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Activation of several key components of the epidermal differentiation pathway in cattle following infestation with the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*

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ABSTRACT

The cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, and the diseases it transmits pose a persistent threat to tropical beef production. Genetic selection of host resistance has become the method of choice for non-chemical control of cattle tick. Previous studies have suggested that larval stages are most susceptible to host resistance mechanisms. To gain insights into the molecular basis of host resistance that occurs during *R. microplus* attachment, we assessed the abundance of proteins (by isobaric tag for relative and absolute quantitation (iTRAQ) and Western blot analyses) and mRNAs (by quantitative reverse transcription PCR (qRT-PCR)) in skin adjacent to tick bite sites from high tick-resistant (HR) and low tick-resistant (LR) Belmont Red cattle following challenge with cattle tick. We showed substantially higher expression of the basal epidermal keratins KRT5 and KRT14, the lipid processing protein, lipocalin 9 (LCN9), the epidermal barrier catalysing enzyme transglutaminase 1 (TGM1), and the transcriptional regulator B lymphocyte-induced maturation protein 1 (Blimp1) in HR skin. Our data reveals the essential role of the epidermal permeability barrier in conferring greater resistance of cattle to tick infestation, and suggest that the physical structure of the epidermal layers of the skin may represent the first line of defence against ectoparasite invasion.

1. Introduction

The cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* is an external parasite present in tropical and sub-tropical areas of America, Africa, Asia and Australia. Tick parasitism remains an important impediment to the efficient raising of cattle on tropical and semi-tropical pastures despite widespread application of acaricides. Tick infestation within both beef and dairy herds can manifest itself through a decrease in fertility, body weight loss and reduced milk production, as well as the transmission of a debilitating blood-borne parasitic cattle disease, tick fever (principally caused by *Anaplasma* and *Babesia*). Furthermore, the cost and excessive use of chemical products and the associated resistance issues present ever increasing challenges to the industry.

To satisfy consumer demand for high-quality products whilst maintaining a clean environment, selection for host resistance has become the method of choice for non-chemical control of cattle ticks in the tropical beef industry. Resistance of bovine hosts to cattle tick infestation is under genetic control. *Bos indicus* cattle breeds are more resistant to *R. microplus* than *Bos taurus* breeds, although considerable variation in resistance exists both between and within breeds (Seifert, 1971). Therefore, highly resistant animals can be selected to progress genetic improvement in tick resistance within a herd (Utech et al., 1978; Utech and Wharton, 1982). Several factors have been shown to influence the resistance of cattle to *R. microplus* (for reviews see Wikel, 1996; Wikel and Bergman, 1997; Mattioli et al., 2000). Protective responses of cattle to *R. microplus* larval infection include grooming behaviour (Bennett, 1969), histamine secretion (Kemp and Bourne, 1980), mast cells and basophil hypersensitivity reaction at the tick bite sites (Hales et al., 1981; Schleger et al., 1981a; Decastro and Newson, 1993), and arteriovenous anastomoses in the skin (Schleger et al., 1981b). Resistance to cattle tick infestation has also been associated with specific immune responses such as differential patterns of specific Ig responses (Kashino et al., 2005) and high concentrations of serum complement (Wambura et al., 1998). Differential immunological responses between high and low resistant animals were supported by recent genome-wide gene expression studies which imply the involvement of non-specific cellular immunity

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and specific immunity mediated by antibodies and T-cells and their products (Piper et al., 2009).

Cattle ticks rely on only one host, that is, all parasitic stages of the life cycle occur on the same animal. Feeding is intermittent but all parasitic stages ingest some blood meal. The parasitic phase of the life cycle lasts approximately 3 weeks from larval attachment through to when the fully engorged females fall to the ground to lay eggs. Earlier studies (Roberts, 1968; Wagland, 1979) investigated the various life stages of ticks in five Brahman steers exhibiting different resistance levels and concluded that tick losses at any stage were generally related to the hosts' level of resistance, but the losses during the first 24 h following tick application were the most important in terms of absolute numbers of ticks lost (up to 90%). Roberts (1968) proposed that rejection of larvae may be due to alteration of the attachment site by host reaction so that it becomes unsuitable for feeding. Studies of larval behaviour, particularly on highly resistant animals, support this conclusion. Kemp et al. (1976) confirmed that a high percentage of larvae in resistant cattle were rejected within 24 h and observed that larvae on highly resistant animals made many more attachments, spent less time at each place and spent more time moving about the skin.

In the present study, we explore the functional significance of skin epidermal barrier function on tick resistance. We performed a comparative proteomic analysis, quantitative reverse transcription PCR (qRT-PCR) and Western blotting to examine the expression of a number of key molecules known to be involved in epidermal terminal differentiation, a process where basal keratinocytes periodically withdraw from the cell cycle and commit to differentiation and migration upward through the spinous and granular layers to eventually form the non-viable cornified stratum corneum (SC) (for reviews, see Segre, 2003 and Candi et al., 2005). The data shows a highly significant up-regulation of basal keratins KRT5 and KRT14, the enzyme transglutaminase 1 (TGM1) and the zinc finger transcription factor B lymphocyte-induced maturation protein 1 (Blimp1) in the skin of high tick-resistant (HR) Belmont Red cattle. The collective findings of this study suggest that the ability of the host to prevent the successful attachment of tick larvae during the early stages of infestation is attributable to changes occurring at the epidermal gene and protein levels, directly altering the skin barrier function.

2. Materials and methods

2.1. Animals

Sixty Belmont Red heifers vaccinated with the attenuated vaccines against Babesiosis, Anaplasmosis and bovine ephemeral fever, were maintained before the experiment at the Belmont Research Station, Rockhampton, Queensland (geographical coordinates: 150°13′East, 23°8′South) where they were field exposed to a low number of *R. microplus* ticks. At 7 months of age (±1 month), animals were housed at J.M. Rendel Laboratories, Rockhampton, in tick-free undercover, concrete floor pens. Animals were allowed 4 weeks to acclimatise to conditions in the animal house before this experiment began. The larvae used for infestation were the N-strain (acaricide susceptible) supplied by the Department of Primary Industries and Fisheries Animal Research Institute in Yeerongpilly, Queensland. Approximately 10,000 (0.5 g egg weight) *R. microplus* larvae were applied along the midline of each animal using a soft brush. Skin biopsies were collected before infestation (time 0 h) and at 3 and 24 h following infestation. At biopsy time, animals were restrained in a crush and local anaesthesia (lignocaine, 2%) was injected (2.5 ml) underneath the skin adjacent to the biopsy site. Biopsy sites were taken from the area where larvae were sighted, although not necessarily attached when sampling. Each biopsy was ~8 mm in diameter and ~4 mm deep and taken from the same area of the animal (near the tail). Tissues were snap frozen in liquid nitrogen and transferred to −80 °C for long term storage. All animal handling protocols were approved by CSIRO (Rendel Laboratory) Animal Ethics Committee.

2.2. Tick counts

Tick counts were undertaken on one side of each animal, as described by Utech et al. (1978). Briefly, female ticks 4.5–8 mm were counted on days 19–21 following larval infestation. Counts (Utech et al., 1978) indicated the number of female ticks surviving to maturity on each animal, as the parasitic life cycle of the cattle tick is around 21 days. Results of overall tick counts from 60 animals showed considerable variation in tick numbers surviving to adults among animals infested with *R. microplus* larvae (Fig. 1). Twelve HR animals (with mean total tick count of 50.5) and 10 low tick-resistant (LR) animals (with mean total tick count of 314.4) were selected as divergent responders for resistance phenotype and used for gene and protein expression comparison (Fig. 1).

2.3. Isobaric tag for relative and absolute quantitation (iTRAQ) analysis – sample preparation and labelling

iTRAQ is a non-gel-based technique used to identify and quantify proteins from different sources in a single experiment. The method is based on the covalent labelling of the N-termini and lysine side-chains of peptides from protein digestions with "iTRAQ tag" of varying mass. These samples are then pooled and analysed by tandem mass spectrometry (MS/MS). A database search is then performed using the fragmentation data to identify the labelled peptides and hence the corresponding proteins. The fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they originated.

Protein extracts obtained from skin at 3 h following tick infestation were used for iTRAQ analysis. Frozen skin tissues were pulverised with a hammer and homogenised in lysis buffer consisting of 40 mM Tris, pH 8.0, 6 M guanidine hydrochloride and 1% DTT. The samples were allowed to solubilise at 4 °C overnight and clarified by centrifugation (100,000*g* for 1 h at 4 °C). Protein samples were then precipitated with methanol overnight at 4 °C. The precipitates were collected by centrifugation (19,000*g* for 30 min at 0 °C) and resuspended in 300 µl of 25 mM triethylammonium bicarbonate, 20 mM sodium carbonate adjusted to pH 8.0 with HEPES. One hundred micrograms of soluble proteins from each sample was sequentially reduced (2 µl of 50 mM tris(2-carboxyethyl)phosphine (TCEP) at 60 °C for 1 h), alkylated (1 µl of 200 mM methyl methanethiosulphonate (MMTS), at room temperature for 10 min), digested (10 µl trypsin (Promega), 1 µg/µl in water, at 37 °C, overnight) and labelled with the four iTRAQ reagents (tags 114, 115, 116 and 117) according to the manufacturer's instructions (Applied Biosystems). Samples derived from two LR cattle (total tick counts of 299 and 282) were labelled with iTRAQ tags 114 and 115, whereas samples from the two HR cattle (total tick counts of 32 and 35) were labelled with tags 116 and 117, respectively.

2.4. Sample fractionation and desalting

The labelled peptides were combined, lyophilised, reconstituted in loading buffer (10 mM KH_2PO_4, pH 3.0, in 25% acetonitrile). Fractionation was achieved using offline strong cation exchange (SCX) chromatography using a PolySulfoEthyl A column on an Ultimate 3000 MDLC system (Dionex). A linear gradient of 0–500 mM KCl (in 25% acetonitrile, pH 3.0) over 20 min at a flowrate of 6 µl/min

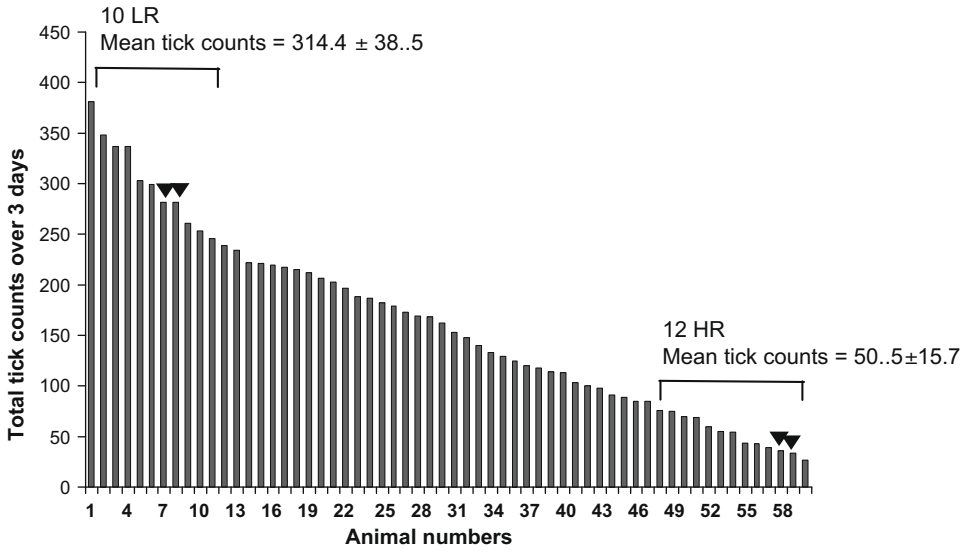

Fig. 1. Mean tick counts of individual Belmont Red cattle. Bars represent the total tick counts over 3 days of all 60 animals in the experiment. Black arrowheads indicate the four animals whose samples were used for isobaric tag for relative and absolute quantitation (iTRAQ) analysis. Bracketed bars indicate the 10 low responder (LR) animals and the 12 high responder (HR) animals selected for analysis.

was employed and 15 SCX fractions were collected. The SCX fractions were acidified with 5% formic acid and injected onto an Ultimate 3000 HPLC system coupled directly to the mass spectrometer. Samples were injected onto a trap column at 15 μL/min and desalted for 3 min prior to valve switching to reverse the direction of flow onto a Dionex C18 PepMap, 3 μm (180 μm × 150 mm) column using a column flowrate of 1 μl/min. A linear gradient from 2% to 42% solvent B over 120 min was employed. Solvent A consisted of 0.1% formic acid and solvent B contained 90% acetonitrile and 0.1% formic acid.

2.5. Mass spectrometry

A QStar® Elite hybrid LC–MS/MS system (Applied Biosystems) equipped with a nano-electrospray ionisation source was utilised for data analysis. MS/MS data-dependent acquisition mode was used in which survey MS spectra were collected (*m/z* 350–1800) for 1 s followed by three MS/MS measurements on the three most intense parent ions (50 counts/s threshold, +2 to +4 charge state, and *m/z* 100–1800 mass range for MS/MS), using the manufacturer's 'smart exit' and 'iTRAQ reagent' method modification settings. Parent ions previously targeted were excluded from repetitive MS/MS acquisition for 30 s (mass tolerance of 50 mDa). Data were acquired and processed using Analyst QS 2.0 software.

2.6. Data analysis

ProteinPilot™ 2.0.1 software (Applied Biosystems) using the Paragon Algorithm (Shilov et al., 2007), was used for the identification of proteins and these were grouped to minimise redundancy. MS/MS data were searched against the IBISS5 database (containing 97,771 non-redundant sequences; http://www.biolives.csiro.au/IBISS5/). Search parameters were defined as cysteine alkylation with MMTS, trypsin as the digestion enzyme and no restrictions were placed on taxonomy. Modifications were set to the "generic workup" and "biological" modification sets provided with this software package, which consisted of 126 possible post-translational modifications. The generic workup modifications set contains 51 potential modifications that may occur as a result of sample handling, for example, oxidation, dehydration and deamidation. Simultaneous quantification of the iTRAQ reporter ions was conducted using ProteinPilot software. All peptides used for the quantification of protein ratios were unique to the given protein; peptides that were common to other isoforms were ignored. A fold-change of each protein expression was calculated by comparing the average iTRAQ ratio of 116 and 117 as the HR-cattle group with the average ratio of 114 and 115 as the LR-cattle group. Proteins quantified with a fold-change of more than 50% (average iTRAQ ratio >1.5 or <0.67) and a *P*-value less than 0.05 were identified as differentially expressed proteins.

2.7. Cattle skin extracts for Western blots

Frozen skin samples were each pulverised with a hammer, placed in 500 μl of cold lysis buffer (30 mM Tris–HCl, pH 8.5, 2 M thiourea, 7 M urea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and homogenised in a hand-held ultra Turrax T8.01 homogenizer (Ika Labortechnik Staufen, Germany). The samples were solubilised overnight (4 °C), then centrifuged at 15,000*g* for 10 min at 4 °C to remove insoluble debris and extracellular material. Samples of the solubilised proteins were used for Western blot analyses.

2.8. Western blot

SDS–PAGE and Western blots were performed according to standard protocols. Twenty micrograms of skin protein extract were separated on 10% SDS–PAGE or 4–12% NuPAGE Bis–Tris gels (Invitrogen) and immobilised onto nitrocellulose membranes. The following antibodies were used: as primary antibody, human cytokeratin 5 monoclonal antibody (clone AE14, 1:1000, Santa Cruz Biotechnology), goat polyclonal antiserum against human TGase 1 (clone C-20, 1:1000, Santa Cruz Biotechnology), and rabbit polyclonal antiserum against human Blimp1/PRDM1 (1:1000; Imgenex). For purposes of loading control and normalisation, the blots were also stained with a goat polyclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone sc-20357, 1:1000, Santa Cruz Biotechnology). Secondary antibodies were peroxidase-conjugated anti-mouse, anti-goat and anti-rabbit secondary antibodies (1:20,000, Kirkegaard & Perry Laboratories, Maryland, USA). SuperSignal West Pico chemiluminescent substrate (Pierce) was used for signal development. Band intensities were quantified by scanning

the non-saturated Western blot image by a high resolution scanner and the image files were analysed using ImageQuant TL software (GE Healthcare).

2.9. Total RNA extraction and quantitative RT-PCR

Total RNA from 0, 3 and 24 h skin biopsies from 10 HR cattle (with mean total tick count of 50.5 ± 15.7) and eight LR cattle (with mean total tick counts of 314.4 ± 38.5) of the 60 animal experiment (Fig. 1) were extracted with TRIzol reagent (Invitrogen) as previously described (Bagnall et al., 2009). RNA samples (2 μg) were treated with DNase (Ambion) and cDNA generated using SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. Each quantitative PCR (qPCR) was carried out in a final volume of 9 μl containing 10 ng cDNA, 4.5 μl 2× SYBR® Green PCR Master Mix (Applied Biosystems) and 900 nM of each forward and reverse primer (Table 1). Four technical replicates were employed per sample for each gene. Standard cycling conditions on an ABI Prism 7900HT Sequence Detection System were used for all reactions. Primers used in the qPCRs were designed using PRIMER3 (http://frodo.wi.mit.edu/) based on their GenBank nucleotide sequence under the Accession Nos. specified in Table 1. Amplicon length ranged between 114 and 233 bp (Table 1). Amplification efficiencies for each primer pair were derived from the slope of standard curves using 10-fold serially diluted cloned plasmid of the respective amplicon. The *E* values for the eight primer pairs (Table 1) ranged between 96% and 99%. For each biological sample, the mean of the cycle threshold (C_T) values for each gene were calculated and normalised against the internal control; acidic ribosomal protein large, P0 (RPLPO), using the QGene software available at http://www.qgene.org/. This software expresses the result in the form of the mean normalised expression ± standard error.

2.10. Statistical analyses

The relative expression analysis was performed using REST 2008 software (Relative Expression Software Tool 2008 at http://gene-quantification.com/rest-2008.html), which compares the expression of each target gene in a sample relative to the internal control, and tests the group differences for significance using randomisation and bootstrapping techniques (http://rest.gene-quantification.info).

3. Results

3.1. Identification of differentially expressed proteins in skin of HR and LR cattle

To delineate the profiles of differentially expressed proteins, we individually labelled 100 μg of digested proteins obtained from 3 h skin of two HR (iTRAQ-116, iTRAQ-117) and two LR (iTRAQ-114, iTRAQ-115) animals with the four isobaric iTRAQ tags. The iTRAQ-labelled peptides were then fractionated by strong cation exchange chromatography and analysed by reverse phase liquid chromatography–electrospray ionisation-tandem mass spectrometry (LC–ESI-MS/MS). Identification of proteins was based on the presence of at least two peptides with peptide confidence values at or above 95% (Aggarwal et al., 2005). In total, 728 proteins were identified from 4,947 unique peptide sequences deduced from 12,498 MS/MS spectra. The number of proteins after grouping to remove protein isoforms resulting from common peptide evidence collapsed to 70 unique proteins (data not shown). The quantification of the peak areas of the reporter ions, *m/z* 114 and 115, or 116 and 117, in the MS/MS spectra illustrate the changes in the abundance of the peptides in HR versus LR proteins. To identify significantly expressed proteins we employed two criteria: (i) *p*-values $\leqslant$0.05, and (ii) protein ratios $\geqslant$1.5-fold increase or $\leqslant$0.67-fold decrease (Applied Biosystems iTRAQ Chemistry Reference Guide), and identified five proteins that were up-regulated in HR skin (Table 2). Notably, we have identified KRT5, KRT14 and lipocalin 9 (LCN9) to be differentially expressed (Table 2). The two keratins are the main structural protein products synthesised by basal keratinocytes which undergo differentiation. The function of LCN9 is not known but it is a member of a large protein family exhibiting great functional diversity which includes lipid binding and processing (Grzyb et al., 2006). As very few abundant proteins were detected as being differentially expressed in our iTRAQ, we conclude protein concentration and the extent of expression changes may be limiting factors for adequate quantification by this approach, in particular with regard to the small sample size used. Thus, it would be prudent to use this technique as a complementary method in conjunction with others such as gene expression analysis and immunoblotting. Despite this, our initial discovery of epidermal proteins KRT5, KRT14 and LCN9 and their possible role in tick resistance make them suitable targets for further investigation.

3.2. Expression of KRT5, KRT14 and LCN9 mRNAs

In order to confirm the changes described at the protein level, we investigated mRNA expression by qRT-PCR. We studied relative mRNA levels of *KRT5, KRT14* and *LCN9* genes in 10 HR animals and eight LR animals. Total RNAs were extracted from 0 to 24 h skin biopsies. The relative mRNA abundance during tick infestation in the HR and LR animal groups correlate with the changes described at the protein levels for *KRT5, KRT14* and *LCN9* (Fig. 2). Measurement of mRNA expression levels 24 h post-infestation indicated a 2-, 10- and 6-fold increase in *KRT5*, *KRT14* and *LCN9* levels, respectively (Fig. 2). At the protein level, iTRAQ analysis revealed a 2.1-, 2.0- and 1.8-fold increase in protein expression after 3 h for KRT5, KRT14 and LCN9, respectively (Table 2). The lower levels of KRT14 and LCN9 proteins observed by iTRAQ (Table 2) were in agreement with the levels of mRNAs at this time which were in general lower than those at 24 h (data not shown). In general, protein levels in the HR and LR skin matched the mRNA expression

Table 1
List of genes whose expression was examined by quantitative real-time PCR (qRT-PCR) comparing high tick-resistant (HR) and low tick-resistant (LR) cattle. Gene names, their symbols and NCBI Accession Nos. are listed. Also shown are forward and reverse primers for the genes and the internal control, 60S acidic ribosomal protein PO (RPLPO), together with the qRT-PCR amplicon sizes.

Gene name	Gene symbol	Accession No.	Forward (5′–3′)	Reverse (5′–3′)	PCR product size (bp)
Keratin 5	*KRT5*	NP_001008663	CAAGGTCCTGGACACCAAGT	TCCAGCTGTCTCCTGAGGTT	114
Keratin 14	*KRT14*	XP_880737	CCTCTGGCTCTCAGTCATCC	CGAACAATCTGCTCGTGTGT	109
Collagen, type I alpha1	*COL1A1*	NM_001046249	TGGTGACAAGGGTGAGACAG	GGGAGACCATTGAGTCCATC	201
Collagen, type III alpha1	*COL3A1*	NM_001098883	ATGGGGTCAAATGAAGGTGA	GTCCGCACGAATTCTTGAT	198
Transglutaminase 1	*TGM1*	NM_001010991	GGCAAGTTCCAGTTCACCAT	GTGGTCAAACTGGCCGTAGT	233
PR domain containing 1 with ZNF domain	*PRDM1*	XM_618588	CCACTGCCACAAGAGCTACA	CCACCAGAGGTCATGTCGATG	194
Acidic ribosomal protein PO	*RPLPO*	AF013214	CAACCCTGAAGTGCTTGACAT	AGGCAGATGGATCAGCCA	220

Table 2
List of proteins that were identified by isobaric tag for relative and absolute quantitation (iTRAQ) analysis to be up-regulated in high tick-resistant (HR) skin. Only proteins that have high tick-resistant/low tick-resistant (HR/LR) ratios ⩾1.5-fold increase are included.

Protein ID	Protein name	Function	Total scores	Unused scores	% Coverage	HR/LR ratio[a]	SD
NP_001008663	Keratin 5 (KRT5)	Structural (epidermis)	18.62	18.62	72.5	2.13	0.33
XP_880737	Keratin 14 (KRT14)	Structural (epidermis)	13.9	13.9	74	2.01	0.25
XP_610233	H2A histone	Structural (chromatin)	6	6	62.2	1.88	0.32
XP_581277	Lipocalin 9 (LCN9)	Lipid metabolic process	5.84	5.84	15.4	1.78	0.38
XP_875882	Keratin 33B (KRTHA 3B)	Structural (hair)	13.59	12.32	53	1.74	0.23

[a] HR/LR ratio corresponds to the relative abundance of a protein in HR and LR skin; the ratio is the mean of two biological replicates.

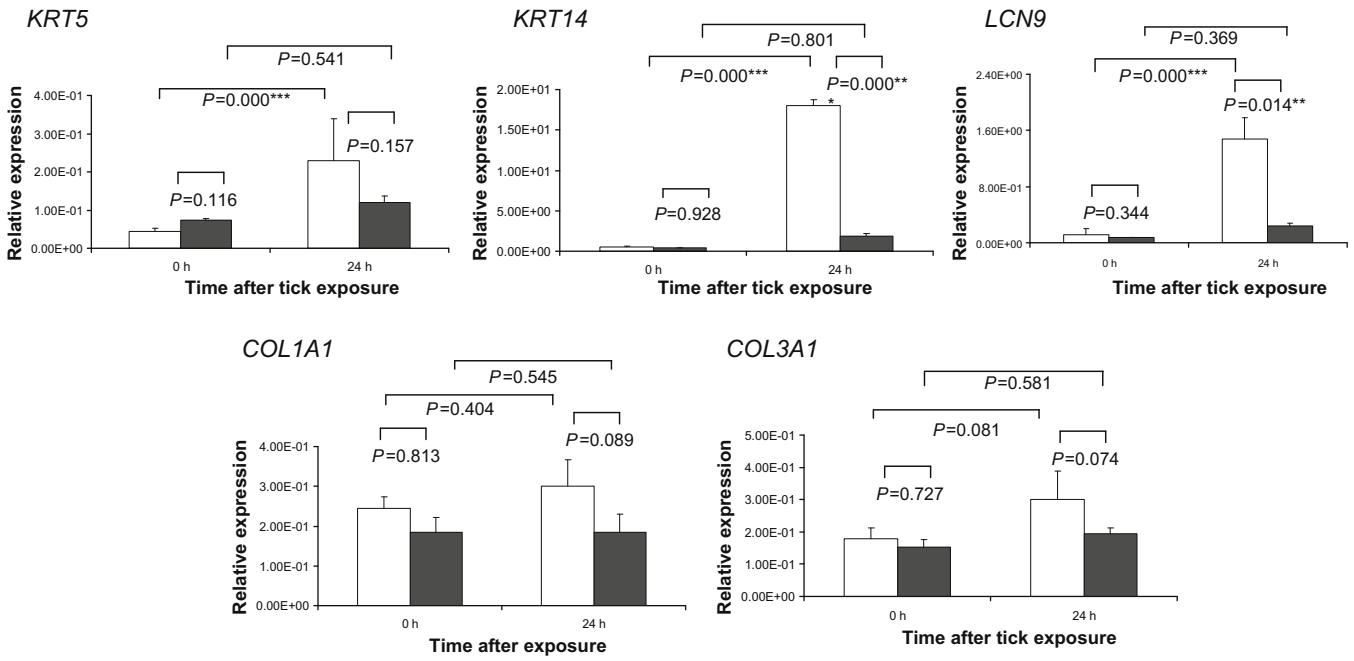

Fig. 2. Comparison of expression of keratin 5, 14 (*KRT5*, *KRT14*), lipocalin 9 (*LCN9*), collagen type 1 alpha1 (*COL1A1*) and collagen type 1 alpha 3 (*COL13A1*) mRNAs in skin of high tick-resistant (HR, white bars) and low tick-resistant (LR, black bars) cattle at 0 and 24 h post-infestation with *Rhipicephalus microplus*. Each bar indicates the mean normalised expression value (n = 10 for HR and n = 8 for LR) with SD from the group mean. Asterisks indicate significance level differences between animals groups, $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$.

course. Moreover, comparing mRNA expression levels over time indicated higher expression of *KRT5*, *KRT14* and *LCN9* at 24 h compared to 0 h in both groups, but the effect was much more pronounced in the HR group (Fig. 2), suggesting that the activation of gene expression may be associated with host resistance.

To investigate whether the up-regulation of specific keratin genes in the HR group following tick exposure was also applied to structural genes in the dermis, we analysed the mRNA expression of two collagen genes, *COL1A1* and *COL3A1* (Fig. 2). In contrast to the keratins, the genes encoding for these two collagens were not significantly up-regulated following tick exposure in either the HR or LR groups (Fig. 2). This is consistent with the earlier iTRAQ result which showed no differential expression of collagens (data not shown). Taken together, the mRNA expression patterns of the structural components of the epidermis, and not the dermis, were more responsive to tick exposure and therefore may have a greater role to play in host resistance.

3.3. Up-regulation of the genes encoding for the catalytic enzyme TGM1 and the transcriptional regulator Blimp1 in HR skin

To further investigate the role of epidermis in host resistance, we examined the expression of two key genes known to function in the establishment of the epidermal permeability barrier, transglutaminase 1 (*TGM1*) and *PRDM1*, the gene encoding for transcription factor Blimp1. Both these genes were found to be affected in mouse mutants with defects in epidermal barrier permeability (Segre, 2003; Magnusdottir et al., 2007). *TGM1*-knockout mice die post-natally, owing to a severe fluid loss, exhibiting dye permeability and a high level of trans-epidermal water loss (Kuramoto et al., 2002; Segre, 2003). Conditional epidermal deletion of *PRDM1* causes severe defects in terminal differentiation of epidermal keratinocytes and delayed stratum corneum (SC) formation and hyperkeratinisation (Magnusdottir et al., 2007). In this study, we were interested to see whether there was any difference in expression of *TGM1* and *PRDM1* genes between the HR and LR animals by assessing the relative mRNA levels of these genes in both HR and LR skin by qRT-PCR. As shown in Fig. 3, both genes showed significantly higher expression following tick exposure in HR animals, but not in the LR animals. The results suggest that the expression of *TGM1* and *PRDM1* was responsive to tick exposure and that the activation of their expression may be required for the development of tick resistance in the Belmont Red breed.

3.4. Corroboration of the gene expression results by Western blot analysis

To confirm the results obtained by qRT-PCR, we compared the protein expression levels of three differentially expressed genes-*KRT5*, *TGM1* and *PRDM1* in HR animals at 0 and 24 h post tick

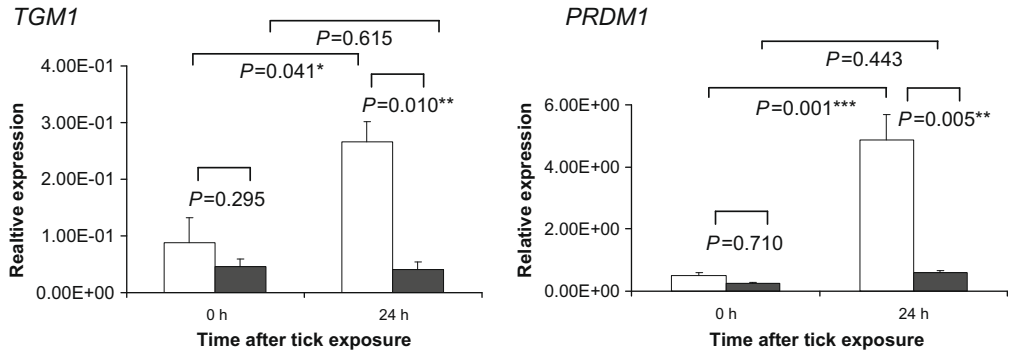

Fig. 3. Expression of transglutaminase 1 (*TGM1*) and Blimp1 (*PRDM1*) mRNAs in skin of high tick-resistant (HR, white bars) and low tick-resistant (LR, black bars) cattle at 0 and 24 h following tick infestation. Results are presented as mean normalised expression values with SD from the group mean. Asterisks indicate significance level differences between animals groups, $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$.

infestation by Western blot analysis. We focused on these three proteins because of the availability of antibodies to their human homologues that also recognise the bovine proteins. In addition, their critical role in the formation of the cornified epidermis and their expression in differentiating keratinocytes make it useful to investigate possible changes in these proteins at the time when host resistance occurs (Fig. 4). GAPDH, a commonly used housekeeping protein, was used as a control for sample loading and other experimental variations. Antibodies against KRT5 (62 kDa), Blimp1 (88 kDa) and GAPDH (36 kDa) enabled visualisation of bands of expected molecular weights in the samples obtained from five HR cattle at both 0 and 24 h time points (Fig. 4). In the case of TGM1, the Western blot detected a faint band at the expected mature protein size (93 kDa) as well as an additional band of ~65 kDa, which may be derived from the proteolysis of the mature TGM1 or may be a TGM1 isoform which is induced in the skin (Candi et al., 2005) (Fig. 4C). Whilst the signals obtained with the antibodies were clearly discernible in all samples and across five individual HR animals, the signals obtained at 24 h samples appeared to be significantly more intense. Gel band densities indicated a 1.8- to 8-fold increase in KRT5 protein levels and 1- to 3-fold increase in the 65 kDa TGM1 and 1.5- to 4-fold increase in Blimp1, respectively, for the 24 h skin samples compared with the five individual HR skin samples taken at 0 h (Fig. 4B, D and F). Although there was no significant augmentation in the levels of the ~100 kDa TGM1 band in the 24 h sample versus the 0 h sample, when the 105 and 65 kDa bands were taken together, a significant increase of up to threefold was observed in the 24 h sample (Fig. 4D). In contrast, GAPDH levels were not as clearly altered (Fig. 4G). Thus, Western blot analysis confirmed the preliminary results produced by iTRAQ analysis for KRT5. Indeed, the fold-change observed by iTRAQ (twofold) is consistent with 1- to 8-fold increase observed in Western blot allowing for animal to animal variation and the different time points at which samples were collected. The Western blot results also validated results obtained from qRT-PCR for *TGM1* and *PRDM1*. Similar Western blots performed on protein extracts from three LR cattle showed no apparent temporal increase in KRT5 and TGM1 protein expression levels (data not shown). Taken together, the results indicate differential expression of key components in the keratinocyte differentiation pathway between the HR and LR groups following tick exposure.

4. Discussion

A key observation of this study was the clear difference between the high and low resistant Belmont Red cattle in their expression of several components of the epidermal differentiation pathway during the attachment phase of cattle tick larvae. The losses of larvae from hosts of high and low resistance were considered as being very significant in the reduction of tick populations on the host, and earlier studies on *B. taurus* cattle showed that larval stages were the most susceptible to the resistance mechanism (Kemp et al., 1971; Roberts, 1971). The rapid stimulation of epidermal differentiation may provide one mechanism that could account for tick detachment. For example, the ability to rapidly restore the epidermal permeability barrier at the tick bite site of the more resistant hosts may result in the failure to establish a feeding lesion, causing stress to the tick and culminating in tick detachment with ramifications in tick survival rates. It is known that larvae on highly resistant hosts take longer to establish stabilised feeding (making several attempts), and that larvae in contact with bovine skin which are prevented from feeding will not survive more than 1 day (Roberts, 1971). Thus it seems that in cattle, a cornified epithelium mediates a broad set of protective "barrier" functions that includes water loss as well as defense against ectoparasite invasion.

Maintenance of a competent epidermal barrier in the face of external and internal stressors requires signals (presently poorly understood) between the uppermost layer of the skin: the SC interface and the underlying basal layer (Fig. 5). These signalling molecules may include c-myc, Notch and CCAAT-enhancer binding protein (C-ERB) and p63 (Dai and Segre, 2004; Aberdam et al., 2008; Romano et al., 2009). Consistent with the literature, our previous work comparing tick-resistant Brahman and tick-susceptible Holstein–Friesian cattle by microarray analysis showed significant (2.7-fold) increase in C-ERB expression in Brahman compared with Holstein at 24 h post tick challenge (Kongsuwan et al., 2008). In addition, it is known that calcium influx plays an essential role in this process. At higher calcium concentrations, the proliferation of keratinocytes is retarded and their differentiation is enhanced (Menon, 2002). Indeed calcium influx activates TGM enzymes to irreversibly cross-link the cornified envelope proteins, creating a tough, insoluble sac that surrounds the keratin fibres (Presland and Dale, 2000; Candi et al., 2005). Consistent with the critical role of calcium in keratinocyte differentiation, qRT-PCR data obtained from the same Belmont Red population used in this study demonstrated higher transcriptional activation of several calcium ion signalling genes in the HR group (Bagnall et al., 2009).

The SC, the matrix of keratinized cells surrounded by lipids (ceramides, cholesterol and free fatty acids), provides an indispensable barrier to the environment. The SC thickness in cattle is approximately 30 μm (Pitman and Rostas, 1981), and there is considerable variability in skin morphology among breeds, both in total skin thickness as well as in the thickness of the various layers (Pitman et al., 1983). In general, ticks have been reported to infest sites with thinner skins and shorter hair (e.g. Mattioli et al., 1997).

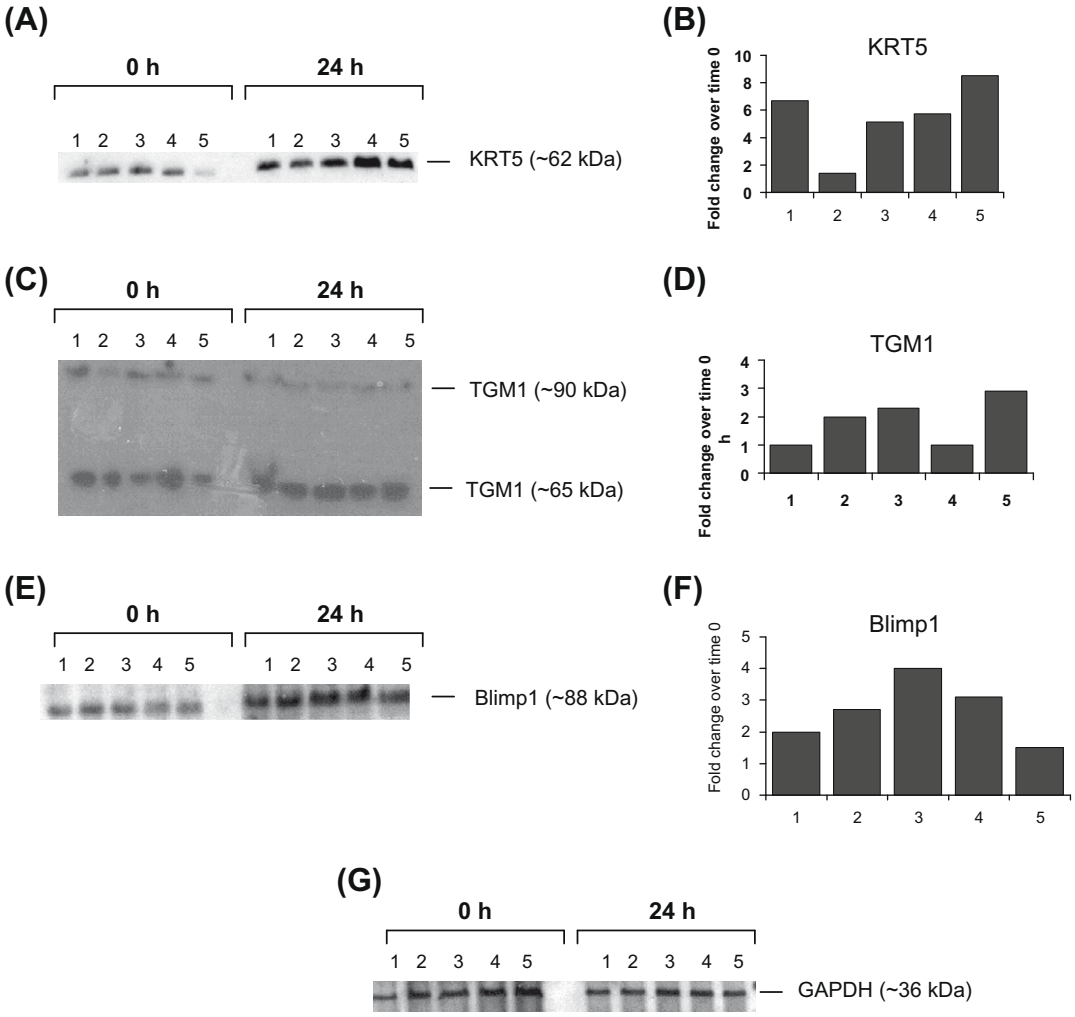

Fig. 4. Comparison of the expression of keratin 5 (KRT5), transglutaminase 1 (TGM1) and B lymphocyte-induced maturation protein 1 (Blimp1) proteins at 0 and 24 h time points. Western blot analyses of protein extracts from skin biopsies of five individual high tick-resistant cattle were performed with anti-human-cytokeratin 5, TGM1 and Blimp1-specific antibodies as described in Materials and methods. Total protein (20 μg loaded per lane) was separated on SDS–PAGE and Western blot was performed. Representative blots for KRT5 (A), TGM1 (C) and Blimp1 (E) with the respective blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (G) were shown. GAPDH, was used as a control for sample loading and other experimental variations. The number in bracket indicates the molecular mass of the detected protein. The protein expression levels of KRT5, TGM1, Blimp1 at 0 and 24 h were estimated by densitometry and normalised against GAPDH signals. The estimated expression at 24 h was expressed as fold-change over time 0 h values, and the results shown in the right-handed histograms (B, KRT5; D, TGM1 and F, Blimp1).

There are some reports using only a small number of animals that suggest the absence of any relationship between cattle tick numbers and total skin thickness (Wilkinson, 1962). However, thickness is not the only measure of physical resistance or toughness of mammalian skin. Another complicating factor is the contribution of lipids on the physical property of the SC. It is known that the permeability of the SC is determined by its lipid composition and organisation (Landmann, 1988). We have no evidence that there was any difference in the lipid composition between the HR and LR animals, but it is worth noting that the putative lipid processing and binding protein, LCN9, was activated in HR skin both at mRNA (Fig. 2) and protein (Table 2) levels. Perhaps this protein assists in the synthesis and processing of key lipids that are required for the formation of new lipid-enriched lamellar bodies (Fig. 5) that are essential for permeability barrier function. In addition, comparing tick-resistant Brahman with the tick-susceptible Holstein–Friesian cattle, we observed that at 24 h following tick exposure the mRNA expression of arachidonate-lipooxygenase 15 (ALOX15; Accession No. NM_001140) and fatty acid binding protein 4 (FABP4; Accession No. NM_001442) exhibited 8- and 4-fold increases, respectively, in Brahman cattle (Kongsuwan et al., 2008). The lipoxygenase (LOX) gene family is known to be critically involved in terminal differentiation of keratinocytes and adipocytes through the PPAR-mediated pathway. LOX inhibitors are known to block calcium induced expression of keratinocyte differentiation markers such as keratin 1 (Thuillier et al., 2002). Hence, there may be a concomitant increase in lipid metabolic gene activity and keratinocyte differentiation in HR skin of tropical cattle in response to parasite invasion.

Hair and wool can also be considered the first barrier to ectoparasite attachment. Cattle hairs are coated with an emulsion of sweat and sebum (oily substance produced by the sebaceous glands) and this emulsion may act as a barrier to prevent tick attachment. Interestingly, Blimp1, whose expression was also found in a small population of cells within the sebaceous gland in mice, was reported to be linked to the production of sebum (Horsley et al., 2006). Moreover, there is evidence that KRT33B, a hair keratin, is more abundant in HR animals (Table 2), which further suggests possible structural differences in hair and sweat glands may exist between HR and LR animals. Such differences may influence the relative humidity at the skin surface, an important factor in the survival of cattle tick larvae.

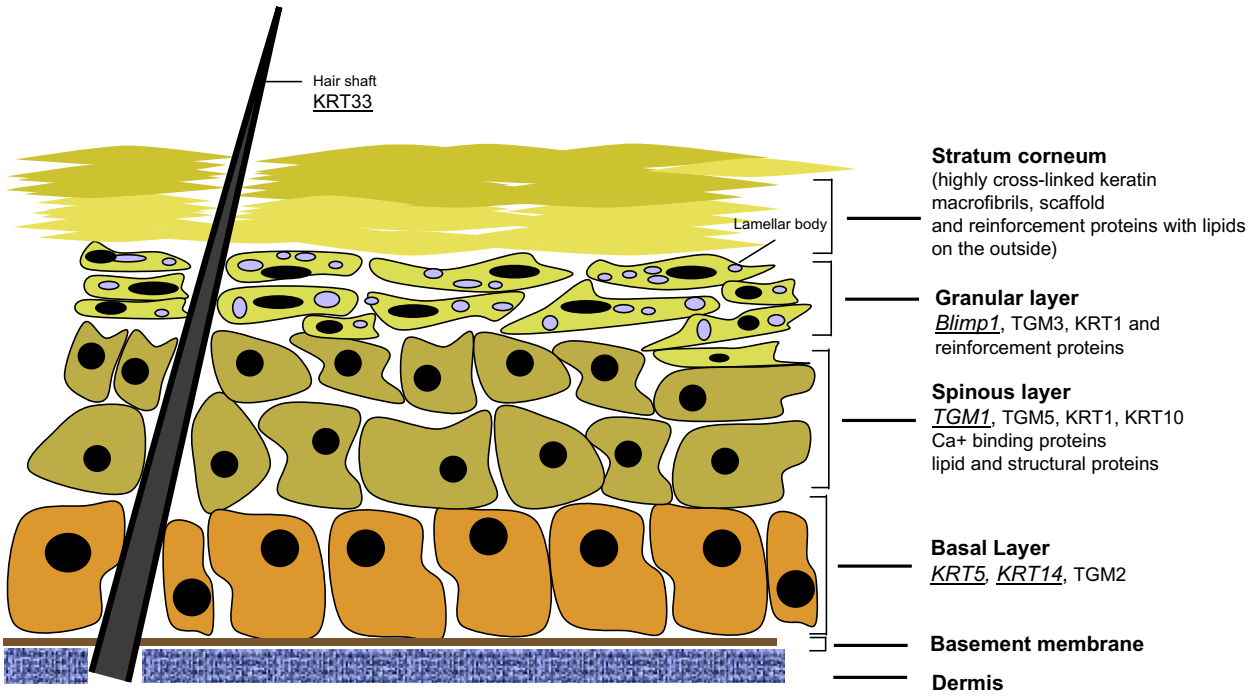

Fig. 5. Schematic illustration of skin epidermis structure showing the differentiation stages of keratinocytes and locations of components (proteins) that contribute to the epidermal barrier and where they are synthesised. Particular locations of various proteins during skin differentiation were obtained from Candi et al. (2005) and Magnusdottir et al. (2007). Epidermal differentiation begins with the migration of keratinocytes from the basal layer, and travel upward to the skin surface in a linear program of terminal differentiation. Spinous and granular layers are intermediate layers between the basal layer and the stratum corneum where the cells remain transcriptionally active, synthesising and assembling cornified envelope framework that provides specific physical properties. Underlines represent proteins that were shown to be up-regulated following tick exposure in the present study.

Molecular interactions taking place at the host–parasite interface will shift the balance between parasite infection and host defense. Host response to the tick bite involves haemostasis, inflammation, and both innate and acquired immunity (Wikel, 1996; Brossard and Wikel, 1997). The tick, too, has developed a unique means of avoiding the host animal's varying responses to the bite (Ramachandra and Wikel, 1992; Mulenga et al., 2007; Francischetti et al., 2009) including saliva rich in proteases, saliva proteins and allergens that activate protease-activated receptors and affect skin permeability, distribution of calcium ions and delay epidermal permeability barrier recovery (Jeong et al., 2008). Serine protease activity in the SC and outer epidermis can down-regulate lamellar body secretion and delay barrier repair (Hachem et al., 2006) and hence the presence of proteases in tick saliva may aid attachment. Because *R. microplus* is a one-host tick that requires sustained (3 weeks) contact with its host, it and the cattle host have evolved a dynamic "co-evolutionary arms race" over millions of years, which probably led to the evolution of robustness in gene signalling networks. It is also possible that such networks can evolve both redundancy and specific abilities allowing them to maintain sustained resistance. In addition, the host responses may differ markedly depending on whether it is early (finding suitable feeding site, penetrating and creating the feeding lesion) or late (following a prolonged feeding period) phase during the tick feeding cycle. Piper et al. (2008) studied changes in gene expression in two cattle breeds (Brahman and Holstein–Friesian) and found no difference in the expression of KRT6A and KRT6L between samples taken at tick attachment sites compared with samples taken from non-attachment sites. However, these samples were taken at days or even weeks following larval application (i.e. at sites where ticks were already attached). It is possible that the epidermal barrier response may not persist post successful tick attachment.

Finally, the genes studied in this work could be considered as candidates for traits associated with tick resistance and beef production and the single nucleotide polymorphisms (SNP) in these genes will help to assess the role of the genes in the genetic control of tick resistance. Using the interactive bovine in silico SNP release 5 (IBISS5) database, built by clustering publicly available bovine expressed sequence tags (EST) and mRNA sequences (Hawken et al., 2004), several putative cis-acting SNPs were detected in 3′-untranslated regions of both *TGM1* and *PRDM1* (data not shown). Interestingly, the 5′-flanking region of the human *TGM1* gene contained potential regulatory motifs, including elements found in other keratinocyte-differentiation genes such as keratin 1. Several candidates (transcription factors) exist that may regulate *TGM1* and other genes involved in terminal keratinocyte differentiation pathway (La Celle and Polakowska, 2001; Romano et al., 2009). The effect of skin barrier function on tick attachment may have utility for anti-tick vaccine design as this pathway is amenable to modulation. A new generation of adjuvants that can modulate the calcium/potassium ions profile (as these ions are known as second messengers effecting barrier homeostasis (Denda, 2000)) or stimulate lipid synthesis in differentiating keratinocytes may be capable of increasing a more robust anti-tick response. As skin barrier provides the first defense against cattle tick larvae, the two-pronged approach to development of anti-tick vaccines which enhances skin barrier function and a recombinant protein aimed at disrupting tick feeding may be more effective than target tick feeding alone.

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