.6 up	0.037#	3.2 up	$<10^{-6}$
PTGS2	363 ± 145	804 ± 257	2.2 up	0.19 (NS)	4.3 up	$<10^{-10}$
IGF1	16 ± 4.3	8 ± 3.2	2.0 down	0.19 (NS)	2.1 down	$<10^{-9}$
DEFB1	2.8 ± 0.70	4.4 ± 0.79	1.6 up	0.19 (NS)	2.3 up	$<10^{-5}$
PDK4	29 ± 10.0	53 ± 8.6	1.8 up	0.10 (NS)	3.2 up	0.04
LAP	24 ± 5.5	45 ± 6.55	1.9 up	0.04	NS	NS
TBXAS1	2.2 ± 0.33	2.8 ± 0.46	1.3 up	0.30 (NS)	NS	NS
DEFB7	51 ± 17.1	76 ± 13.6	1.5 up	0.28 (NS)	NS	NS
DEFB5	52 ± 19.1	128 ± 47.9	2.5 up	0.20 (NS)	NS	NS
IGFBP6	5.8 ± 1.12	3.0 ± 0.89	1.9 down	0.08 (NS)	NS	NS
Housekeeping genes						
RPL19	28 ± 2.3	24 ± 4.5		0.81		
GAPDH	19 ± 2.6	27 ± 7.1		0.36		
18SrRNA	72079 ± 9463	72187 ± 9939		0.99		

*Comparison was by t test, those indicated by # used log-transformed data to normalise variances.

+ 2 probes on array.

NS, not significantly different.

Fig. 1.


MNEB
SNEB
IGF-I (ng/ml)
60
40
20
*
*
PGFM (ng/ml)
0.8
0.6
0.4
0.2
0.0
SAA (ng/ml)
250
200
150
100
50
0
0 to 2
3 to 5
6 to 8
9 to 11
12 to 14
Days

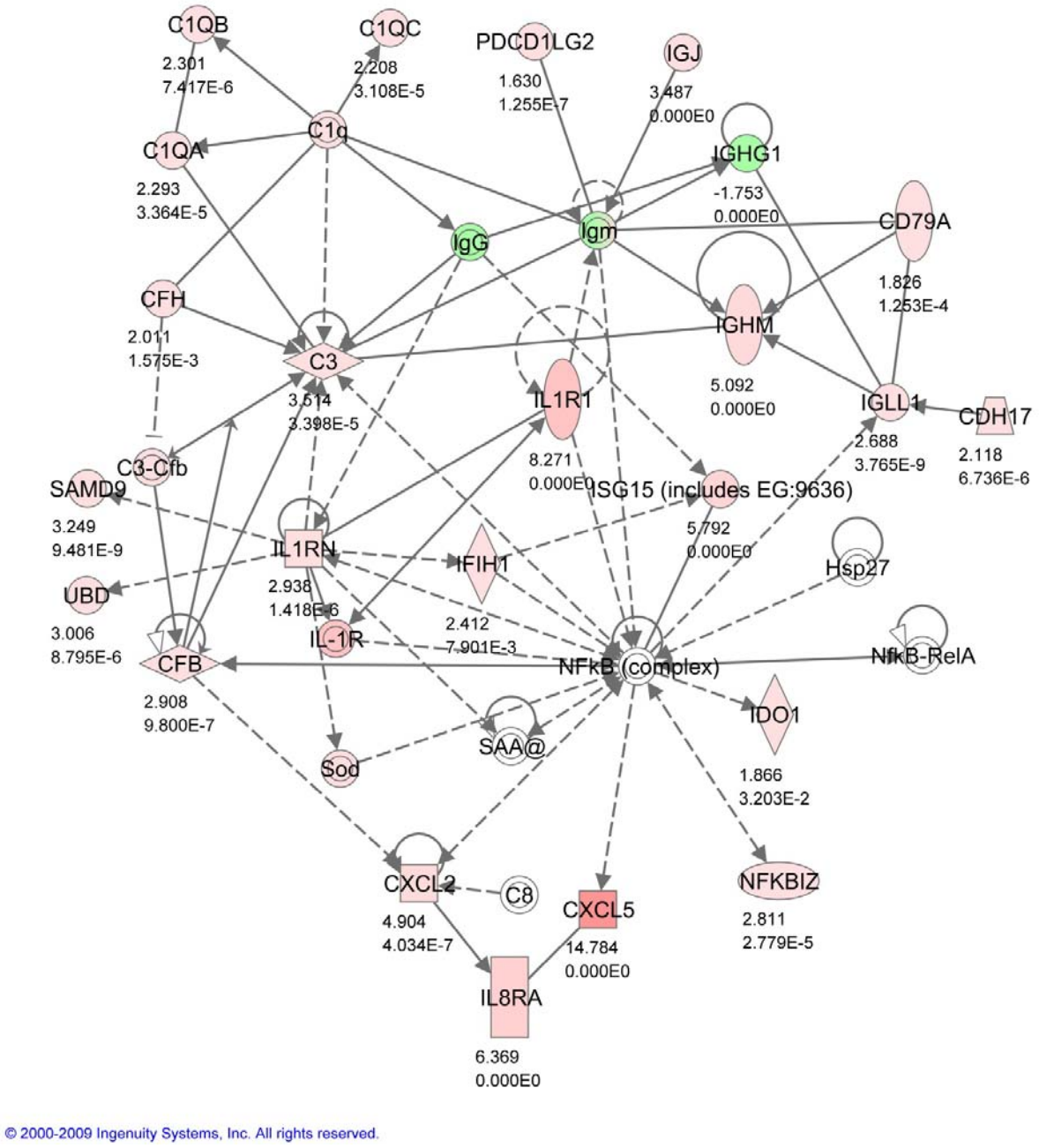


Fig. 2. Network 1.

□ Cytokine, ◇ enzyme, ▯ G-protein coupled receptor, ▽ kinase, ◇ peptidase, ⬭ transcription regulator, ⬯ transmembrane receptor, ⏢ transporter, ◎ complex/group, → acts on, ⟶ translocates, ⊣ inhibits AND acts on, ___ direct interaction, ... indirect interaction.

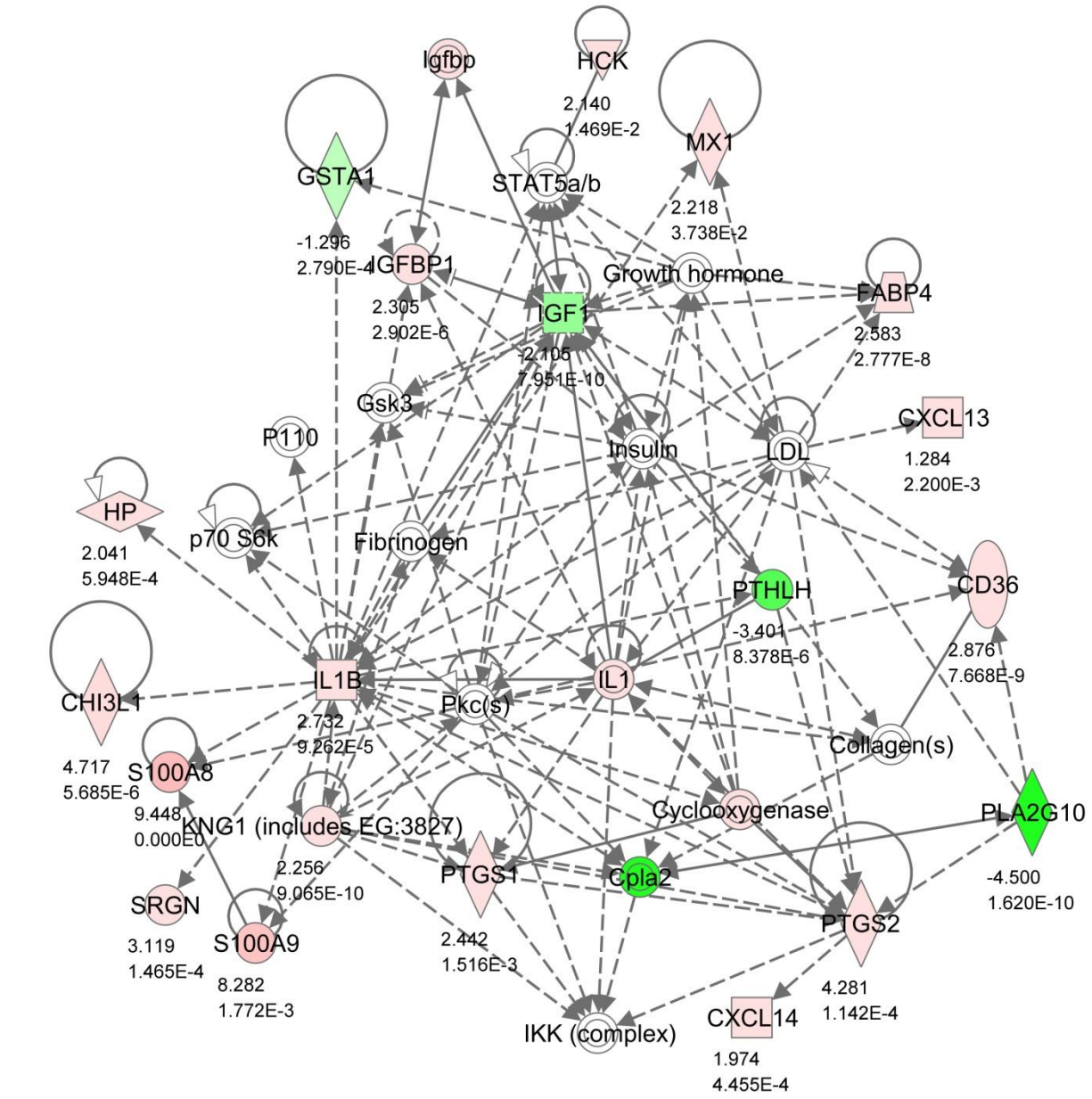


Fig. 3. Network 2.

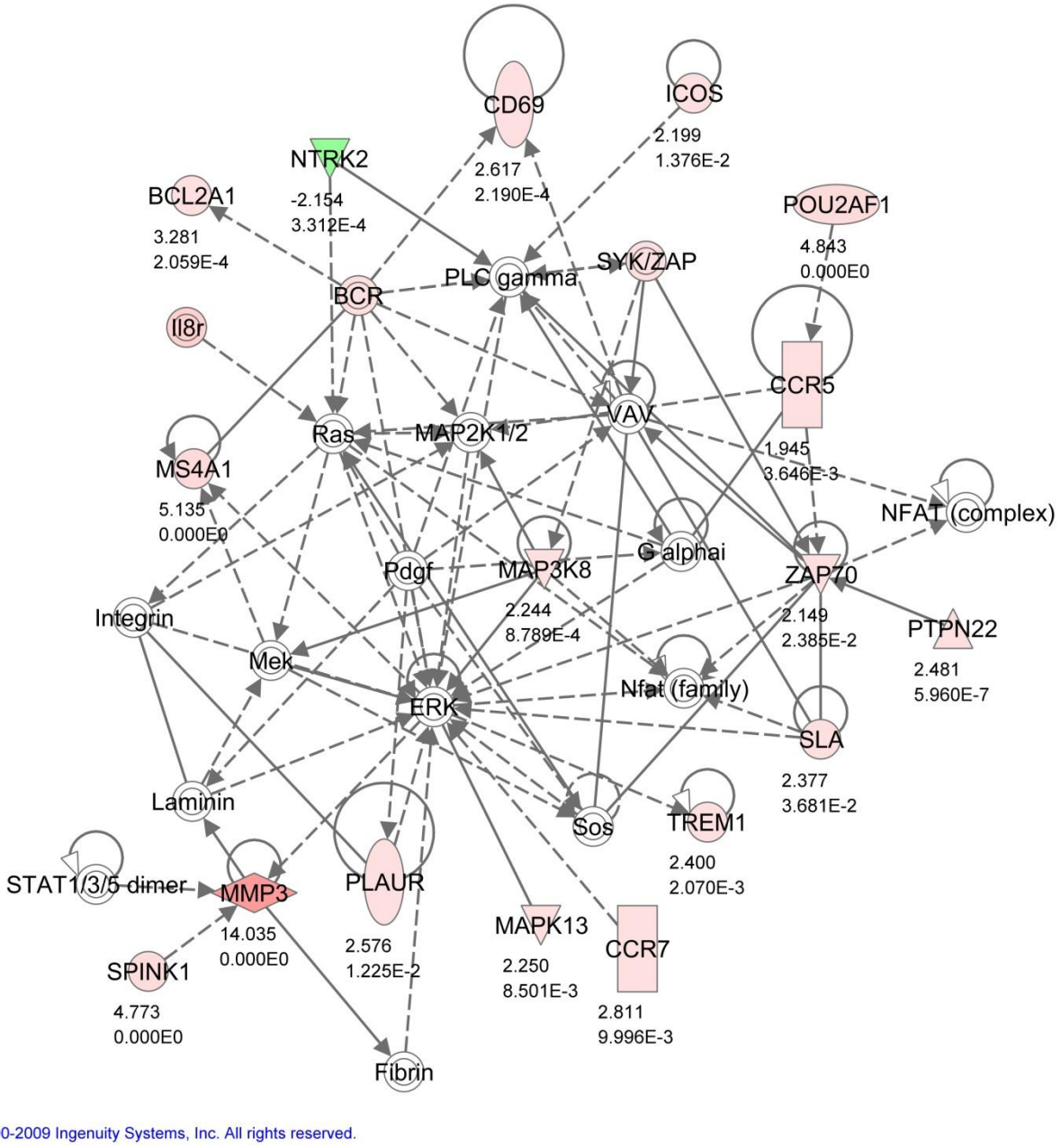


Fig. 4. Network 3.

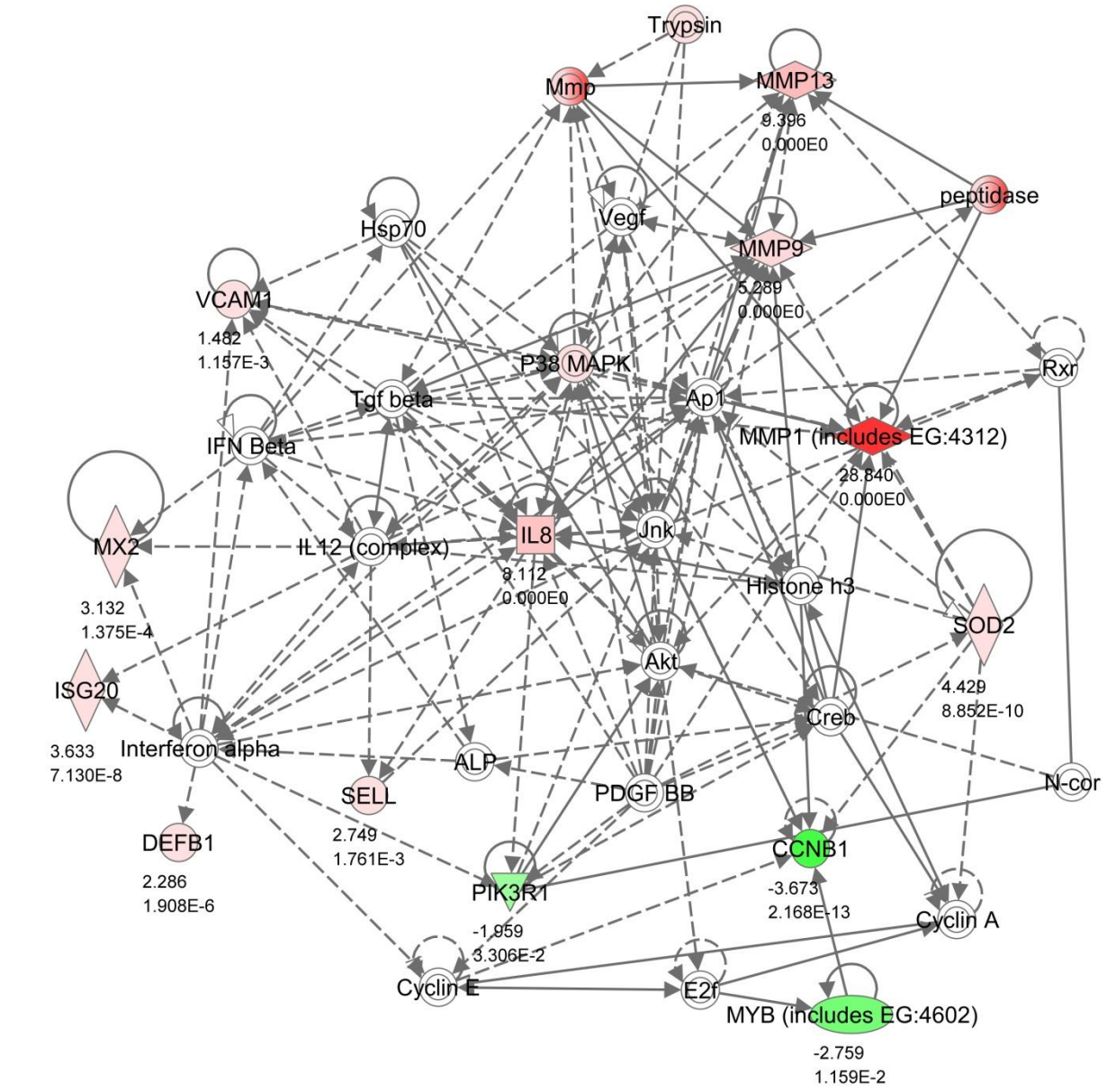


Fig. 5. Network 4.