Research paper

Living with the enemy or uninvited guests: Functional genomics approaches to investigating host resistance or tolerance traits to a protozoan parasite, *Theileria annulata*, in cattle

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ABSTRACT

Many breeds of cattle with long histories of living in areas of endemic disease have evolved mechanisms that enable them to co-exist with specific pathogens. Understanding the genes that control tolerance and resistance could provide new strategies to improve the health and welfare of livestock. Around one sixth of the world cattle population is estimated to be at risk from one of the most debilitating tick-borne diseases of cattle, caused by the protozoan parasite, *Theileria annulata*. The parasite mainly infects cells of the myeloid lineage which are also the main producers of inflammatory cytokines. If an infectious or inflammatory insult is sufficiently great, inflammatory cytokines produced by macrophages enter the circulation and induce an acute phase proteins (APP) response. The *Bos taurus* Holstein breed produces higher and more prolonged levels of inflammatory cytokine induced APP than the *Bos indicus* Sahiwal breed in response to experimental infection with *T. annulata*. The Sahiwal exhibits significantly less pathology and survives infection, unlike the Holstein breed. Therefore, we hypothesised that the causal genes were likely to be expressed in macrophages and control the production of inflammatory cytokines. A functional genomics approach revealed that the transcriptome profile of the *B. taurus* macrophages was more associated with an inflammatory programme than the *B. indicus* macrophages. In particular the most differentially expressed gene was a member of the signal regulatory protein (SIRP) family. These are mainly expressed on myeloid cell surfaces and control inflammatory responses. Other differentially expressed genes included bovine major histocompatibility complex (MHC) (BoLA) class II genes, particularly BoLA DQ, and transforming growth factor (TGF)B2. We are now exploring whether sequence and functional differences in the bovine SIRP family may underlie the resistance or tolerance to *T. annulata* between the breeds. Potentially, our research may also have more general implications for the control of inflammatory processes against other pathogens. Genes controlling the balance between pathology and protection may determine how livestock can survive in the face of infectious onslaught. Next generation sequencing and RNAi methodologies for livestock species will bring new opportunities to link diversity at the genome level to functional differences in health traits in livestock species.

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Despite the many advances over the last few decades in ameliorating the impact of infectious pathogens on livestock species, the control of infectious disease still remains one of the most important global priorities for effective and efficient livestock management. This is true even where there is adoption of good management practices and increasing availability of vaccines and other therapeutics. Breeding for resistance or tolerance to

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endemic and non-endemic pathogens could provide an additional and complementary approach that would be sustainable in the long term and could lead to the use of animals that were beneficial to the environment as well as avoid the introduction of potential contaminants into the environment. However, there are many hurdles to be overcome if this solution is to become feasible. In this review, we explore some of these issues in relation to one of the major tick borne pathogens of cattle, *Theileria annulata* which is endemic in many countries from North Africa to China. It is also found in Southern Europe. We focus on a comparison of a cattle breed which is likely to have co-evolved with the pathogen, with a breed which has not previously encountered *T. annulata*, and discuss how greater knowledge of the genetic differences between breeds could inform new control strategies. However, the ideas and approaches are likely to be of relevance to other host–pathogen interactions in relation to adapted and non-adapted species and breeds. Thus indigenous breeds of livestock across the globe may harbour many beneficial gene variants for disease resistance or tolerance. For example rare but ancient breeds of sheep are clearly resilient to nematode infections *i.e.* they survive and thrive despite the presence of parasite worms (Piedrafita et al., 2010). However for the main part, they do not possess production traits attractive to breeders and farmers. Discovering ways to combine both types of trait could generate new breeds fit for the challenges of the 21st century.

1. Co-evolution of hosts and pathogens

Over prolonged periods of time, hosts and pathogens tend to become co-adapted, enabling both types of population to survive and reproduce in the presence of the other. This is often referred to as a co-evolutionary arms race (Durand and Coetzer, 2008). Following first encounter with a pathogen, the host population evolves new defence mechanisms against the pathogen, leading to improved disease resistance or tolerance. This in turn results in the pathogen developing ways to overcome these defences. Thus, the host populations undergo corresponding waves of fitness levels (in terms of reproductive success) across time (Durand and Coetzer, 2008). This is because defence against prevailing micro-organisms is believed to have a cost in terms of fitness, although the evidence is equivocal. The host may make a compromise between making a strong immune response, leading to pathology, and making a weak immune response leading to repeated or chronic infections in the host. The result may be a trade-off or balancing act between fitness and host defence traits (Sheldon and Verhulst, 1996; Romanyukha et al., 2006; van der Most et al., 2011). Evidence of this can potentially be seen in the host genome, with changes in the frequency of genetic variants that confer greater fitness in the host populations. These genes may also display signatures of positive (advantageous) selection (Barreiro and Quintana-Murci, 2010).

Infection with non-endemic micro-organisms often results in pathology and even fatality in non-adapted species and breeds. In many cases, the response is unregulated inflammation associated with a cytokine storm.

For example, the viral pathogens including severe acute respiratory syndrome (SARS) coronavirus, influenza and Ebola can all cause devastating consequences in humans (Nagata et al., 2010; Beigel et al., 2005; Wauquier et al., 2010, respectively), yet not in their wild-life reservoir hosts (Graham and Baric, 2010; Kalthoff et al., 2010; Calisher et al., 2006, respectively). In livestock populations, African swine fever is a current threat to farmed pig populations yet is apparently benign in warthogs (Costard et al., 2009). The two main viruses causing malignant catarrhal fever are a serious problem in cattle, yet the closely related ruminant species, sheep and wildebeest, respond to infection with remarkably little effect (Russell et al., 2009). All of these pathogens induce similar clinical signs including fever, anaemia, inappetence, and cachexia and are often fatal, within a few days of infection. These clinical signs in the non-adapted hosts are similar to those induced by high levels of pro-inflammatory cytokines, akin to a cytokine storm (Zhang et al., 2004; de Jong et al., 2006; Wauquier et al., 2010; Gomez Del Moral et al., 1999). Similar clinical signs are seen in cattle in response to the tick-borne protozoan parasite, *T. annulata*. This pathogen induces a severe inflammatory response, and ultimately high levels of fatality in susceptible *Bos taurus* cattle (Glass et al., 2003), yet in the *Bos indicus* breed, the Sahiwal, which originates in a *T. annulata* endemic region, the inflammatory response is controlled and the breed survives (Glass et al., 2005). It is believed that *T. annulata* probably originated in Asian water buffalo (*Bubalus bubalis*) which in contrast to cattle, generally have milder clinical signs (Robinson, 1982).

These milder host responses to endemic pathogens are likely to reflect a long history of co-adaptation. Perhaps the best studied example is that of human populations exposed to the protozoan parasites of *Plasmodium* spp. which cause malaria. These populations have higher frequencies of haemoglobin and other red cell related polymorphisms than non-exposed populations (Durand and Coetzer, 2008). It appears that malaria has a very strong selective pressure on the human genome (Quintana-Murci and Barreiro, 2010). Amongst the better known examples of haemoglobin mutations there is evidence that gene variants related to host immune defence are also associated with malaria resistance (Kwiatkowski, 2005). Given the observation that non-adapted host species often respond to newly emerging pathogens by inappropriate over-stimulation of inflammation, it is possible that genes regulating such processes would be under strong selection pressure (Fig. 1). Interestingly, in a survey of human SNPs associated with immune-related phenotypes, Barreiro and Quintana-Murci (2010) found evidence of recent positive selection (*i.e.* apparently advantageous) in genes associated with autoimmune or inflammatory disease. They suggest that this may have arisen because in the past, such genes may have conferred greater immunity in human populations from prevailing pathogens. It is possible that the milder reactions to *T. annulata* infection by water-buffalo (Robinson, 1982) and *B. indicus* (Glass et al., 2005) may reflect a longer association between these host and pathogen populations, compared to *B. taurus*. Interestingly, *Theileria lestoquardi* is highly pathogenic in many sheep and goat breeds, and likely evolved relatively recently from a
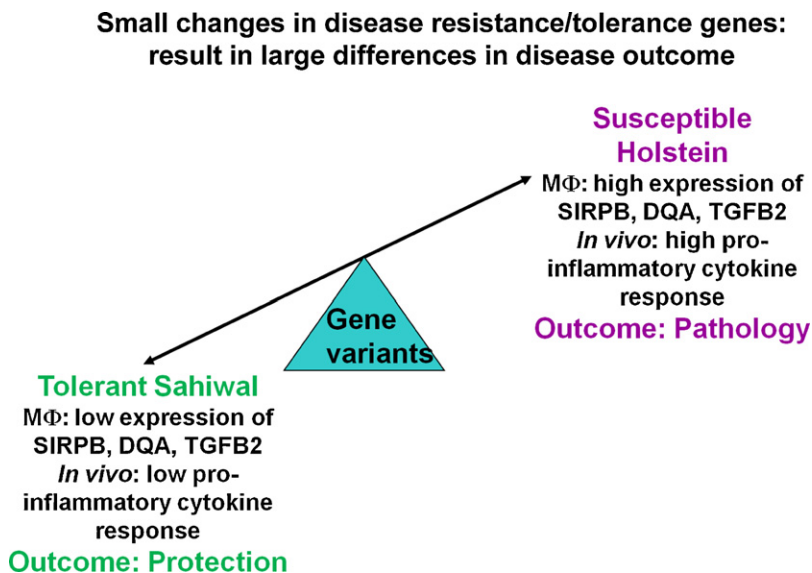
Small changes in disease resistance/tolerance genes: result in large differences in disease outcome

Fig. 1. Schematic diagram illustrating the role of gene variants controlling resistance or tolerance traits, and determining the balance between pathology and protection in response to *T. annulata*. Holstein MΦ express high levels whereas Sahiwals express low levels of genes such as SIRPB, DQA and TGFB2. The gene variants that regulate the expression of these genes are currently unknown. See text for more details. The expression differences in these genes leads to differences in the production or induction of pro-inflammatory cytokines, which in turn results in distinct outcomes.

common ancestor with *T. annulata*, at least as far as can be ascertained from sequence comparisons analysed to date (Katzer et al., 1998).

2. Disease resistance and tolerance

Co-evolution of hosts and parasites is dependent on background mutations with natural selection of gene variants favourable to the propagation of the species. Thus, the frequency of "resistance or tolerance" conferring genes in any population would reflect its previous history of infections. It follows that essentially all host populations will harbour a variety of polymorphic genes some of which must underlie the considerable variation observed among individuals in their response to pathogens. It is not our intention to provide a comprehensive overview of the literature on disease resistance *per se* as this has been extensively discussed elsewhere (for recent reviews see: humans (Vannberg et al., 2011), model organisms (Gruenheid and Gros, 2010) and livestock species (Davies et al., 2009) including cattle (Glass et al., 2012). The underlying genetics of disease resistance or tolerance traits are likely to be complex with many genes of small to moderate effect contributing to the observed phenotype of the host, rather than single genes of large effect (Glass, 2004; Glass and Jensen, 2007). Identifying relevant gene variants that determine resistance or tolerance could ultimately lead to selectable markers for breeding more resistant or tolerant cattle. Markers for genes conferring resistance could enable these genes to be specifically bred (introgression) into more productive livestock, as suggested by Hanotte et al. (2003) for trypanotolerance traits in cattle. Furthermore, discovery of such gene variants may also identify novel pathways and mechanisms that control protection and/or pathology of

relevance for vaccine and therapeutic development (Glass, 2001, 2004; Gruenheid and Gros, 2010; Jarosikova, 2011).

However, identifying the genes underlying variation in responses to pathogens is not straightforward as in general, relevant phenotypic data linked to genotypes are required from large numbers of individuals. Apart from complex genetics, the logistics and cost entailed, the most relevant phenotypes (correlates of protection) that can be readily measured in large numbers of animals are often unclear (Glass et al., 2012). Furthermore, the level of protection reflects the final outcome of a cascade of complex interactions that are initiated and most likely shaped by the innate immune response. Since the genes expressed at this early stage of an infection are germ-line encoded, genetic resistance is likely to be determined by polymorphisms of innate immune genes (Lazarus et al., 2002).

In addition, it has been suggested that tolerance *i.e.* maintenance of "fitness" of the host in the face of pathogen challenge may be controlled by different genes than those that control resistance *i.e.* prevention of infection or control of pathogen levels and fitness. Indeed these genes could potentially be antagonistic (Raberg et al., 2007; Schneider and Ayres, 2008). It is difficult to measure resistance *versus* tolerance traits in large animals such as cattle. However the fact that the *B. indicus* breed becomes infected and then is able to control the pro-inflammatory cytokine dependent acute phase response to *T. annulata* (Glass et al., 2005) might suggest that the underlying trait is related more to tolerance than resistance. Studies on growth and production following challenge in both breeds would provide more evidence of the nature of the trait. Carrier Holstein cattle have been reported to have significantly lower milk yield than non-carrier cattle (Gharbi et al., 2006), but no breed comparisons have been conducted so far. Importantly, given the negative correlation between the presence

of systemic pro-inflammatory cytokines, pathology and production traits in many domestic species (Johnson, 1997; Eckersall, 2000; Colditz, 2002; Clapperton et al., 2005; O'Boyle et al., 2006; Clapperton et al., 2009; van der Most et al., 2011), it would seem likely that genes that control the balance of cytokines induced during infection would be tolerance traits (Fig. 1).

3. Role of pro-inflammatory cytokines and macrophages in pathology induced by *T. annulata*

The parasite, *T. annulata*, exists in a number of distinct life-stages which are adapted to dwelling in its two hosts, ruminants and ticks (mainly *Hyalomma anatolicum anatolicum*) (Bishop et al., 2009). The sporozoite stage develops in the tick salivary gland and is injected into the ruminant host, where it invades host macrophages (MΦ) in the draining lymph nodes (DNL) of the tick bite sites (Campbell et al., 1995). *T. annulata* then differentiates into the macroschizont stage and this intracellular stage induces host cell proliferation both *in vivo* and *in vitro*, a feature it shares with some of its close relatives, *Theileria parva* and *T. lestoquardi*, although not with other *Theileria spp* or other species of *Apicomplexa* (Bishop et al., 2009). Although the mechanisms by which these parasites commandeer host cell division is not clear (Shiels et al., 2006), several signalling pathways involved in cell proliferation, apoptosis and inflammation such as NK-κB are constitutively activated (reviewed in Dobbelaere and Rottenberg, 2003). The sporozoite stage of all three *Theileria* species also induce severe pathology in susceptible hosts, with clinical signs similar to the cytokine storms induced by the pathogens discussed above. During the differentiation process in the DLN to the macroschizont stage, the parasite appears essentially hidden from the host immune system. However the exit of infected cells around day 7 following infection (Nichani et al., 1999), is associated with a rise in body temperature and development of anaemia and cachexia. Infected cells are disseminated around the animal and foci of proliferating cells appear in lymphoid and non-lymphoid organs, which are associated with tissue damage (Forsyth et al., 1999; Preston et al., 1999).

However, it is possible to induce protection to these three Theileria species. In the case of *T. annulata* and the closely related sheep parasite *T. lestoquardi* attenuated cell line vaccines produced by prolonged tissue culture of infected macrophage lines are protective (Ali et al., 2008; Darghouth, 2008). Attenuation is associated with reduced expression of pro-inflammatory cytokines as well as the matrix metalloproteinase MMP-9 (Graham et al., 2001; Ali et al., 2008), which are linked to the invasive capacity of these cell lines (Adamson and Hall, 1998; Forsyth et al., 1999).

In the case of *T. annulata* infection of a susceptible *B. taurus* breed, the Holstein, the intra-MΦ macroschizont stage is associated with a temperature rise, leukopenia, cachexia, and anaemia, both temporally and in terms of severity (reviewed in Glass and Jensen, 2007). Levels of acute phase proteins (APP) are elevated and correlate with the degree of disease severity and clinical outcome (Glass et al., 2003). APP only appear in the blood in response

to systemic release of pro-inflammatory cytokines, which are associated with and generally derived from infected and activated MΦ (Baumann and Gauldie, 1994). We have shown that the parasite up-regulates gene expression of interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNFα) in infected MΦ (McGuire et al., 2004) and the presence of TNFα protein in infected cells has been detected *in vivo* (Forsyth et al., 1999) and is secreted in its active form, at least *in vitro* (Guergnon et al., 2003). Furthermore infected cell line clones selected on the basis of pro-inflammatory cytokine mRNA level induced significantly distinct responses *in vivo*. Greater pathology was seen in animals immunised with clones expressing high mRNA levels compared to those immunised with low mRNA level clones (Graham et al., 2001). Thus, it seems likely that infected cells are the main producers of pro-inflammatory cytokines, although it is possible that other cell types are an additional source of these mediators. Parasite infected cells induce a contact and soluble factor dependent aberrant polyclonal T cell proliferation and activation both *in vitro* and *in vivo* (Campbell et al., 1995, 1997). The soluble factor may be IL-1α, as mRNA levels of this pro-inflammatory cytokine in parasite infected cells correlates with the level of proliferation induced in naïve T cells (Brown et al., 1995). It is known that these aberrantly activated T cells produce high levels of interferon γ (IFNγ) (Campbell et al., 1997; Nichani et al., 1999). It is possible that these T cells are also producing pro-inflammatory cytokines. Potentially a positive feedback loop may then ensue between infected cells and these T cells, leading to a cascade of pro-inflammatory cytokines.

In contrast to the pathological sequelae associated with infected MΦ, it is likely that innate immunity involving MΦ plays a role in protection against *T. annulata* (Preston et al., 1999; Ahmed et al., 2008). An earlier study had also indicated that stimulation of the innate immune system provided short-term protection against *T. annulata* infection, with evidence that this may have been mediated by MΦ (Manickam et al., 1983).

MΦ are part of the innate immune system and exhibit a broad range of different phenotypes, indicative of their great functional plasticity, which is dependent on their microenvironment. They have major functions in patrolling the host, sensing invasion by micro-organisms and danger signals induced by inflammation (Plueddemann et al., 2011), and as effector cells with major roles in phagocytosis, scavenging and cytotoxicity (Gordon and Taylor, 2005; Auffray et al., 2009). They also interact with other cells of the immune system by producing regulatory factors, chemokines and pro-inflammatory cytokines (Parameswaran and Patial, 2010; Valledor et al., 2010). MΦ link to the adaptive immune system through antigen presentation and regulation (Hume, 2008). They receive adaptive immune signals that modulate their function, for example IFNγ up-regulates their effector mechanisms and secretion of pro-inflammatory cytokines (Murray and Wynn, 2011). MΦ are also involved in immunosuppression, wound healing and tissue repair (Pastula and Marcinkiewicz, 2011; Brancato and Albina, 2011).

Infection with *T. annulata in vivo*, induces suppressor or regulatory MΦ which are capable of inhibiting both

the proliferation of *T. annulata* infected cells (Preston and Brown, 1988) as well as the polyclonally activated T cells which are induced by infected cells (Preston et al., 2002). It is not clear what the mechanisms for such inhibition are, although at least a component appears to involve indomethicin sensitive prostaglandin (Preston et al., 2002). Nor is it clear if these ill-defined MΦ populations play protective or pathogenic roles during infection with *T. annulata* parasite. Interestingly, in Holsteins infected with *T. annulata*, the presence of MΦ capable of inhibiting T cell proliferation was more pronounced and prolonged than in the Sahiwals infected with *T. annulata* (Preston et al., 2002). Furthermore, Holstein MΦ produced both indomethicin sensitive and non-sensitive suppressive factors whereas Sahiwal MΦ only produced soluble indomethicin sensitive factors. The nature of the additional Holstein-derived factor is unclear. The effects on T cells by these MΦ populations have some similarity to myeloid-derived suppressor cells (MDSC) which are immunoregulatory in both adaptive and innate immunity (Pastula and Marcinkiewicz, 2011). MDSC appear to be associated with chronic inflammation such as that induced by superantigen mediated polyclonal T cell activation (Cauley et al., 2000) and cancer (Talmadge, 2007). Chronic inflammation, T cell dysregulation and (reversible) transformation are also features of *T. annulata* pathogenesis. Thus it appears that the innate immune response and MΦ in particular play a role in both protection and pathology in *T. annulata* infection. In addition, it seems likely that control of the level of pro-inflammatory cytokines produced or induced by MΦ is the key to the breed differences in disease susceptibility (Fig. 1). There is a need to understand how to balance these processes which could inform new and complementary strategies to control infection. Identifying the early host–pathogen interactions that determine breed differences in resistance and susceptibility, could ultimately lead to more effective ways to manipulate responses to the benefit of the host, either through breeding for resistance or developing superior vaccines.

4. Manipulation of the host transcriptome

By targeting MΦ, where it resides in the cytoplasm, *T. annulata* is able to orchestrate changes in the host transcriptome (Jensen et al., 2008). By manipulating gene expression, *T. annulata* is able to subvert the host immune response, leading to its survival and proliferation.

In particular, macrophage transcription factors are down-regulated including the "master" macrophage transcription factor, proviral integration oncogene spi1 or Pu.1 (Jensen et al., 2009). Pu.1 appears to act on the genome as a macrophage lineage commitment and maintenance regulator of gene expression (Heinz et al., 2010; Natoli, 2010). This, suggests that by targeting this transcription factor, the parasite may also fundamentally change the macrophage epigenome. In addition in the same study, infection with *T. annulata* also reduced levels of Musculoaponeurotic fibrosarcoma oncogene (MAF) transcription factors, MafB and c-MAF (Jensen et al., 2009), which together control macrophage proliferation (Aziz et al., 2009). It is possible that regulation of these genes may be a major mechanism

by which the parasite induces proliferation of the host cell. Furthermore, these alterations in transcription factor levels may then lead directly to the phenotypic and functional changes in infected cells, including up-regulation of pro-inflammatory cytokines (McGuire et al., 2004) and down-regulation of cell surface molecules, CCR1, chemokine (C–C motif) receptor 1 and integrin B7 (ITGB7). Infection of MΦ is also accompanied by loss of markers of mature macrophages as well as effector function and the cells appear to enter a dedifferentiated state (Sager et al., 1999).

Unlike the *B. taurus* susceptible breeds, in the *B. indicus* breed, the Sahiwal, the host undergoes a significantly shorter and less severe fever response, rapidly controls the schizont stage, limits the production APPs and returns to a normal phenotype (Glass et al., 2005). This suggested that the *B. indicus* and *B. taurus* breeds differed in their ability to control inflammatory responses to *T. annulata*. However, infected MΦ cell lines derived from both breeds had no difference in their mRNA levels of the pro-inflammatory cytokines, IL-1, IL-6 and TNFα (McGuire et al., 2004). Although this seems contradictory, we reasoned that there must be differences between the breeds *in vivo* that were not reflected in the *in vitro* cell lines. This would suggest that interactions between infected cells and other immune cells and/or mediators are the key to the breed differences. There are a number of possible scenarios. Both IL-1 and TNFα undergo post-translational modification in order for them to be secreted as active cytokines and their activities are regulated by many different receptors and other molecules (Levine, 2008). In addition other cell types such as the abnormal T cells described above may play a significant role. It is of interest to note that the differences between breeds only becomes apparent around day 8, following infection with *T. annulata* sporozoites (Glass et al., 2005). This is after the parasite develops into the macroschizont stage and the aberrantly activated T cells start to appear in the DLN (Campbell et al., 1997). Around that time the proliferating infected and non-infected cells also leave the DLN (Nichani et al., 1999). In addition, if additional stimuli present *in vivo* but not *in vitro* are necessary for release of pro-inflammatory cytokines, this could also explain the correlation between cytokine gene expression in the *in vitro* clones and pathology seen *in vivo* (Graham et al., 2001).

Nonetheless it is possible that pro-inflammatory cytokine gene expression in infected MΦ is irrelevant to the breed differences. However, our evidence to date would still support the hypothesis that regulation of gene expression in MΦ is the key to the differences in resistance and susceptibility. In order to explore the apparently contradictory results between the pro-inflammatory cytokine transcript levels in *T. annulata* infected MΦ, and the acute phase response to *in vivo* infection, we decided to take an unbiased global view of MΦ gene expression, with the aim of identifying underlying gene variants that might underpin the differences in the breeds' responses. First, we created a MΦ specific cDNA microarray (Jensen et al., 2006). We used this array to identify gene expression differences between MΦ (both uninfected and infected) from both breeds (Jensen et al., 2008). Among the statistically significant gene expression differences identified, 89 genes

Table 1

Top fold difference in gene expression in uninfected MΦ from the Holstein (H) and Sahiwal (S) breed as measured by transcriptome analysis with a MΦ enriched cDNA microarray (FDR < 0.05).

Gene name[a]	H > S[b] fold difference	Gene name	S > H fold difference
SIRPB1[c]	25	AOX1[c]	9
A2M	8	FN1[c]	7
CXCL3	8	LOC339745	4
LTB4DH[c]	7	Unique 1[c,d]	3
CD9	7	PPARBP	3
CCL4L2	6	LRRK2	3
EGR2	5	CR2	3
Unknown 1[e]	5	GPR155[c]	3
DQA[c]	5	Unique 2[f]	3
C1R	4	FCAR	2
TNFAIP3	4	FOLR2	2

[a] HUGO nomenclature where possible;.

[b] H > S refers to the fold difference in expression of the gene, where Holstein (H) MΦ expression is greater than Sahiwal (S) expression as measured by microarray analysis (False Discovery Rate (FDR) ≤ 0.05) (Jensen et al., 2008), and confirmed by qRT-PCR.

[c] These genes maintained their differential expression over 72 h following infection with *T. annulata* and in response to Lipopolysaccharide and IFNγ (FDR ≤ 0.05).

[d] Unique 1 = Accession number: AJ817886.

[e] Unique 2 = Accession number: AJ819634.

[f] Unknown 1 = Accession number: AJ819256.

were expressed at higher levels in uninfected MΦ from the Holstein breed compared to the Sahiwal breed. Of these 26 were expressed at more than two-fold higher level in Holstein MΦ (False Discovery Rate (FDR) < 0.05). Uninfected Sahiwal MΦ expressed 51 genes at significantly higher levels in uninfected MΦ compared to the MΦ from the Holstein breed. This included 15 distinct transcripts expressed at more than two-fold higher in Sahiwal MΦ (FDR < 0.05). In terms of functional categories, nine of the 11 top genes which were expressed at higher level in the Holstein MΦ were either related to inflammation or immune response whereas this bias was not seen in the Sahiwal MΦ (Table 1) (Jensen et al., 2008). Furthermore, several of these genes maintained their differential expression following infection with *T. annulata*. This suggests that MΦ from the two breeds may have unique signatures of expression, and the Holstein MΦ may be particularly responsive to inflammatory signals. It would thus appear that these differences are inherent and not related to responses to specific pathogens. Thus, it is possible that MΦ from each breed may in general respond differently to other pathogens. Although there is currently no direct evidence for this, these breed difference patterns were maintained in response to the bacterial ligand lipopolysaccharide (LPS), a TLR4 agonist, and IFNγ (Jensen et al., 2008).

In addition to these intrinsic differences between the breeds, there were also differences in their responses to *T. annulata*, particularly at an early time point (2 h post infection) (Jensen et al., 2008). This suggests that the inherent transcriptome differences in Holstein and Sahiwal MΦ may be fundamentally important for early host–pathogen interactions. One particularly interesting gene was the pattern recognition receptor toll-like receptor (TLR)10. This was up-regulated in Sahiwal MΦ more than 8-fold higher than in Holstein MΦ following infection with *T.*

annulata. LPS and IFNγ also induced greater up-regulation of TLR10 in Sahiwal MΦ. TLR10 is present in the human and bovine genomes but not the mouse genome (McGuire et al., 2006). It is highly expressed in immune cells, and it has recently been suggested that it can form heterodimers with TLR1 or TLR2 as well as homodimers. These molecules may recognise lipopeptides, although their biologically relevant ligands remain unknown (Govindaraj et al., 2010) and may not signal through the usual TLR pathways *via* NFkB with induction of pro-inflammatory cytokines (Guan et al., 2010). In fact although many PRRs have been identified for bacteria and viruses, very few have been discovered that interact with protozoan derived ligands.

Interestingly many of the transcripts associated with these breed differences remain unannotated or are unique to the MΦ library used to generate the microarray (Jensen et al., 2008), despite the more recent publication of the bovine genome (The Bovine Genome Sequencing Consortium, 2009). It is to be hoped that further annotation, especially of immune-related genes and transcripts, will result in better understanding of the interaction between *T. annulata* and bovine MΦ, as well as responses to pathogens in general.

Although the MΦ microarray was enriched for clones representing transcripts from stimulated and infected MΦ, it is now clear that many relevant transcripts are missing from this array, including some of the transcription factors described above. With the advent of next generation sequencing for RNA transcripts (Mortazavi et al., 2008), together with genome sequences, some of the limitations of microarrays for livestock species will be overcome. Furthermore, additional and functionally important information can be obtained through RNA-seq technologies including alternative splicing, gene and exon boundary mapping and even novel transcript discovery. For example, it would be interesting to see if MΦ transcription factors are differentially expressed. We mainly concentrated on early time points following MΦ infection with *T. annulata* (at 2 and 72 h post-infection *in vitro*) and it will be important to explore the functional consequences of the breed differences in gene expression. In the next sections, some of these breed differences in MΦ gene expression are explored in more detail, in particular, genes that may play a role in determining the cytokine levels produced or induced by MΦ: Signal Regulatory Protein (SIRP)B, BoLA DQA and Transforming Growth Factor (TGF)B2 (Fig. 1).

5. Signal regulatory proteins (SIRPs)

The most differentially expressed gene between the breeds was originally identified as a transcript variant of signal regulatory protein alpha, SIRPA (Jensen et al., 2008). However, since the paper was published, further analysis and more recent annotation of the transcript in the bovine genome (Btau4.0) have identified it as SIRPB1. SIRPs are a family of glycoproteins, mainly expressed on immune cells, particularly those of the myeloid lineage, and neuronal cells. The majority of SIRPs are cell surface receptors with three similar extracellular immunoglobulin like domains (Barclay and Brown, 2006). They have variable transmembrane and cytoplasmic domains and regulate inflammatory

responses (Barclay and Brown, 2006). SIRPA, also known as macrophage fusion receptor, CD172a or SHPS-1, consists of 3 extracellular domains and a long intracellular tail which contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). SIRPA recognises a self-ligand, CD47, which is ubiquitously expressed, and their interaction results in an inhibitory signal which inhibits phagocytosis and TNFα production (Barclay, 2009). Cattle were one of the first species for which SIRPA was described (Brooke et al., 1998). SIRPA is also reported to be a marker for rat (OX41) (Yrlid and MacPherson, 2003), bovine (Hope et al., 2001) and ovine (Contreras et al., 2010) afferent dendritic cell subsets which are associated with high antigen presenting capacity. In cattle afferent lymph SIRPA+ DC also secrete IL-1 in response to stimulation (Hope et al., 2001). There are several SIRPB molecules including SIRPB1, SIRB2 and SIRPB3 which differ from SIRPA by having only short cytoplasmic tails and positively charged transmembrane amino acids. These interact with DAP12 and/or possibly other adapters (Liu et al., 2005) to initiate a positive signal for phagocytosis and inflammation (Hayashi et al., 2004). Although SIRPBs appear to have similar extracellular domains to SIRPA, they do not appear to interact with CD47 and their ligands are unknown (Barclay and Hatherley, 2008). Dendritic cells have been reported to express different levels of SIRPBs as well as SIRPA in different subsets (Lahoud et al., 2006) although their functional significance is as yet unclear. SIRPG has a similar structure to SIRPB, but has no positively charged residue, yet does interact with CD47 though weakly. It is expressed by T cells and NK cells rather than myeloid cells and appears to be involved in the regulation of T cell proliferation (Piccio et al., 2005). SIRPD consists of only a single Ig-like domain and is presumed to be soluble; its function is unknown but may be expressed in lung and sperm (van Beek et al., 2005). Although SIRPs are highly conserved and variants are found in most vertebrate species (van Beek et al., 2005), SIRPs in general are highly diverse with gene duplication, polymorphism and splice variants reported in different species (van Beek et al., 2005; Barclay and Hatherley, 2008). Furthermore, they also show positive selection with high dN/dS ratios (Barclay and Hatherley, 2008). All of which suggests that they are rapidly evolving genes, and it is conceivable that the selective pressures are pathogen driven and it has been suggested that the SIRPB ligands may be pathogen ligands (Barclay and Hatherley, 2008). Despite the considerable within and between species diversity, no specific diseases have been associated with specific SIRP polymorphisms.

No previous studies have reported on the presence and expression of SIRP genes in cattle apart from SIRPA, and our own data on SIRPB1. qRT-PCR of Holstein and Sahiwal uninfected and *T. annulata* infected MΦ with specific primers revealed that in fact, SIRPA was more highly expressed in Sahiwals, whereas SIRPB1 was more highly expressed in Holstein than Sahiwal MΦ (Jensen et al., 2008). With the sequencing of the bovine genome (The Bovine Genome Sequencing Consortium, 2009), we have now been able to undertake a more thorough analysis of the SIRP genes present in the bovine genome. There are two separate clusters on BTA 13, one cluster contains genes for SIRPA, SIRPB3, two apparently duplicated genes

for SIRPB1 and a pseudogene for SIRPG, whereas the other cluster contains SIRPB2, a SIRPB2 pseudogene and two apparently duplicated genes for SIRPD. Preliminary analysis of expression of these genes in a variety of bovine tissues and cells has revealed that SIRPA is the most ubiquitously expressed gene, although SIRPB1 and SIRPB2 are also widely expressed. In contrast SIRPG transcripts were mainly found in gut and immune related tissues and transcripts for SIRPD were only found in buffy coat samples. Resting and LPS and IFNγ activated monocytes and monocyte-derived MΦ were positive for SIRPA, SIRPB1, SIRPB2 and SIRPG. In addition, evidence was found for the expression of a splice variant of SIRPB1, the significance of which is still to be determined. However, SIRPB3 was only expressed in *T. annulata* infected MΦ. Furthermore, additional breed differences were observed in the expression of SIRPBs. SIRPB2 was also more highly expressed in *T. annulata* infected Holstein MΦ compared to Sahiwal infected MΦ, although it is as yet unclear if the detected transcripts are specific for SIRPB2 or the SIRPB2 pseudogene. SIRPB3 is expressed in 6/6 Holstein *T. annulata* infected MΦ tested to date but in only 1/6 Sahiwal infected MΦ. These results together suggest that the differential expression of SIRPs in Holstein and Sahiwal MΦ may lead to differences in response to inflammatory signals as well as infection and further studies are now underway to explore the consequences of this differential expression.

6. Bovine major histocompatibility complex (MHC) (BoLA) class II

In addition to the differences in SIRPA and SIRPB expression between MΦ of the two breeds, another immune related transcript for bovine major histocompatibility complex (MHC) (BoLA) DQA was also found to be significantly differentially expressed (Table 2) (Jensen et al., 2008). Further analysis revealed that both BoLA DQA and DQB mRNA were expressed at higher levels in Holstein MΦ compared to Sahiwal MΦ (Jensen et al., 2008). We have now been able to confirm these differences at the protein level using anti DQA and DQB specific monoclonal antibodies (mAB) and flow cytometry (Russell et al., 2000) (Fig. 2; Table 2). By using QIFIKIT® beads (Dako, UK) we were able to quantify the level of specific antibody binding sites per cell (SABC), which roughly equates to molecules per cell. Furthermore, additional differences in BoLA DR and BoLA class I protein expression were found (Table 2). This revealed that essentially all uninfected Holstein and Sahiwal circulating MΦ expressed BoLA class I, DR and DQA and there was no significant difference in the level of expression of BoLA DR, whereas Sahiwal MΦ expressed lower levels of BoLA DQA ($p < 0.05$) and higher levels of BoLA class I ($p < 0.05$) compared to Holstein monoctyes. In contrast there was a marked difference between the breeds in DQB expression, with very few Sahiwal MΦ expressing DQB unlike the Holstein MΦ which virtually all expressed DQB ($p < 0.001$). It is unusual to express DQA without DQB. Thus, the difference in Sahiwal MΦ might have been due to the anti-DQB antibody not recognising the Sahiwal DQB. An additional complexity of cattle DQ, is that DQA and DQB can be duplicated. Thus, it could be that the Sahiwals have duplicated

Table 2

BoLA class I, DR and DQ cell surface expression on uninfected MΦ and *T. annulata* infected MΦ from Holsteins and Sahiwals (% positivity and quantified mean fluorescence intensity).

Specificity of antibody[a]	Uninfected MΦ				*T. annulata* infected MΦ			
	Sahiwal		Holstein		Sahiwal		Holstein	
BoLA	%[b]	SABC[c]	%	SABC	%	SABC	%	SABC
DR	99	58	98	108	5[*]	17[*]	34	55
DQα	97	17[*]	94	35	0[*]	NA[d]	13	18
DQβ	4[***]	NA	91	21	0[*]	NA	6	11
Class I	100	77[*]	100	153	80[**]	338[**]	67	176

[a] Specificity of antibody: Anti-DR antibody = J11, anti-DQα antibody = VPM36 and anti-DQβ = VPM44 (confirmed as specifically recognising the BoLA equivalents by Russell et al., 2000); anti-class I antibody = IL-A19 (Bensaid et al., 1989).

[b] Mean %.

[c] SABC: specific antibody binding capacity/cell ($\times 10^3$) $\sim$ molecules/cell; mean SABC (determined from the mean fluorescence intensity) of MΦ expressing the BoLA molecule (mean of biological replicates (animals) for Holsteins (4) and Sahiwals (5)).

[d] NA = not applicable.

[*] $P < 0.05$, as calculated by Student's T-test (breed comparisons between uninfected or *T. annulata* infected MΦ).

[**] $P < 0.01$, as calculated by Student's T-test (breed comparisons between uninfected or *T. annulata* infected MΦ).

[***] $P < 0.001$, as calculated by Student's T-test (breed comparisons between uninfected or *T. annulata* infected MΦ).

haplotypes whereas the Holsteins have single haplotypes. However, Sahiwal B cells expressed similar levels of DQB to the Holstein DQB (results not shown). This suggests that these possibilities were unlikely to be the case, and furthermore, suggesting that the lack of expression of DQB in the Sahiwal breed was MΦ specific.

T. annulata infected cell lines derived *ex vivo* from peripheral blood of both breeds following infection, showed reduced expression of DR, DQA and DQB, although the down-regulation of these molecules was more profound in the Sahiwal breed ($p < 0.05$) (Table 2). This is in contrast to earlier reports in which DR was expressed at higher levels in *T. annulata* infected cell lines than in uninfected MΦ (Glass and Spooner, 1990; Brown et al., 1995). However these earlier cell lines were generated *in vitro*, and these differences in phenotypic profiles may indicate that regulation of gene expression *in vivo* is influenced by the *in vivo* environment, whether it involves other cells and/or factors.

In contrast, although BoLA class I was also expressed on fewer numbers of infected cells in both breeds, it is

Fig. 2. BoLA DQA cell surface molecules are more highly expressed by uninfected Holstein MΦ (black line) than Sahiwal MΦ (grey line). Flow cytometry histogram of log fluorescence with anti BoLA DQ antibody (VPM 36) of breed typical MΦ ($P < 0.05$).

notable that the level of BoLA class I increased dramatically (over 4-fold) on those Sahiwal MΦ positive for BoLA class I compared to both uninfected Sahiwal MΦ as well as Holstein *T. annulata* infected MΦ (Table 2). BoLA DR and DQ expression are associated with T cell activation, and it is tempting to speculate that the lower expression of these molecules on Sahiwal MΦ would result in lower levels of the aberrant T cell activation that occurs during the early stages of *T. annulata* infection *in vivo* (Campbell et al., 1995). These cells together with proliferating infected cells contribute to the expansion of lymph nodes draining the site of infection. It is notable that following *in vivo* infection with *T. annulata*, Sahiwals have significantly less lymph node enlargement for a shorter time than Holsteins (Glass et al., 2005). The significant up-regulation of BoLA class I on Sahiwal MΦ following infection with *T. annulata*, may indicate that this breed has overcome a parasite dependent mechanism for down-regulating class I and thus, avoiding the attention of cytotoxic T cells which are likely to play a role in protection against *T. annulata* (Preston et al., 1999). MHC gene and protein expression are regulated by many mechanisms involving transcription factors as well as post-transcriptional and translational regulation. Unlike MHC class I, MHC class II expression is not ubiquitous, but in most species is confined to antigen presenting cells such as MΦ, dendritic cells and B cells, and can be upregulated in response to inflammatory cytokines such as IFNγ on other cell types. The class II transactivator (CIITA) is considered to be the master regulator, which together with several other enhanceosome proteins controls the transcription of all of the classical MHC class II genes. However, other factors clearly play a role including polymorphisms in the promoter elements (Ting and Trowsdale, 2002). In addition there must be additional regulators of DQ genes as differential expression between DR and DQ has been reported in cattle (Taylor et al., 1993), mice and humans (Handunnetthi et al., 2010). Not surprisingly many eQTL have been mapped to the MHC locus in humans (Vandiedonck and Knight, 2009) and the levels of MHC class II expression have been associated with susceptibility to inflammatory and autoimmune diseases (Handunnetthi et al., 2010). It will be of
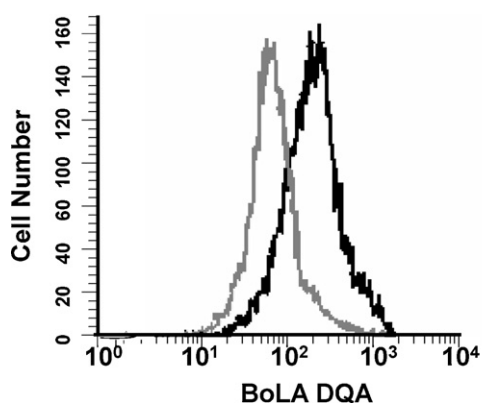
Table 3

Association of TGFB2 expression with *in vitro* invasiveness and *in vivo* virulence of *T. annulata* infected cell lines.

T. annulata cell line	Phenotype of *T. annulata* infected cell lines		
	Expression of TGFB2[a]	*In vitro* invasiveness[b]	*In vivo* virulence[c]
Holstein[d]	+++[e]	+++	NA[f]
Sahiwal[d]	+	+	NA
Sahiwal + rboTGFB2[g]	+	+++	NA
Low passage vaccine line[h]	+++	+++	+++
High passage vaccine line[h]	+	+	+

See Chaussepied et al. (2010) for more details.

[a] as measured by qRT-PCR (Chaussepied et al., 2010).

[b] as measured using Matrigel migration chambers (Lizundia et al., 2006).

[c] according to clinical signs in cattle immunised with high and low *in vitro* passage of Ode vaccine cell line (Singh, 1990).

[d] Holstein and Sahiwal MΦ cell lines were derived *ex vivo* and passaged *in vitro* between 3–5 times (see McGuire et al., 2004 for further details).

[e] + and +++ represent low and high scores associated with the phenotype.

[f] NA: not ascertained.

[g] Sahiwal infected MΦ cell line incubated with recombinant bovine TGFB2 (Chaussepied et al., 2010).

[h] *T. annulata* infected vaccine cell line of low or high *in vitro* passage associated with virulence and attenuation *in vivo*, respectively (Singh, 1990).

interest to determine which if any of these pathways are influenced by *T. annulata* in different ways in Holstein and Sahiwal MΦ.

7. Transforming growth factor β2

More recently, further intriguing differences in gene expression between the breeds have come to light. These were not originally detected by the functional genomics approach, because the transcripts were either not represented on the cDNA array or the gene probe failed to work under the hybridisation conditions used. However, we and others have ascribed important functional consequences to these differences. Following *T. annulata* infection of Holstein and Sahiwal MΦ, transcript levels of transforming growth factor (TGF)B2 are expressed at much higher levels in Holstein MΦ than in Sahiwal MΦ (Table 3). In contrast, levels of the related factors, TGFB1 and TGFB3, and the receptors for these 3 factors, TGFBR1, TGFBR2 and TGFBR3 are all expressed at similar levels in MΦ of both breeds (Chaussepied et al., 2010). TGFB factors are part of a family of cytokines and growth factors that form a complex network of cell signals and interactions, which lead to transcriptional changes mainly through the Sma- and Mad-related (SMAD) pathways (reviewed in Ross and Hill, 2008). Although it may seem counter-intuitive that the Holstein MΦ have higher levels of a cytokine associated with anti-inflammatory responses, in fact at early time points following infection both breeds up-regulated TGFB2, but in the Sahiwal infected MΦ, the level of TGFB2 was then down-regulated and remained low, whereas in the Holstein MΦ, TGFB2 remained highly expressed (Chaussepied et al., 2010). It is possible that the continuing high levels of TGFB2 in Holsteins are related to a prolonged induction of a negative feedback loop associated with the Holstein's higher inflammatory phenotype. However, the TGFB family are also associated with metastasis (Huang and Huang, 2005), and virulence of *T. annulata* infected MΦ cell lines are also associated with invasive capacity (Adamson and Hall, 1998). Our study has linked the expression of TGFB2 with an invasiveness phenotype *in vitro* as well as virulence *in vivo*, but not with proliferation or survival of infected cells (Chaussepied et al., 2010). We showed that Holstein

T. annulata infected MΦ had greater capacity to traverse matrigel chambers than Sahaiwal infected MΦ, and these differences mirrored those seen with low passage and high-passage vaccine cell lines, which were associated with virulence and attenuation respectively (Table 3). Addition of recombinant TGFβ2 or supernatant derived from Holstein infected cells to Sahiwal infected cell lines increased their invasive capacity, and conversely with the invasive capacity of Holstein infected cells was significantly reduced in the presence of a TGFβ-receptor 1 inhibitor (Chaussepied et al., 2010). This suggests that at least one of the mechanisms operational in Sahiwals is the ability to regulate the expression of TGFB2 in infected MΦ, which results in a lower propensity of these cells to metastasise (Table 3). TGFB2 is also a compelling candidate for the indomethicin resistant suppressive factor which is only associated with Holstein and not Sahiwal infected MΦ described by Preston et al. (2002).

However, it has been suggested that protozoan parasites evade or prevent an effective protective immune response by acting as apoptotic body mimics, through their expression of phosphatidylserine on their cell surfaces (de Freitas Balanco et al., 2001; Seabra, 2004; Wanderley et al., 2006; DaMatta et al., 2007). This in turn leads to the up-regulation of TGFB and downstream signalling in MΦ, resulting in a suppressive phenotype. Possibly *Theileria* parasites may do the same. Pretending to be apoptotic bodies would be a good strategy from an evolutionary point of view, as it could make MΦ more "tolerogenic" and/or dampen down inflammatory responses. Again, the Sahiwal breed may have evolved a counter-mechanism that prevents this type of response. Intriguingly TGFB2 has also been identified as a candidate gene for susceptibility in humans to another Apicomplexan parasite, *Plasmodium falciparum* (Sambo et al., 2010).

8. Concluding remarks

In conclusion, in support of the hypothesis that control of the inflammatory cytokine "storm" is the key to the Sahiwals ability to cope with invasion by the protozoan parasite, *T. annulata*, the broad transcriptome analysis of the two breeds of cattle MΦ revealed that

the most differentially expressed genes were associated with inflammation and immune response, with the most extreme expression seen in SIRPB1 which was more highly expressed in Holstein MΦ and may endow them with a more inflammatory phenotype. In addition TGFB2, which was also more highly expressed in Holstein MΦ, is also likely to play a role in transcriptional regulation of inflammatory processes, but particularly appears to be involved in enhancing invasiveness of the parasite infected cells. BoLA DQ in contrast is expressed at much lower levels in Sahiwals than Holsteins, and this may indicate that Sahiwal MΦ are more refractory to external inflammatory signals such as IFNγ and/or relate to a host mechanism for inhibiting the "nonspecific" T cell activation and proliferation that occurs early in infection. The genetic variation underlying these gene expression differences may also be relevant to disease resistance and tolerance to other pathogens, and could potentially provide new insights into the control of inflammation. Our research has also uncovered considerably more variation in the form of splice variants and these add to the complexity of the transcriptome. In the past, our knowledge of the immune-related transcriptome in livestock was limited by the databases of expressed sequence tags (ESTs) which mostly derived from non-immune tissue or tissue that had not undergone an immune response. Given that immune system genes probably undergo the greatest modulation compared to the rest of the genome, this has been a major hindrance in our understanding of livestock immunity, let alone what underlies disease resistance and tolerance. Today, with the advent of livestock genomes to map onto, we can begin to look forward to employing RNA-seq (Hawkins et al., 2010) to investigate livestock transcriptomes with theoretically the ability to sequence and quantify every transcript within a cell. Combined with ChiP-Seq data, this should provide us with new understandings of how gene expression is coordinated and regulated both at a species level and across species. The extent of cross-reactivity with mouse and human reagents is particularly low for immune-related proteins in livestock species compared to other proteins, presumably because pathogen pressure has induced significantly more diversity in proteins with roles in immunity. This problem remains to be addressed and limits the ability to conduct meaningful functional studies on livestock immunity and disease resistance. Advances in proteomics may also help provide some solutions to these issues, but greater investment in generating appropriate livestock reagents is needed. In any case, the information is likely to revolutionise our understanding of how complexity is generated from a limited set of genes.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

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