1 **TGF-β2, catalase activity, H₂O₂ output and metastatic** 2 **potential of diverse types of tumour**

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20 **Abstract:**

21 *Theileria annulata* is a protozoan parasite that infects and transforms bovine
 22 macrophages causing a myeloid-leukaemia-like disease called tropical theileriosis.
 23 TGF- β 2 is highly expressed in many cancer cells and is significantly increased in
 24 *Theileria*-transformed macrophages, as are levels of Reactive Oxygen Species (ROS),
 25 notably H₂O₂. Here, we describe the interplay between TGF- β 2 and ROS in cellular
 26 transformation. We show that TGF- β 2 drives expression of *catalase* to reduce the
 27 amount of H₂O₂ produced by *T. annulata*-transformed bovine macrophages, as well as
 28 by human lung (A549) and colon cancer (HT-29) cell lines. *Theileria*-transformed
 29 macrophages attenuated for dissemination express less catalase and produce more
 30 H₂O₂, but regain both virulent migratory and matrigel traversal phenotypes when
 31 stimulated with TGF- β 2, or catalase that reduce H₂O₂ output. Increased H₂O₂ output
 32 therefore, underpins the aggressive dissemination phenotype of diverse tumour cell
 33 types, but in contrast, too much H₂O₂ can dampen dissemination.

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34 Introduction

35 TGF-β (Transforming Growth Factor beta) is a pleiotropic cytokine that is involved in
 36 diverse cellular processes such as proliferation, apoptosis and motility (1 , 2). In
 37 advanced cancer, TGF-β acts as an oncogenic factor that promotes tumour
 38 progression. Three isoforms have been defined of which TGF-β2 is highly expressed
 39 in many cancer cell lines, especially those showing a high dissemination potential.
 40 *Theileria annulata* parasitizes bovine macrophages and transforms them into
 41 disseminating tumours that cause a myeloid-leukemia-like disease called tropical
 42 theileriosis. However, *T. annulata*-transformed macrophage dissemination can be
 43 attenuated by multiple *in vitro* passages and attenuated macrophages are used as live
 44 vaccines in countries endemic for tropical theileriosis (3).

45 TGF-β2 levels are high in *Theileria*-transformed macrophages and this correlates with
 46 susceptibility to disease (4). Upon attenuation *in vitro*, the Ode vaccine line displays
 47 both reduced TGF-β2 expression and dissemination, which are re-established by
 48 addition of exogenous TGF-β (4); observations consistent with a pro-metastatic role
 49 for TGF-β2 in the virulence of *Theileria*-transformed macrophages. Furthermore,
 50 excessive cellular oxidative stress diminishes the virulence of *Theileria*-transformed
 51 macrophages, as attenuated macrophages produce more H₂O₂ (5). However,
 52 heightened reactive oxygen species (ROS) have been reported to increase TGF-β
 53 expression and stimulate release of TGF-β from latent complexes (6, 7). Although this
 54 might contribute to TGF-β2 production by virulent macrophages it does not explain
 55 why attenuated macrophages that produce more ROS express less TGF-β2 (4, 5).
 56 TGF-β2-signalling induces the transcription factor CREB in *Theileria*-transformed
 57 macrophages and CREB activity diminishes upon attenuation of macrophage

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58 virulence (8). The cyclic AMP response element-binding protein (CREB) is a
59 transcription factor of general importance in diverse cell types (9). CREB signalling is
60 associated with cancer development and poor clinical outcome in leukemogenesis
61 (10), but it is not known if CREB is a player in ROS regulation.

62 Elevated rates of ROS production have been described for human cancer cells, where
63 excessive ROS underpins many aspects of tumour development and progression (11).
64 However, tumours also express increased levels of antioxidant proteins to detoxify
65 ROS such as superoxide dismutases (SODs), catalase, peroxiredoxins, the glutathione
66 system that includes glutathione (GSH), glutathione reductase and glutathione
67 peroxidases (GPx) (11). Here, we focus on catalase that detoxifies hydrogen peroxide
68 (H_2O_2) by turning it into water and oxygen. We report that TGF- β 2 induces CREB
69 transactivation to promote catalase transcription that leads to increased catalase
70 activity and reduction in H_2O_2 levels. We provide evidence that TGF- β 2-driven
71 catalase activity regulates the H_2O_2 redox balance that impacts directly not only on
72 the hyper-dissemination phenotype of *Theileria*-transformed macrophages, but also
73 on the metastatic potential of human lung and colon cancer cell lines.

74 **Results**

75 ***T. annulata*-transformed macrophages attenuated for dissemination display**
76 **significantly less catalase activity compared to virulent hyper-invasive**
77 **macrophages.**

78 We previously showed that attenuation of *Theileria*-transformed macrophage
79 virulence correlates with an increase of H_2O_2 output (5). This appeared counter-
80 intuitive, as one imagined that a decrease in infected macrophage virulence would be

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81 accompanied by a reduction in their oxidative stress status. However, accumulation of
82 H₂O₂ can occur either due to an increase in superoxide dismutase (SOD) activity that
83 produces H₂O₂, or reduced detoxification of H₂O₂ leading to its accumulation. In
84 order to discriminate between these two possibilities SOD and catalase activities were
85 measured in virulent (V) and attenuated (A) *Theileria*-transformed macrophages
86 (Figure 1). No significant change in SOD activity was detected between virulent and
87 attenuated macrophages (Fig. 1A), whereas catalase activity was significant
88 diminished in attenuated macrophages (Fig. 1B). Moreover, decreased catalase
89 protein levels underpinned the reduced catalase activity of attenuated macrophages
90 (Fig. 1C). Catalase activity was also measured in *Theileria*-transformed macrophages
91 isolated from disease-resistant Sahiwal cattle and found to be lower (Fig.S1A). Thus,
92 attenuated macrophages isolated from disease-susceptible animals resemble
93 transformed macrophages isolated from disease-resistant animals with respect to
94 catalase activity.

95 **TGF β -2 stimulates *catalase* transcription leading to an increase in protein levels** 96 **and catalase activity.**

97 We examined if the decrease in TGF- β 2 levels underpinned loss of catalase
98 transcription and activity. When attenuated macrophages were stimulated with
99 recombinant TGF- β 2 *catalase* transcription is increased, but not that of *glutathione*
100 *peroxidase*, coding for another antioxidant enzyme (Figure 2A). Stimulation of
101 attenuated macrophages with recombinant TGF- β 2 increased catalase activity (Fig.
102 2B) and decreased H₂O₂ output (Fig. 2C). Taken together, TGF- β 2-signalling clearly
103 activates *catalase* transcription leading to increased amounts of catalase and greater
104 catabolic activity towards H₂O₂.

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105 **TGF- β 2-signalling activates *catalase* transcription via CREB.**

106 CREB is a transcription factor that binds DNA on CRE sites (cAMP response
107 element) to regulate transcription of target genes, and in *Theileria*-transformed
108 macrophages TGF- β 2-signalling activates *CREB* transcription (8). Consistently,
109 bioinformatic analyses detected CRE sites in the promoter region of the *catalase* gene
110 (data not shown). Inhibition of CREB-mediated transcription with a specific CREB-
111 CBP interaction inhibitor decreased *catalase* transcription and catalase activity to
112 levels characteristic of attenuated macrophages (Figure 3A & B). Consequently,
113 virulent macrophages produce H₂O₂ equivalent to attenuated levels and conversely,
114 decreased H₂O₂ output occurred upon activation of CREB-mediated transcription
115 following stimulation of attenuated macrophages with membrane-permeable db-
116 cAMP (Fig. 3C). Importantly, pre-treatment of attenuated macrophages with the
117 CREB inhibitor prevented the drop in H₂O₂ levels provoked by addition of db-cAMP
118 (Fig. 3C). Thus, TGF- β 2-signalling via CREB activates *catalase* transcription leading
119 to increased catalase activity and reduced H₂O₂ output.

120 **Virulence and attenuation of *Theileria*-transformed macrophages depends on** 121 **their redox balance.**

122 *T. annulata* transforms host macrophages into tumour-like cells that have heightened
123 motility and invasiveness two traits that are typical of metastatic/disseminating cancer
124 cells. Figure 4 shows that detoxifying H₂O₂ by adding catalase, or TGF- β 2 to
125 attenuated macrophages induces a regain in cell migration by *Theileria*-transformed
126 macrophages. Boiling catalase to inactive the enzyme ablated its ability to reduce
127 H₂O₂ levels that stimulate migration. By contrast, increasing H₂O₂ output by virulent
128 macrophages via SB431542 blockade of TGF-R attenuated their migration (Fig. 4).

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129 Similarly, TGF-R blockade with SB431542 or inhibition of catalase activity with
130 AminoTriazole (AT) reduced matrigel traversal of virulent macrophages to levels
131 typical of attenuated macrophages (Fig.4B). Therefore, modifying transformed
132 macrophage redox balance via TGF- β 2 stimulation, or manipulating catalase activity
133 changes a virulence trait (heightened migration) of *Theileria*-transformed
134 macrophages.

135 **TGF β -2 stimulation also increases catalase activity and metastatic potential of** 136 **human lung and colon cancer cell lines.**

137 In order to extend our observations on *Theileria*-transformed macrophages to other
138 cancer cell types, we treated HT-29 (human colorectal adenocarcinoma) and A549
139 (adenocarcinomic human alveolar basal epithelial) cell lines with TGF-R and/or
140 CREB-CBP interaction inhibitors. Both HT-29 and A549 H₂O₂ levels increase
141 following inhibitor treatment due to a corresponding drop in catalase activity (Figure
142 5 A& B), similar to *Theileria*-transformed macrophages (Figures 2, 3 & FigS1).
143 Moreover, inhibition of TGF-R and/or CREB signalling decreases matrigel traversal
144 of A549 cells (Fig. 5C). The ensembles lead to the conclusion that TGF- β 2 regulation
145 of catalase activity via CREB-mediated transcription and their impact on H₂O₂-type
146 oxidative stress is common to different cancer cell types of human and bovine origin.

147 **Discussion**

148 In this study, we have demonstrated that TGF- β 2 induces CREB to drive *catalase*
149 transcription, leading to more catalase enzyme and hence activity to detoxify H₂O₂.
150 *Theileria*-transformed macrophages with attenuated dissemination potential are
151 characterized by decreased TGF- β 2 production, and consequently, reduced catalase
152 activity and increased H₂O₂ output. Stimulating attenuated macrophages with

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153 exogenous TGF- β 2 increased catalase activity and decreased H₂O₂ output leading to a
154 regain in their capacity to migrate and traverse matrigel. In contrast, blockade of
155 either TGF-R or CREB binding to CBP decreased catalase activity and increased
156 H₂O₂ output led to a reduced migratory and matrigel traversal capacity of virulent
157 macrophages. These observations are not restricted to *Theileria*-transformed bovine
158 macrophages, but were shared by human A549 and HT-29 cancer cell lines, where
159 inhibition of TGF- β 2-signalling and/or CREB-mediated transcription again decreased
160 catalase activity, increased the H₂O₂ output and reduced their capacity to traverse
161 matrigel. Thus, our demonstration that catalase activity and hence H₂O₂ levels are
162 regulated by TGF- β 2-signaling can be generalized to different types of tumours.

163 Our study is consistent with a pro-metastatic role for TGF- β 2, since adding back
164 recombinant TGF- β 2 to attenuated macrophages resulted in a regain in their migratory
165 and dissemination potentials. Clearly, one way that TGF- β 2 promotes virulent
166 dissemination is by inducing CREB transactivation to activate catalase and detoxify
167 excess H₂O₂. Tumours produce large amounts of ROS that cause damage to DNA,
168 proteins and lipids and we propose that infected macrophages attenuated for
169 dissemination have countered the tumorigenic effect of *Theileria* by producing higher
170 levels of H₂O₂. In virulent macrophages with high TGF- β 2 levels CREB induction
171 activates catalase that dampens H₂O₂ output and similarly TGF- β 2-mediated changes
172 H₂O₂ output and catalase activity impact on the metastatic potential of human A549
173 and HT-29 cancer cell lines. This argues that many tumours of different origins
174 exploit TGF- β 2-driven *catalase* expression to control their H₂O₂ redox balance.

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175 MATERIALS AND METHODS

176 **Cell culture:** virulent Ode macrophage line (12) corresponds to passage 62 and
 177 attenuated macrophages correspond to passage 309. All macrophages were maintained
 178 in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-
 179 glutamine, 100U penicillin, 0.1 mg/ml streptomycin, and HEPES. The cell lines S2,
 180 S3 and H7–H8 have been described previously (13). Cells were maintained in the
 181 above culture medium with 50 μ M of β -mercaptoethanol. The human colon
 182 adenocarcinoma cell line HT-29 was maintained in McCoy's medium supplemented
 183 with 10% FBS. A549 human lung adenocarcinoma cells (ATCC, CCL-185) were
 184 cultured in DMEM/RPMI (1:1) with 10% fetal bovine serum. All the cells were
 185 incubated at 37°C with 5% CO₂.

186 **Total RNA extraction and reverse transcription:** Total RNA of *Theileria*-infected
 187 macrophages was isolated using the RNeasy mini kit (Qiagen) according to the
 188 manufacturer's instructions. The quality and quantity of RNA was measured by
 189 Nanodrop spectrophotometer. For reverse transcription, 1 μ g isolated RNA was
 190 diluted in water to a final volume of 12 μ L, warmed at 65°C for 10 min, then
 191 incubated on ice for 2 min. Afterwards, 8 μ L of reaction solution (0.5 μ L random
 192 hexamer, 4 μ L 5x RT buffer, 1.5 μ L 10mM dNTP, 1 μ L 200U/ μ LRT-MMLV
 193 (Promega) and 1 μ L 40U/ μ L RNase inhibitor (Promega) was added to get a final
 194 reaction volume of 20 μ L and incubated at 37°C for 2 h. The resultant cDNA was
 195 stored at -20°C.

196 **Quantitative polymerase chain reaction (qPCR):** mRNA expression levels were
 197 estimated by qPCR on Light Cycler 480 (Roche) using SYBR Green detection
 198 (Thermo). *GAPDH* was used as internal control to normalize for mRNA levels. The

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199 detection of a single product was verified by dissociation curve analysis and relative
200 quantities of mRNA calculated using the method described (14).

201 **Pharmaceutical inhibition and activation:** TGF- β signalling was inhibited using the
202 TGF-Receptor I/ALK5 inhibitor SB431542, 10 μ M (Sigma #S4317) and activated by
203 adding 5ng/ml of recombinant bovine TGF- β 2 (NIBSC, Potters Bar. UK). Cells were
204 treated for 24 h at 37°C. Catalase activity was ablated with a selective inhibitor
205 Aminotriazole (AT) (Sigma, A8056), and restored by adding bovine catalase (Sigma,
206 C4963-2MG). Cells were treated overnight at a concentration of 1200 μ M with AT
207 and 80U/ml of bovine catalase. For inhibiting CREB transcription, a cell-permeable
208 naphthamide compound that effectively blocks the interaction between the KIX
209 domain of CBP and the KID domain of CREB was used (Calbiochem, CAS 92-78-4)
210 at a concentration of 25 μ M for 1 h at 37°C with 5% CO₂.

211 **Western Blotting:** Cells were harvested and extracted by lysis buffer (20mM Hepes,
212 Nonidet P40 (NP40) 1%, 0.1% SDS, 150mM NaCl, 2mM EDTA, phosphatase
213 inhibitor cocktail tablet (PhosSTOP, Roche) and protease inhibitor cocktail tablet
214 (Complete mini EDTA free, Roche). Protein concentration was determined by the
215 Bradford protein assay. Cell lysates were subjected to Western blot analysis using
216 conventional SDS/PAGE and protein transfer to nitrocellulose filters (Protran,
217 Whatman). The membrane was blocked by 5% non-fat milk-TBST (for anti-catalase),
218 or 3% non-fat milk-PBST (for anti-actin antibody) for 90 min at room temperature
219 (RT).

220 Antibodies used in immunoblotting were as follows: rabbit polyclonal antibody anti-
221 catalase (Cell Signaling) and goat polyclonal antibody anti-actin (I-19, Santa Cruz
222 Biotechnology). Membranes were incubated with peroxidase-conjugated secondary
223 antibody (rabbit anti-IgG and goat anti-IgG (Santa Cruz biotechnology). After

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224 washing, proteins were visualized with ECL western blotting detection reagents
225 (Thermo Scientific) on X-ray films. The level of β-actin was used as a loading control
226 throughout.

227 **Dynamic monitoring of cell migration with the xCELLigence system:** Cell
228 migration assay was assessed using the xCELLigence system (Roche). Medium was
229 added to the bottom chamber of the CIM-Plate 16. The CIM-Plate 16 was assembled
230 by placing the top chamber coated with Matrigel (BD) onto the bottom chamber and
231 snapping the two together. Serum-free medium was placed in the top chamber to
232 hydrate and the membrane was pre-incubated for 1 hour in the CO2 incubator at 37
233 °C. Once the CIM-Plate 16 has equilibrated, it is placed in the RTCA DP station and
234 the background cell-index values are measured. The CIM-Plate 16 is then removed
235 from the RTCA DP station and the cells passaged 24h in serum free medium were
236 added to the top chamber. The CIM-Plate 16 is placed in the RTCA DP station and
237 migration is monitored for several hours.

238 **Matrigel chambers assay:** The invasive capacity of transformed cells was assessed
239 *in vitro* using Matrigel migration chambers, as described (15). Culture coat 96-well
240 medium BME cell invasion assay was obtained from Culturex instructions (3482-096-
241 K). After 24 h of incubation at 37°C, each well of the top chamber was washed once
242 in buffer. The top chamber was placed back on the receiver plate. 100μL of cell
243 dissociation solution/Calcein AM were added to the bottom chamber of each well,
244 incubated at 37°C for 1 h to fluorescently label cells and dissociate them from the
245 membrane before reading at 485 nm excitation, 520 nm emission using the same
246 parameters as the standard curve.

247 **Intracellular levels of hydrogen peroxide (H₂O₂):** 1×10⁵ cells were seeded in 96
248 well plates and incubated 18h in complete medium. Cells were washed in PBS and

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incubated with 100μL per well of 5μM H₂-DCFDA diluted in PBS (Molecular Probes). H₂O₂ levels were assayed by spectrofluorimetry on a fusion spectrofluorimeter (PackardBell). Fluorescence intensity was recorded every hour over a period of 5 h. Excitation and emission wavelengths used for H₂O₂ were 485 and 530nm. The number of cells was evaluated by the crystal violet assay. Cells were stained in 0.05% crystal violet and 2% ethanol in PBS for 30 min at room temperature. After four washes in PBS, the stain was dissolved in methanol and measured at 550nm on Fusion. The level of H₂O₂ was calculated in each sample as follows: reactive oxygen species rate (arbitrary unitsmin⁻¹10⁵ cells⁻¹) = [fluorescence intensity (arbitrary units) at T300 minutes – fluorescence intensity (arbitrary units) at T0] per 60min per number of cells as measured by the crystal violet assay.

Catalase activity assay: A dry pellet of 1×10⁵ cells was lysed in 50μL PBS, 1% NP40. 50μL of lysate, 50μL of anti-peroxydase antibody (1/2000, Sigma) and 50μL H₂O₂ (1/4000, Sigma) were added to a 96-well plate and incubated for 10 min at 37°C in 5% CO₂. 50μL of OPD (SIGMAFAST™, #P9187) was then added and the absorbance immediately read at 405nm. Catalase activity assay was assayed on Fusion. Catalase measurement was reported to the amount of protein in each sample (bovine serum albumin microbiuret assay, Pierce, Bezons, France).

SOD activity: Superoxide dismutase (SOD) activities of cells were evaluated by the nitroblue tetrazolium reduction technique according to Beauchamp and Fridovich (16). SOD measurements were reported to the amount of protein in each sample (Bradford method was used).

Statistical Analysis: Data were analyzed with the Student's t-test. All values are expressed as mean+/-SEM. Values were considered to be significantly different when p values were < 0.05.

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324 **Figure legends:**

325 **Figure 1: Catalase activity is decreased in attenuated *Theileria*-transformed**
 326 **macrophages. A.** SOD activity doesn't change between virulent and attenuated
 327 macrophages. **B.** Catalase activity is diminished in attenuated compared to virulent
 328 macrophages. **C top,** Catalase expression is high in virulent macrophages compared
 329 to attenuated macrophages. **C bottom,** Actin expression is unchanged between
 330 virulent and attenuated macrophages. ROS measurements in A and B were done
 331 independently (n = 3) and in triplicate. **p<0.005 compared to attenuated
 332 macrophages.

333 **Figure 2: TGF- β 2 activates *catalase* transcription in *Theileria*-transformed**
 334 **macrophages. A.** The transcription of *catalase* and *GPx* is decreased in attenuated
 335 compared to virulent macrophages. Adding recombinant TGF- β 2 restores
 336 transcription of *catalase* in attenuated macrophages to virulence levels. No effect was
 337 observed in the transcription level of *GPx*. **B.** Left panel, Catalase activity is down
 338 regulated in attenuated macrophages and rescued by exogenous TGF- β 2 stimulation.
 339 **C.** Right panel, H₂O₂ levels are increased in attenuated compared to virulent
 340 macrophages and is dampened by addition of exogenous TGF- β 2. All experiments
 341 were done independently (n = 3) and in triplicate. * p<0.05 compared to virulent
 342 macrophages; ** p<0.005 compared to virulent macrophages; *** p<0.001 compared
 343 to virulent macrophages; ## p<0.005 compared to attenuated macrophages and ###
 344 p<0.001 compared to attenuated macrophages.

345 **Figure 3: CREB drives *catalase* transcription. A.** *Catalase* transcription is higher in
 346 virulent (V) than attenuated (A) macrophages. Catalase transcription is diminished to
 347 attenuated levels when CREB-mediated transcription in virulent macrophages is

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348 ablated with the specific CREB-CBP interaction inhibitor (25μM 1 h at 37°C). **B.**
 349 Catalase activity is higher in virulent (V) compared to attenuated (A) macrophages,
 350 and CREB-CBP-mediated inhibition of CREB in virulent macrophages reduces
 351 catalase activity to attenuated levels. **C.** H₂O₂ levels are higher in attenuated (A)
 352 compared to virulent (V) macrophages. CREB-CBP-mediated inhibition of CREB-
 353 driven transcription in virulent macrophages increases the level of H₂O₂, while adding
 354 db-cAMP to attenuated macrophages decreases H₂O₂ output. Treatment with CREB-
 355 CBP interaction inhibitor abolishes *catalase* expression and ablates the increase in
 356 H₂O₂ induced by db-cAMP stimulation. All experiments were done independently (n
 357 = 3) and in triplicate. ** p<0.005 compared to virulent macrophages; *** p<0.001
 358 compared to virulent macrophages and # p<0.05 compared to attenuated
 359 macrophages.

360 **Figure 4: TGF-β2 levels regulate both oxidative stress and metastatic potential of**
 361 ***Theileria*-transformed macrophages. A.** Cell migration index of virulent (V)
 362 macrophages is greater than that of attenuated (A) macrophages, but is reduced upon
 363 TGF-R blockade with SB431542. TGF-β2 stimulation of attenuated macrophages
 364 restores their cell migration index, as does addition of active catalase. Boiled catalase
 365 fails to restore attenuated macrophages migration index. **B.** Matrigel traversal is
 366 higher for virulent (V) than attenuated (A) macrophages. Blocking TGF-R-signalling
 367 with SB431542 diminishes traversal of V macrophages, as does AT-induced
 368 inhibition of catalase activity. All experiments were done independently (n = 3) and in
 369 triplicate. * p<0.05 compared to virulent macrophages; ** p<0.005 compared to
 370 virulent macrophages; *** p<0.001 compared to virulent macrophages; # p<0.05
 371 compared to attenuated macrophages and # p<0.05 compared to attenuated
 372 macrophages.

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Figure 5: Observations on *Theileria*-transformed macrophages can be extended to human A549 and HT-29 cancer cell lines. A. H₂O₂ levels are lower in HT-29 and A549 compared to HT-29 and A549 treated with TGF-R inhibitor SB431542. **B.** Catalase activity is higher in HT-29 and A549 compared to HT-29 and A549 treated with SB431542. **C.** The metastatic potential as reflected by matrigel traversal of A549 decreased following CBP-induced inhibition of CREB and TGFR blockade by SB431542. * p<0.05 compared to HT-29 and HCT-116.

Figure S1:
TGF- β 2 regulates oxidative stress in *Theileria*-transformed macrophages. A and B. Catalase activity is augmented in independent clones (H7 & H8) of macrophages isolated from disease-susceptible Holstein-Friesian (H) animals compared to independent clones (S2 & S3) of macrophages isolated from disease-resistant Sahiwal (S) animals. SB431542 blockade of TGF-R abolished heightened catalase expression by independent clonal lines of H macrophages, whereas adding TGF- β 2 to independent clonal lines of S macrophages increased catalase activity. **C.** H₂O₂ output is higher in disease-resistant S macrophages compared to disease-susceptible H macrophages. Blockade of TGF-R-signalling in H macrophages increased levels H₂O₂, while stimulating independent clonal lines of S macrophages with TGF- β 2 decreased H₂O₂ levels. ** p<0.005 compared to Holstein macrophages and ## p<0.05 compared to Sahiwal macrophages.

A **B**

A **B** **C**

A **B** **C**

A

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B

C

A

B

C

A

B

C