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The protozoan parasite *Theileria annulata* alters the differentiation state of the infected macrophage and suppresses musculoaponeurotic fibrosarcoma oncogene (MAF) transcription factors

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**Abstract**

The tick-borne protozoan parasite *Theileria annulata* causes a debilitating disease of cattle called Tropical Theileriosis. The parasite predominantly invades bovine macrophages (mφ) and induces host cell transformation by a mechanism that has not been fully elucidated. Infection is associated with loss of characteristic mφ functions and phenotypic markers, indicative of host cell de-differentiation. We have investigated the effect of *T. annulata* infection on the expression of the mφ differentiation marker c-maf. The up-regulation of c-maf mRNA levels observed during bovine monocyte differentiation to mφ was suppressed by *T. annulata* infection. Furthermore, mRNA levels for c-maf and the closely related transcription factor maB were significantly lower in established *T. annulata*-infected cell-lines than in bovine monocyte-derived mφ. Treatment of *T. annulata*-infected cells with the theileriacidal drug buparvaquone induced up-regulation of c-maf and maB, which correlated with altered expression of down-stream target genes, e.g. up-regulation of integrin B7 and down-regulation of IL12A. Furthermore, *T. annulata* infection is associated with the suppression of the transcription factors, Pu.1 and RUNX1, and colony stimulating factor 1 receptor (CSF1R) which are also involved in the regulation of monocyte/mφ differentiation. We believe these results provide the first direct evidence that *T. annulata* modulates the host mφ differentiation state, which may diminish the defence capabilities of the infected cell and/or promote cell proliferation. Musculoaponeurotic fibrosarcoma oncogene (MAF) transcription factors play an important role in cell proliferation, differentiation and survival; therefore regulation of these genes may be a major mechanism employed by *T. annulata* to survive within the infected mφ.

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1. Introduction

The tick-borne apicomplexan parasite *Theileria annulata* is the causative agent of the cattle disease Tropical Theileriosis, which is of major economic importance in countries in Northern Africa and Asia. The infectious sporozoite stage of the parasite exhibits cell tropism, predominantly invading bovine macrophages (mφ) and to a lesser extent B lymphocytes (Glass et al., 1989). Within these cells the parasite differentiates into the multinucleated schizont stage, which is associated with much of the disease pathology.

A unique feature of *T. annulata*, and the closely related parasite *Theileria parva*, is the ability of the schizont stage to induce host cell transformation without involving the integration of parasite DNA into the host genome. This leads to continuous proliferation of the infected cell and parasite (Spooner et al., 1989). These cells disrupt the morphology and function of the lymph node draining the site of infection (Campbell et al., 1995). In addition, infected cells disseminate through lymphoid tissue and non-lymphoid organs including the heart, lungs and brain, causing haemorrhagic lesions (Forsyth et al., 1999). The mechanism behind the transformation event has been extensively studied, but is not fully understood (reviewed Dobbelnaere and Kuenzi, 2004; Heussler et al., 2006; Plattner and Soldati-Favre, 2008). This work has revealed an extremely complex network of interactions between the parasite and host cell, involving many cellular pathways, including: NF-κB (Heussler et al., 2002), c-myc (Dessauge et al., 2005), SRC-related tyrosine kinase (Eichhorn and Dobbelnaere, 1994), c-jun NH2-terminal kinase (JNK) (Galley et al., 1997; Chaussepied et al., 1998), phosphoinositide-3 kinase (Baumgartner et al., 2000), protein kinase A (Guergnon et al., 2006) and Notch (Chaussepied et al., 2006).

*Theileria annulata* infection alters the phenotype and function of the host mφ (reviewed by Glass and Jensen, 2007). Phenotypic changes include the down-regulation of mφ markers CD14 and CD11b, together with increased surface expression of CD2 and bovine major histocompatibility complex (BoLA) class II genes (Glass and Spooner, 1990; Brown et al., 1995; Sager et al., 1997;
Glass and Jensen, 2007). Several mφ functions are impared by T. annulata, including phagocytosis and the production of nitric oxide, superoxide and pro-inflammatory cytokines in response to stimulation by phorbol 12-myristate 13-acetate and lipopolysaccharide (LPS) (Sager et al., 1997). Transformation of the infected mφ is reversible and requires the presence of live parasites (Sager et al., 1997). Treatment of infected cells with the thiolericidial drug buparvaquone results in a decrease in cell-cycling, increased surface expression of CD14 and CD11b, and the reacquisition of phagocytosis function (Sager et al., 1997).

Mφ are an end product of the differentiation of myeloid progenitor cells via blood monocytes. Monocytes have the potential to differentiate into different subsets of mφ and dendritic cells (DC), which play a multitude of important roles in the immune response (Iwasaki and Akashi, 2007; Geissmann et al., 2008; Serbina et al., 2008). It has been postulated, from the phenotypic and functional changes associated with T. annulata infection, that the parasite induces the mφ to revert back to a de-differentiated state, which may be a strategy utilized by the parasite to subvert the mφ defence response (Sager et al., 1997). However, T. annulata-infected cells exhibit up-regulation of BoLA class II molecules and pro-inflammatory cytokines (Brown et al., 1995; McGuire et al., 2004), enhanced antigen-presenting capabilities (Glass and Spooner, 1990) and induce neighbouring T lymphocytes, including naive T cells, to activate and proliferate (Campbell et al., 1995). Therefore the infected mφ does not resemble monocytes or myeloid progenitor cells.

The differentiation of mφ from myeloid progenitor cells is a tightly controlled process regulated by transcription factor activity (reviewed by Hume and Himes, 2003; Friedman, 2007). Transcription factors involved include the Ets family member spleen focus forming virus (SFV) proviral integration oncogene sp1 (Pu.1), the core binding family member runt-related transcription factor 1 (RUNX1) and two members of the musculoaponeurotic fibrosarcoma oncogene (MAF) transcription factor family, c-MAF and MAFB. Expression of human c-MAF increases progressively from myeloid progenitor cells to mφ and is, therefore, a good marker of human mφ differentiation (Martinez et al., 2006; Liu et al., 2008). Transcriptome profiling of bovine monocytes following infection with T. annulata revealed that c-MAF was one of the most differentially regulated genes (Jensen et al., 2008). In addition to its role in differentiation, c-MAF was originally identified as an oncogene (Kataoka et al., 1993) and therefore may play a role in the transformation of the T. annulata-infected cell. Therefore we have further investigated the effect of T. annulata infection on the expression of c-MAF and other transcription factors in bovine monocytes and mφ. The study has revealed that the expression of both MAF transcription factors and other transcription factors involved in the regulation of monocyte/mφ differentiation are suppressed by the presence of T. annulata. This supports the hypothesis that the parasite alters the differentiation state of the host cell, which may be essential for parasite-induced survival and proliferation.

2. Materials and methods

2.1. Animals

Peripheral monocytes were isolated from four Sahiwal cattle (Bos indicus) maintained off pasture at the Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh, UK, which have been described previously (Jensen et al., 2006). Cells were also collected from 12 female Holstein-Friesian cattle (Bos taurus) maintained at The Roslin Institute, University of Edinburgh, UK. These Holstein-Friesian cattle were between 3 and 5 years of age and kept on pasture. All experimental protocols were authorized under the UK Animals (Scientific Procedures) Act, 1986.

2.2. Isolation of bovine monocytes

Peripheral blood was collected aseptically into acid citrate dextrose (ACD) and immediately stored on ice. Peripheral blood mononuclear cells (PBMC) were separated under cold conditions by density gradient centrifugation as described previously (Jensen et al., 2006). Peripheral monocytes were isolated from PBMC by positive selection using the monoclonal antibody IL-A24, which recognizes signal-regulatory protein alpha (SIRPA) (Brooke et al., 1998), and the MACS system (Miltenyi Biotec) as described previously (Jensen et al., 2006). Fluorescence-activated cell sorting (FACS) analysis confirmed that the cell purity exceeded 95% (data not shown).

2.3. Infection and stimulation of bovine monocytes

Resting peripheral monocytes from five cattle were resuspended at 4 x 10⁶ cells/ml in RPMI-1640 medium supplemented with 20% FBS and aliquoted into a 12-well plate. One half of each sample were infected with T. annulata (Ankara) sporozoites in homogenized infected tick (Hyalomma anatolicum) preparations (kindly provided by Dr. Patricia Preston, University of Edinburgh, UK), as described previously (Jensen et al., 2008). Briefly, an equal volume of sporozoite suspension at 0.25 tick equivalents/ml in RPMI-1640 medium supplemented with 40% FBS was added to the monocytes and incubated at 37 °C in a 5% CO₂ incubator. The sporozoite concentration had been previously optimized to maximize the percentage of monocytes being infected (data not shown). As a medium-only negative control, an equal volume of RPMI-1640 medium supplemented with 40% FBS was added to the other half of each monocyte sample. Cells were harvested at 0 and 72 h post stimulation and RNA was immediately isolated from the cells.

In a separate experiment, monocytes from four cattle were purified as described above and stimulated with homogenized uninfected H. anatolicum (kindly provided by Dr. Alan Walker, University of Edinburgh, UK), prepared using a similar protocol to that used to generate infected tick preparations (Brown, 1987). Cells were harvested at 0 and 72 h post stimulation and RNA was immediately isolated from the cells.

2.4. Preparation of bovine monocyte-derived macrophages

Bovine mφ were generated from the peripheral blood of eight Holstein-Friesian cattle as described previously (Jungi et al., 1996). Briefly, blood was collected aseptically into ACD and buffy coats were separated by centrifugation. The resulting cells were washed with citrate buffer (30 mM citric acid, 0.6% NaCl, 3 mM KCl, 4.3 mM Glucose) to remove fibrinogen, followed by hypotonic lysis of erythrocytes. PBMC were separated by density gradient centrifugation on Lymphoprep (Axis-Shield) and resuspended at 4–5 x 10⁶ cells/ml in Iscove’s modified Dulbecco’s medium (IMDM) (Invitrogen) supplemented with GlutaMax™ (Invitrogen), 25 mM Hepes, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM sodium pyruvate, 1% minimum essential medium (MEM) vitamins (Invitrogen), 1% non-essential amino acids (Invitrogen), 50 µM l-mercaptoethanol and 20% FBS. The purified PBMC were cultured in non-adherent Teflon bags for 7 days at 37 °C in a 5% CO₂ incubator, during which time the monocytes differentiated into mφ (Jungi et al., 1996). Cells were resuspended in fresh medium supplemented as above, except that the FBS concentration was
reduced to 2%. Mo were purified by selective adherence overnight to 6-well plates.

2.5. Cell lines

Two sets of *T. annulata*-infected cell-lines were used in this study. The first set comprised 10 previously established *T. annulata* (Hisar) infected cell-lines, between passages 4 and 7, which were established ex vivo from the peripheral blood of Sahiwal and Holstein-Friesian calves following experimental infection (McGuire et al., 2004). The second set comprised five *T. annulata*-infected cell-lines generated from in vitro infection of purified Holstein-Friesian or Sahiwal peripheral monocytes, prepared as described above. The cells were infected with the Ankara, Hisar or Garh strains of *T. annulata*. All cell-line cultures, between passages 4 and 8, were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 50 μM β-mercaptoethanol and cultured at 37 °C in a 5% CO2 incubator.

The parasites were eliminated from cells by treatment with the theileriacidal drug buparvaquone. The cells, at 1 × 10⁶ cells/ml, were treated with 25 ng/ml buparvaquone (kindly supplied by Dr. MaHugh, CTVM) diluted in 10 mM potassium hydroxide (KOH) in 100% ethanol for 72 h. In addition negative control samples, treated with an equal volume 10 mM KOH in 100% ethanol, were prepared.

2.6. Total RNA extraction

Total RNA was extracted from all monocyte, bovine mő and *T. annulata*-infected cell-line samples using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. The resulting RNA was treated with DNA-free (Ambion) according to the manufacturer’s instructions to remove any contaminating genomic DNA. The quality and quantity of the resulting RNA was determined by gel electrophoresis and using a Nanodrop spectrophotometer. First strand cDNA was reverse transcribed from 0.5 μg total RNA using oligo(dT) primer and Superscript II (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA was diluted 1:25 for quantitative reverse transcription-PCR (qRT-PCR) analysis.

2.7. Quantitative RT-PCR

Oligonucleotides were designed for each gene using Primer3 (Rozen and Skaletsky, 2000) and Netprimer (Biosoft International) software (Table 1). The mRNA levels of each transcript were quantified by qPCR using the Platinum SYBR Green qPCR Supermix UDG kit (Invitrogen). Reactions were carried out in 20 μl vols. containing: 1 x supermix (SYBR Green, Platinum taq DNA polymerase, dNTPs, uracil DNA glycosylase (UDG) and stabilizers), 0.4 μl Rox dye, 1 μl forward and reverse primers at predetermined optimal concentrations and 5 μl diluted cDNA. Amplification and detection of products was carried out using a Mx3000P PCR machine (Stratagene) with the following cycle profile: 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The detection of a single product was verified by dissociation curve analysis. Each PCR experiment was carried out in triplicate and contained several non-template controls and a log₁₀ dilution series of activated monocyte cDNA or plasmids containing the sequence of interest. The relative quantities of mRNA were calculated using the method described by Pfaffl (2001). The qRT-PCR results for zinc finger protein 828 (2NF828), previously known as Chromosome 13 open reading frame 82, were used to calculate differences in the template RNA levels and thereby standardize the results for the genes of interest. 2NF828 was previously selected from microarray and qRT-PCR analyses as a constitutively and moderately expressed gene in activated, *T. annulata*-infected and resting Holstein-Friesian- and Sahiwal-derived monocytes (Jensen et al., 2006). The relative quantity values were transformed on the log₂ scale before statistical analyses to stabilize the variance. The effects of cell differentiation, infection and buparvaquone treatment were examined by t-test analysis (Genstat 10.2, Lawes Agricultural Trust, Rothamsted).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene symbol</th>
<th>Orientation</th>
<th>Primer sequence (5′–3′)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C–C motif) receptor 1</td>
<td>CCR1</td>
<td>F</td>
<td>AAA TGA GAA GAA GGC CAA ACC</td>
<td>140</td>
</tr>
<tr>
<td>Colony stimulating factor 1 receptor</td>
<td>CSF1R</td>
<td>R</td>
<td>TGC TCT GCT CAC ACT TAC GG</td>
<td></td>
</tr>
<tr>
<td>Colony stimulating factor 2</td>
<td>CSF2</td>
<td>R</td>
<td>ACC TTC ACA TTG CAG CCT GA</td>
<td>149</td>
</tr>
<tr>
<td>Integrin, beta 7</td>
<td>ITGB7</td>
<td>R</td>
<td>CAG CCC AGA ACT GAA GCA G</td>
<td>116</td>
</tr>
<tr>
<td>IL10</td>
<td>IL10</td>
<td>R</td>
<td>TGG TGC TCT TCG TCG TCT CTC</td>
<td>120</td>
</tr>
<tr>
<td>IL12A(b) (p35)</td>
<td>IL12A</td>
<td>R</td>
<td>AGG TTC TTG GGT GGG TCT G</td>
<td>214</td>
</tr>
<tr>
<td>Runx-related transcription factor 1</td>
<td>RUNX1</td>
<td>R</td>
<td>CCA GCC AAC TCT CAT TCG</td>
<td>183</td>
</tr>
<tr>
<td>Sp1 transcription factor</td>
<td>SP1</td>
<td>R</td>
<td>TGC AGT ACT TTA AGG GGT AC</td>
<td></td>
</tr>
<tr>
<td>Spleen focus forming virus (SFFV) proviral integration oncogene sp1</td>
<td>Pu.1</td>
<td>R</td>
<td>AGG GCA GAA AGC CAT GAC</td>
<td></td>
</tr>
<tr>
<td>v-maf Musculoaponeuetic fibrosarcoma oncogene homolog</td>
<td>c-maf</td>
<td>R</td>
<td>TCC TGG CTA CAG CAA CTA</td>
<td>148</td>
</tr>
<tr>
<td>v-maf Musculoaponeuetic fibrosarcoma oncogene homolog B</td>
<td>mafB</td>
<td>R</td>
<td>TGG CGG AGG CAA GAA GGA</td>
<td></td>
</tr>
<tr>
<td>Zinc finger protein 828</td>
<td>ZNF828</td>
<td>R</td>
<td>AGG AGC CAA CAG CAG GT</td>
<td>205</td>
</tr>
</tbody>
</table>

F and R denote forward and reverse primers, respectively.
3. Results

3.1. c-MAF is a marker of bovine macrophage differentiation

The expression of c-MAF has previously been shown to be a marker of human mφ differentiation (Martinez et al., 2006; Liu et al., 2008). To investigate whether this was also true of bovine mφ, the expression of c-MAF and MAFB was measured in bovine mφ and compared with that found in freshly isolated, resting monocytes. Bovine mφ were generated from peripheral monocytes by culturing for 7 days in Teflon bags as described previously (Jungi et al., 1996) and exhibited typical mφ phenotypic characteristics, e.g. morphology and cell marker expression (data not shown). The expression of c-MAF and MAFB was measured by qRT-PCR and found to be consistent between bovine monocyte samples (Fig. 1). The level of c-MAF expression was up-regulated in bovine mφ, by an average 43-fold, compared with freshly isolated monocytes (P < 0.001). MAFB expression in bovine mφ did not differ significantly from that observed in resting monocytes. This result confirms that c-MAF, but not MAFB, is a marker of bovine monocyte to mφ differentiation.

3.2. In vitro T. annulata infection suppresses the transcriptional up-regulation of c-MAF induced by monocyte differentiation

The schizont stage of T. annulata, which is the stage associated with host cell proliferation, develops within 2 days after sporozoite infection of the mφ (Jura et al., 1983). To investigate whether the regulation of mφ differentiation is required for host cell transformation, c-MAF mRNA levels were measured in monocytes cultured in the presence or absence of T. annulata sporozoites for 3 days, before parasite-induced host cell proliferation becomes apparent. The bovine monocytes were cultured for 3 days in tissue culture plates, which induces an intermediate differentiation state in human monocytes (Martinez et al., 2006; Lehtonen et al., 2007). The expression of c-MAF was observed to increase by an average 481-fold after this period (Fig. 2A, M), which was significantly greater than observed after 7 days culture in Teflon bags (Fig. 1). This discrepancy may be due to the different culture conditions or may result from c-MAF levels decreasing in the latter stages of differentiation.

Compared with the medium-only control c-MAF levels rose by statistically significantly less (P < 0.001), on average 62-fold, when cultured for 3 days in the presence of T. annulata sporozoites (Fig. 2A, Ta). The sporozoites were prepared from infected ticks, which may contribute to the suppression of c-MAF expression. However, monocytes cultured for 3 days in medium and uninfected tick preparations exhibited similar c-MAF levels to cells cultured in medium only (Fig. 2A, U). We believe these results provide the first direct evidence that T. annulata infection affects the expression of the differentiation marker c-MAF, which suggests that the parasite has affected the differentiation of monocytes to mφ induced by cells being in culture. However, due to possible down-regulation of c-MAF levels during the latter stages of mφ differentiation, it is unclear whether T. annulata has inhibited or accelerated mφ differentiation.

3.3. Theileria annulata infection does not affect MAFB expression during monocyte differentiation

The effect of T. annulata infection, medium and tick debris on MAFB expression was also investigated. After 3 days in culture MAFB mRNA levels were moderately up-regulated, between 3- and 9-fold by all three stimuli (Fig. 2B), similar to levels seen previously (Fig. 1). However, there was no significant difference between the responses to the three stimuli. The wide variation observed between the biological replicates results from the use of cells isolated from different animals from an out-bred population.
and the large scale of the graph. Therefore, c-MAF and MAFB transcrip-
tion are differentially regulated during monocyte to macrophage dif-
férentiation and there is no evidence that T. annulata modulates the expression of MAFB during early infection.

3.4. c-MAF and MAFB mRNA levels are lower in T. annulata infected cell-lines than in uninfected macrophages

The expression of c-MAF and MAFB in 10 ex vivo-derived T. annulata-infected cell-lines was compared with that measured in resting monocytes and macrophages by qRT-PCR (Fig. 3). Levels of c-MAF mRNA in the T. annulata-infected cell-lines were not statistically different from those measured in resting monocytes. However, there was a statistically significant difference in c-MAF levels measured in differentiated macrophages and T. annulata-infected cell-lines (P < 0.001). In addition, MAFB mRNA levels were statistically significantly lower (P < 0.001) in T. annulata-infected cell-lines than in resting monocytes and macrophages, exhibiting an average 9900-fold decrease in expression. The T. annulata-infected cell-lines are believed to be of monocyte origin and therefore these results provide evidence that transcription of both c-MAF and MAFB is suppressed by T. annulata infection.

3.5. Elimination of T. annulata induces up-regulation of c-MAF and MAFB

The previous results suggest that T. annulata suppresses c-MAF and MAFB expression at the transcriptional level. However, the T. annulata-infected cell-lines used in this study were of polyclonal origin, cultured from the blood or lymph of infected animals. Therefore, it is possible that the cell-lines were derived from cells of B lymphocyte origin, which may account for the low expression of MAF transcription factors. To investigate this, c-MAF and MAFB mRNA levels were measured in five in vitro-derived T. annulata-infected cell-lines of known monocyte origin. To confirm that T. annulata suppresses c-MAF and MAFB expression, the mRNA levels of these transcription factors were measured by qRT-PCR after culturing the cell-lines in the presence or absence of the theileria-cidal drug buparvaquone. After 72 h the buparvaquone-treated cell-lines exhibited decreased proliferation compared with the control cell-lines (data not shown), indicative that the parasite had been killed. c-MAF mRNA levels were up-regulated in four of the T. annulata-infected cell-lines, by on average 11-fold (Fig. 4) compared with medium only controls. In addition, buparvaquone treatment resulted in MAFB mRNA levels being up-regulated in all five T. annulata-infected cell-lines by on average 39-fold (Fig. 4).

The T. annulata-infected cell-lines were generated from monocytes isolated from two breeds of cattle; Holstein-Friesian and Sahiwal, which are susceptible and tolerant to T. annulata infection, respectively (Glass et al., 2005). Although the sample size was small, there was no detectable breed difference in c-MAF and MAFB mRNA levels nor in the up-regulation of the MAF transcription factors in response to buparvaquone treatment. The cell-lines were generated from in vitro infections with three strains of T. annulata: Ankara, Hisar and Gharb. The up-regulation of MAFB upon buparvaquone treatment was similar in all three parasite strains. However, c-MAF was not up-regulated at 72 h post-buparvaquone treatment in the Ankara cell-line. A second Ankara strain cell-line was generated on a separate occasion from infection of purified monocytes from the same animal and buparvaquone treatment also failed to modulate c-MAF mRNA levels in this cell-line (data not shown). At this time it is not possible to determine whether the parasite strain or the animal accounts for the different c-MAF response.

To confirm that the up-regulation of c-MAF and MAFB was due to the elimination of the parasite and not an additional action of the drug buparvaquone, purified monocytes from three animals were cultured for 72 h with and without buparvaquone. There was no significant effect of buparvaquone treatment on c-MAF or MAFB mRNA levels (data not shown).

3.6. Buparvaquone treatment induces the transcription of genes regulated by MAF transcription factors

An increase in mRNA levels does not necessarily have a biological consequence, due to potential regulation at the protein level. Unfortunately anti-bovine MAF antibodies were not available and attempts to consistently detect MAF proteins using anti-human MAF antibodies failed. Therefore, to investigate whether the increase in c-MAF and MAFB mRNA levels was associated with downstream effects, the mRNA levels of seven genes known to be regulated by MAF transcription factors were measured by qRT-PCR. The expression of IL10 and CD14 in the five in vitro-derived T. annulata-infected cell-
lines was not consistently altered by buparvaquone treatment (Fig. 5A) and exhibited no correlation with c-MAF or MAFB expression. However, the average mRNA levels for chemokine (C–C motif) receptor 1 (CCR1), CD14, colony stimulating factor 2 (CSF2), integrin B7 (ITGB7) increased 7.7- and 9.0-fold, respectively, following buparvaquone treatment (Fig. 5A). There was a statistically significant correlation between ITGB7 up-regulation and that measured for c-MAF (Fig. 5B), with a correlation coefficient of 0.85 \((P < 0.01)\). In addition, there was a statistically significant correlation between CCR1 up-regulation and that measured for MAFB, with a correlation coefficient of 0.92 \((P < 0.01)\) (data not shown). Furthermore, the average IL12A mRNA level decreased 2-fold following buparvaquone treatment (Fig. 5A) and there was a statistically significant correlation between IL12A down-regulation and c-MAF up-regulation, with a correlation coefficient of \(-0.85\) \((P < 0.01)\) (Fig. 5C). However, a similar correlation was not observed for IL12B, which was up- or down-regulated in different cell-lines in response to buparvaquone treatment. In addition to IL12A, there was also a significant, negative correlation between c-MAF up-regulation and the down-regulation of colony stimulating factor 2 (CSF2) following buparvaquone treatment, with a correlation coefficient of \(-0.83\) \((P < 0.02)\) (data not shown).

3.7. Other transcription factors regulating monocyte/macrophage differentiation and the mΦ marker CSF1R are suppressed by T. annulata infection

The maturation of myeloid progenitor cells to mΦ is coordinated by the activity of several transcription factors, including c-MAF and MAFB. The effect of buparvaquone treatment on the expression of three of these transcription factors; Pu.1, RUNX1 and Sp1, was measured in the five in vitro-derived T. annulata-infected cell-lines (Fig. 6A). Furthermore, the expression of colony stimulating factor 1 receptor (CSF1R), which plays an essential role in mΦ development and is regulated by transcription factor activity, was also investigated. There was considerable variation in the expression of CSF1R in the cell-lines following buparvaquone treatment, with either increased and reduced expression in different cell-lines. In contrast, the expression of Sp1 transcription factor was not significantly altered by buparvaquone treatment. Increased expression of Pu.1 and RUNX1 was induced by buparvaquone treatment, by an average 7.2- and 3.2-fold, respectively. This result provides further evidence that T. annulata modulates the differentiation state of the mΦ at the transcriptional level. However, the suppression of these transcription factors was statistically significantly less than observed for MAFB \((P < 0.02)\).

To further investigate the involvement of T. annulata in the regulation of Pu.1 and RUNX1 mRNA levels their expression, together with that of CSF1R, was compared in bovine monocytes, mΦ and T. annulata-infected cell-lines (Fig. 6B). RUNX1 mRNA levels were statistically significantly higher in mΦ compared with monocytes, with on average 3.6-fold higher expression \((P < 0.05)\). RUNX1 expression in T. annulata-infected cells was not significantly different from that measured in monocytes, but was statistically significantly lower than the mRNA level detected in mΦ \((P < 0.001)\). There was no significant difference in CSF1R and Pu.1 mRNA levels in monocytes or mΦ (Fig. 6B) and CSF1R expression was very variable between mΦ samples. However, both were expressed at significantly lower levels in T. annulata-infected cells compared to

![Fig. 5. Down-stream targets of the musculoaponeurotic fibrosarcoma oncogene (MAF) transcription factors c-MAF and MAFB are up-regulated in Theileria annulata-infected cell-lines upon parasite elimination. (A) The average log2 mRNA fold change in chemokine (C–C motif) receptor 1 (CCR1), CD14, colony stimulating factor 2 (CSF2), integrin B7 (ITGB7), IL10, IL12A and IL12B levels following treatment of T. annulata-infected cell-lines with 25 ng/ml buparvaquone for 72 h compared with that detected in cells cultured for 72 h in an equal volume of the solution used to dilute the buparvaquone. The error bars indicate the standard error for five in vitro-derived T. annulata-infected cell-lines. (B) Visualization of the positive correlation between c-MAF and ITGB7 mRNA up-regulation observed in the five in vitro-derived T. annulata-infected cell-lines from a representative experiment. (C) Visualization of the negative correlation between c-MAF and IL12A expression changes induced by buparvaquone treatment observed in the five in vitro-derived T. annulata-infected cell-lines from a representative experiment. The correlation coefficient \((r)\) is indicated.](image-url)
monocytes and macrophages, Pu.1 mRNA levels were 10-fold lower in infected cells than in monocytes ($P < 0.001$), whilst CSF1R expression was over 6900-fold lower than that measured in monocytes ($P = 0.001$) (Fig. 6B).

4. Discussion

To establish and survive within mammalian cells, intracellular parasites need to modulate the host cell. In the case of *T. annulata* and *T. parva*, this includes preventing the host cell from entering apoptosis pathways and inducing their uncontrolled cellular division. The mechanisms involved have been extensively studied, but have not been fully elucidated (reviewed by Dobbelaar and Kuenzi, 2004; Heussler et al., 2006; Plattner and Soldati-Favre, 2008). The transformation of bovine monocyte by *T. annulata* infection is associated with loss of characteristic monocyte functions and phenotypic markers, which has led to the hypothesis that the host cell has de-differentiated (Sager et al., 1997). A previous investigation of early events during *T. annulata* infection revealed that the transcription factor c-MAF, a known marker of monocyte differentiation (Martinez et al., 2006; Liu et al., 2008) and an oncogene (Kataoka et al., 1993), was modulated and therefore may play a role in the transformation event (Jensen et al., 2008). We believe our further studies, reported here, have provided the first direct evidence that *T. annulata* modulates the monocyte differentiation state, by suppressing c-MAF at the RNA level. Furthermore, RUNX1, which is also a marker of bovine monocyte to macrophage differentiation, was suppressed in *T. annulata*-infected cells together with Pu.1 and MAFB, other transcription factors involved in monocyte differentiation.

The progression of monocyte and macrophage differentiation is orchestrated by the coordinated activity of transcription factors, including Pu.1, RUNX1, CSF1R, enhancer binding proteins (CEBPs), Sp1, MAFB and c-MAF. The expression of CEBPs was not investigated due to difficulties designing suitable primers. RUNX1 principally plays an essential role in regulating very early events in haemopoiesis (Friedman, 2002, 2007; Rosenbauer and Tenen, 2007). However, conditional ablation of RUNX1 was associated with mild myeloproliferation, including increased numbers of myeloid progenitor cells (Gowney et al., 2005), supporting a role for RUNX1 in later stages of the myeloid differentiation pathway. In contrast, Pu.1 is expressed throughout the differentiation programme, from early progenitor cell to monocyte (Rosenbauer and Tenen, 2007). Both c-MAF and MAFB are important in the development of monocyte/macrophage differentiation (Hegde et al., 1999; Kelly et al., 2000). Both MAF transcription factors act partly by repressing the expression of non-monocyte/macrophage genes (Sieweke et al., 1996; Hegde et al., 1998). The transcription factor Sp1, which plays a limited role in monocyte differentiation (Hume and Himes, 2003), was found not to be affected by *T. annulata* infection. In contrast, CSF1R was profoundly down-regulated in *T. annulata*-infected cells compared with uninfected monocytes, which is essential for monocyte survival (reviewed by Barreda et al., 2004) and is therefore paradoxical that its receptor is down-regulated to such an extent in a transformed monocyte cell-line.

The level of *T. annulata*-induced suppression differed considerably amongst the transcription factors investigated, with the greatest effect on the MAF transcription factors. However, c-MAF levels only increased in four out of the five tested *T. annulata*-infected cell-lines after buparvaquone treatment, while MAFB levels increased in all of the cell-lines. This result suggests that MAFB is the more important target for suppression by *T. annulata* and indeed MAFB exhibited the greatest suppression in *T. annulata*-infected cells. However, there is functional redundancy between c-MAF and MAFB (Aziz et al., 2006), which may explain the parasite-induced suppression of c-MAF.

The variable regulation of transcription factors involved in monocyte/macrophage differentiation may account for the novel phenotype of *T. annulata*-infected cells, which are distinct from any monocyte progenitor cell. Therefore, it is more accurate to describe the effect of the parasite as 'modulation of differentiation' rather than de-differentiation. The balance of Pu.1 and MAFB expression has been shown to specify monocyte or DC cell fate (Bakri et al., 2005). High Pu.1 levels relative to MAFB were associated with DC differentiation from human peripheral monocytes, whilst higher MAFB levels were associated with monocyte differentiation (Aziz et al., 2006). Whilst the balance between MAFB and Pu.1 was not measured in this study, MAFB is down-regulated to a much greater extent than Pu.1 in *T. annulata*-infected cells, over 9,900-fold and 10-fold, respectively, compared with levels in monocytes, which would alter the balance between the transcription factors and may be associated with the infected cells developing DC-like characteristics, e.g. enhanced antigen presenting capabilities and up-regulation of B7A class II molecules (Glass and Spooner, 1990; Brown et al., 1995).
In addition to their involvement in monocyte/mо development, c-MAF and MAFB are involved in a range of cellular events, which may explain why *T. annulata* appears to specifically target these genes. For example, c-MAF prevents the nuclear translocation of the c-REL component of NF-κB, which potentially affects the expression of many NF-κB target genes (Homma et al., 2007). NF-κB is constitutively activated in *T. annulata*-infected cells due to the degradation of IκBs, the cytoplasmic inhibitors of NF-κB, by the IκB signalosome, which is recruited in large activated foci around the schizont surface (Heussler et al., 2002). The composition of NF-κB complexes has not been investigated in *T. annulata*-infected cells. However, T lymphocytes infected with *T. parva*, which also constitutively activate NF-κB, do express c-REL. Furthermore, c-REL-containing NF-κB complexes are translocated to the host cell nucleus (Machado et al., 2000). The prevention of nuclear translocation of NF-κB complexes containing c-REL by c-MAF would partly nullify the activity of the IκB signalosome and suggest that the suppression of MAF transcription factors is important for *T. annulata* and *T. parva* survival within the host cell. However, it is not known at this time if MAF transcription factors are suppressed in *T. parva*-infected T and B lymphocytes.

MAF transcription factors were originally identified as onco-genes and chromosomal translocation events in multiple myelomas frequently lead to the over-expression of c-MAF and MAFB (Hurt et al., 2004; Fabris et al., 2005). Moreover, the over-expression of c-MAF induces cell transformation (Kataoka et al., 1993). Therefore, it seems counter-intuitive that the expression of MAF transcription factors, e.g. AP-1, which are regulated by c-MAF (Hegde et al., 1999), was not consistently up-regulated by c-MAF (Baylis et al., 1995). Similarly, CD14 expression, which is induced by c-MAF (Hegde et al., 1999), was not consistently up-regulated by c-MAF (Baylis et al., 1995). The reason for this discrepancy is unclear at this time, but may relate to the difference in cell type or the involvement of other genes modulated by *T. annulata*.

The mechanism behind *T. annulata* induced suppression of the transcription factors identified in this study is unknown at this time. The transcription factor genes may be the direct target of parasite proteins or their suppression could result from the regulation of up-stream events in the monocyte/мφ differentiation pathway. Whilst considerable efforts have been made to understand the mechanisms behind the reversible transformation event from the perspective of the bovine cell, less effort has been made to identify the parasite proteins responsible for hijacking the bovine signalling pathways. The *T. annulata* genome has now been sequenced (Pain et al., 2005) and 244 parasite-encoded proteins have been identified as candidate effector molecules (Shiels et al., 2006). These fulfil several criteria, being: unique to *Theileria*, common to both *T. annulata* and *T. parva*, specifically expressed in schizonts and containing a putative peptide sequence for translocation across the parasite membrane into the bovine cell. Of these candidates only one family, the TashATs, have been shown to localize to the bovine nucleus and modulate gene expression (Shiels et al., 2004; Oura et al., 2006). Transfection of one family member, SuAT1, altered the morphology of the bovine мφ cell line BoMac and modulated the expression of cytoskeletal proteins, including actin (Shiels et al.,...
2004). Interestingly, MAFB \( ^{\sim} \) m\( ^{\sim} \) also exhibit an altered morphology, associated with the up-regulation of genes involved in actin organization (Aziz et al., 2006). However, the modulation was dis-similar to that observed in SuATI-transfected BoMac cells; for example SuATI expression decreased actinin levels, whereas this was enhanced in MAFB \( ^{\sim} \) m\( ^{\sim} \) (Aziz et al., 2006). However, there are 17 TashAT family members and the functions of the majority are unknown. In summary, the data presented here support the hypothesis that \( T. \) annulata modifies the differentiation state of the host m\( ^{\sim} \) to a novel phenotype which is beneficial for parasite survival. In particular, \( T. \) annulata regulates the MAF transcription factors, which play an important role in m\( ^{\sim} \) proliferation, differentiation and survival. The multiple functions of these transcription factors may explain why the parasite specifically targets these genes. Therefore, further investigation of the MAF transcription factors may lead to novel control strategies in the future.

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