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c-Myc activation by *Theileria* parasites promotes survival of infected B-lymphocytes

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Theileria **parasites infect and transform bovine lymphocytes, but host cell immortalization is reversible, as upon parasite death the lymphocytes rapidly die of apoptosis. Infection leads to a marked augmentation in the levels of lymphocyte c-Myc, and the parasite achieves this by inducing increased c-*** ***myc*** **transcription and by prolonging the half-life of the transcription factor. Reduction in c-Myc turnover can be ascribed to CK2-mediated phosphorylation of the transcription factor. A parasite-dependent GM-CSF autocrine loop activates a JAK2/STAT3 signalling pathway that contributes to heightened c-*** ***myc*** **transcription, and inhibition of the pathway leads to caspase 9 activation and apoptosis that can be directly ascribed to a reduction in c-Myc. An antiapoptotic role for c-Myc was clearly demonstrated by specific inhibition of c-*** ***myc*** **expression with antisense oligonucleotides, and this correlates with loss of the antiapoptotic protein Mcl-1, and, consistently, ectopic expression of c-Myc abrogates B-cell death induced upon JAK2 inhibition. Thus, *** ***Theileria*** **parasites ensure the survival of their host lymphocytes via specific activation of c-Myc.**
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Introduction

The c-*myc* proto-oncogene codes for the c-Myc transcription factor that is the cellular homologue of the viral oncogene *v-myc* and deregulated expression of c-*myc* have been observed in a high percentage of human cancers (for recent reviews, see Pelengaris *et al*., 2002; Nilsson and Cleveland, 2003). This implies that c-*myc* deregulation contributes to tumorigenesis by modulating specific target genes. The c-Myc transcription factor is a helix–loop leucine zipper protein that binds, in collaboration with its obligate binding partner Max, to specific sites called E-boxes (5′-CACGTG-3′) in the promoters of at least 1382 different genes (Zeller *et al*., 2003; http://www.myccancergene.org/site/mycTargetDB.asp). The large number of c-*myc* target genes probably explains the wide range of diverse functions that c-Myc appears to regulate *in vivo*. Given its well-established role in cell cycle progression, particularly entry into G1, it was a surprise when ectopic expression of c-*myc* in cell lines induced apoptosis (Evan *et al*., 1992). This apparent contradiction gave rise to the concept that cancer cells must have escaped from programmed cell death for tumours to arise, and that proliferation and apoptotic pathways are somehow linked. Thus, proliferation occurs when survival signals predominate over death-promoting ones, the specific players depending on the cellular context. In contrast to proliferation, the c-*myc* target genes that directly mediate apoptosis have not been convincingly identified. However, it is well established that c-*myc* indirectly regulates both the Bcl-2 family and Arf-Mdm2-p53 pathways and in this way influences the balance between survival and apoptosis (see reviews and references therein; Pelengaris *et al*., 2002; Nilsson and Cleveland, 2003).

Increased levels of c-Myc can involve both transcriptional upregulation as well as increased protein stability, since under normal conditions c-Myc is turned over rapidly. Many of the signal transduction pathways that are induced in activated cells lead to c-*myc* transcription and this often involves growth factors/cytokines. For example, pathways leading to E2F activation can result in E2F-mediated transactivation of c-*myc*, and, in addition, E2F itself is a c-*myc* target gene (Albert *et al*., 2001; Leone *et al*., 2001). B-cell growth can be controlled by phosphatidylinositol-3-kinase (PI3-K)-mediated activation of NF-κB-regulated c-*myc* transcription (Grumont *et al*., 2002), and platelet-derived growth factor (PDGF) induces c-*myc* expression through a JNK- and AP-1-dependent signalling pathway (Iavarone *et al*., 2003). Furthermore, STAT3 is required for gp130-mediated full activation of c-*myc* (Kiuchi *et al*., 1999) and STAT3-mediated c-*myc* expression is necessary for Src transformation (Bowman *et al*., 2001). Activation of STAT3 following engagement of gp130-containing cytokine receptors is often

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mediated by JAK2 and inhibition of JAK2 by AG490 has been used to treat acute lymphoblastic leukaemia (ALL), with no deleterious effect on normal haematopoiesis (Meydan *et al*., 1996). Interestingly, constitutive activation of STAT3 signalling also confers resistance to apoptosis in myeloma cells (Catlett-Falcone *et al*., 1999) and its inhibition leads to apoptosis of leukaemic large granular lymphocytes (Epling-Burnette *et al*., 2001). Phosphorylation of c-Myc appears to both positively and negatively regulate its half-life, as threonine 58 and serine 62 in the amino-terminus are substrates for both glycogen synthase kinase-3 (GSK3) and mitogen-activated protein kinases (MAPK) that accelerate its degradation by the proteasome (Henriksson *et al*., 1993). In contrast, casein kinase II (CK2) phosphorylation of the C-terminal PEST domain protects c-Myc from proteasomal degradation (Channavajhala and Seldin, 2002). Thus, the overall level of c-Myc within a cell is the sum of these different transcriptional and post-translational events.

Theileria are protozoan parasites that invade and immortalize bovine lymphocytes, but the transformed state can be reserved by killing the parasite with a specific drug (BW 720c, or Buparvacone) that has no effect on noninfected host lymphocytes (for a review, see Dobbelaere and Heussler, 1999). The transformation process is associated with the constitutive activation of CK2 (ole-MoiYoi *et al*., 1992) and although *Theileria*-infected T cells were the first example where increased CK2 activity was associated with cellular transformation, the consequences of increased CK2 activity on parasite-provoked lymphocyte immortalization have yet to be elucidated. In contrast, survival of a significant proportion of infected lymphocytes has been ascribed to the parasite permanently activating the NF-κB signalling pathway, probably through physical association with the IκB-signalosome (Heussler *et al*., 2002), but the actual mechanism of NF-κB-mediated protection remains elusive. Nonetheless, it is clear that the presence of live intracellular parasites leads to constitutive induction of a number of antiapoptotic proteins (Kuenzi *et al*., 2003) and a decrease in proapoptotic ones (Guergnon *et al*., 2003a), suggesting that *Theileria* somehow alters the balance of these key regulators in favour of survival. Infected lymphocytes also use autocrine loops to augment their proliferation and we have shown that a TNF-autocrine loop contributes to NF-κB activation, yet inhibition of TNF did not provoke apoptosis (Guergnon *et al*., 2003a). This implies that death receptor signalling is not manipulated by the parasite to assure host lymphocyte survival and, consistently, upon parasite death one observes strong induction of an intrinsic (caspase 9 to caspase 3) pathway of programme host cell death (Guergnon *et al*., 2003b). Another autocrine loop operative in *Theileria*-transformed B cells involves GM-CSF and this leads to sustained PI3-K activation and AP-1 induction (Baumgartner *et al*., 2000). Constitutive Src activity also characterizes *Theileria*-transformed B cells and this occurs by perpetual exclusion of the negative regulator Csk from Hck-positive rafts/GEMS (Baumgartner *et al*., 2003). Since *Theileria* transformation induces a GM-CSF autocrine loop, we asked, could it activate a JAK2/STAT3 signalling pathway leading to c-Myc transactivation and if so, does c-Myc provoke survival of *Theileria*-transformed lymphocytes? We show here that *Theileria* induces a strong and sustained induction of c-Myc by intervening with both c-*myc* transcription and c-Myc stability, and that infection rapidly translates into a c-Myc-mediated antiapoptotic response that involves Mcl-1.

Results

Theileria *infection of B cells induces constitutive phosphorylation of STAT3, as well as c-Myc and Mcl-1 induction*

As *Theileria* infection leads to immortalization of host lymphocytes and a GM-CSF autocrine loop (Baumgartner *et al*., 2000) contributes to uncontrolled B-cell proliferation, we asked if this was associated with activation of a signalling pathway resulting in c-Myc induction. One activator of c-*myc* transcription is STAT3, which in *Theileria parva*-infected B cells is constitutively phosphorylated compared to noninfected B-lymphocytes (Figure 1a). Constitutive phosphorylation of STAT3 in B cells transformed by *T. parva* occurs without any increase in STAT3 protein levels. Moreover, c-Myc protein can readily be detected in *Theileria*-transformed B cells, whereas it is undetectable in noninfected B-lymphocytes (Figure 1a). To confirm that constitutive phosphorylation of STAT3 and c-Myc induction is directly due to *Theileria* infection, transformed B cells were treated with a parasitocidic drug (BW 720c) and the changes in STAT3 phosphorylation and c-Myc expression were followed over time (Figure 1b). Within 24 h, endogenous c-Myc levels rapidly decrease and loss of STAT3 phosphorylation becomes marked between 48 and 72 h, a time at which Mcl-1 (a c-Myc target) levels decrease. No changes in the overall amounts of STAT3 were observed.

As STAT3 signalling to c-*myc* can involve the JAK2 kinase, we inhibited JAK2 specifically using low doses of AG490 and monitored both STAT3 phosphorylation and c-Myc induction (Figure 1c). As little as 6 h treatment with 3.12 μM AG490 resulted in complete ablation of STAT3 phosphorylation, and at 12.5 μM there was a significant drop in endogenous c-Myc. Over the time course studied, blocking JAK2 signalling induced a more rapid and pronounced downregulation of STAT3 phosphorylation than direct killing of *Theileria* (compare panels b and c). One possible explanation is that culture supernatants take time to become depleted in JAK2-dependent cytokines following parasite death. Conversely, BW 720c-induced parasite death reduced lymphocyte c-Myc levels within 24 h, arguing that other *Theileria*-dependent signal transduction pathways contribute to c-Myc induction, and reduced c-Myc half-life upon parasite death likely also contributes to rapid c-Myc loss (see below and Discussion).

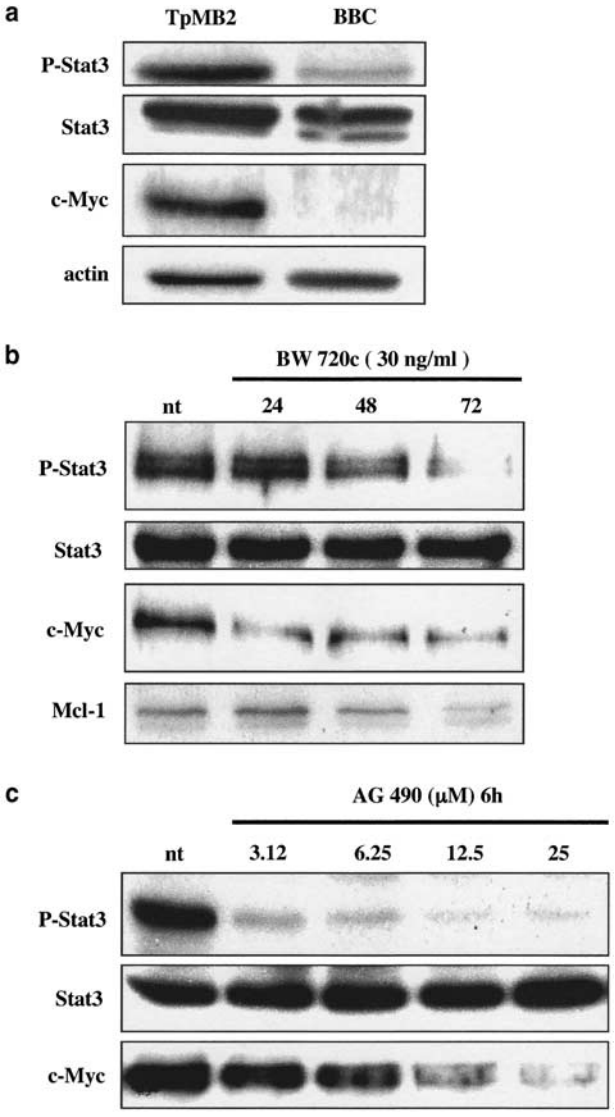

Figure 1 *Theileria* transformation of B lymphocytes involves constitutive JAK2/STAT3 activation and c-Myc and Mcl-1 induction. (**a**) The levels of phospho-STAT3, STAT3, c-Myc and actin were compared in total cell extracts of infected (TpMB2) and noninfected bovine CD21 + B cells (BBC). *Theileria* transformation induces phosphorylation of STAT3 and induction of c-Myc. (**b**) Drug-induced elimination of *Theileria* leads to loss of STAT3 phosphorylation and c-Myc and Mcl-1 induction. To eliminate the parasite, TpMB2 cells were incubated with BW 720c (30 ng/ml) for 0, 24, 48 or 72 h and cells extracts were analysed by Western blotting. (**c**) TpMB2 cells were treated with increasing, but low, doses (3.12, 6.25, 12.5 and 25 μM) of AG490 for 6 h and the levels of phospho-STAT3, STAT3 and c-Myc determined. Inhibition of JAK2 leads to loss of STAT3 phosphorylation and c-Myc induction

Theileria-*dependent post-translational stabilization of c-Myc involves CK2*

As increased c-Myc levels can result from reduced turnover of the transcription factor, we examined whether parasite infection affected c-Myc protein stability. Infected B cells were pretreated for 18 h with BW 720c to induce parasite death and then initiation of transcription was arrested by 10 μg/ml cycloheximide (CHX) treatment of both infected, non-BW 720c-treated and drug-treated B cells, and the quantity of c-Myc compared to actin followed in time (Figure 2a). In lymphocytes where short-term BW 720c treatment has initiated parasite death, c-Myc levels were essentially unchanged prior to CHX addition, but 50 min after the block in *de novo* protein synthesis c-Myc levels were almost as low as that associated with noninfected lymphocytes. In contrast, in B cells harbouring live intracellular parasites, c-Myc half-life was significantly prolonged and protein levels appeared unchanged, even 50 min after the block in *de novo* protein synthesis.

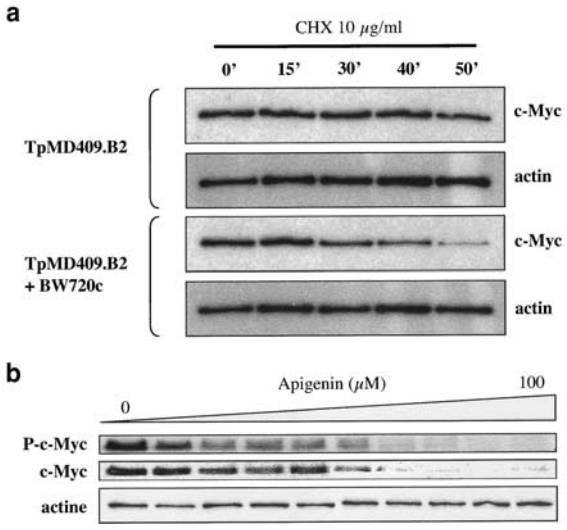

Figure 2 *Theileria*-induced post-translational stabilization of c-Myc involves CK2. (**a**) Infected B cells were pretreated for 18 h with BW 720c to induce parasite death and then initiation of transcription was arrested by 10 μg/ml CHX treatment of both infected, non-BW 720c-treated and drug-treated B cells and the quantity of c-Myc compared to actin followed in time. From 30 min onwards post-addition of CHX, there is a decrease only in c-Myc in BW 720c-treated B cells such that at 50 min c-Myc has return to normal levels. (**b**) Inhibition of CK2 by increasing doses of Apigenin (from 0 to 100 μM in increments of 10 μM) ablates c-Myc phosphorylation and proteins levels, with actin remaining unchanged. Thus, live intracellular parasites augment the stability of c-Myc via CK2-mediated phosphorylation

It has been known for some time that *Theileria* infection leads to an increase in lymphocyte CK2α activity (ole-MoiYoi *et al*., 1992) and CK2α has been demonstrated to increase the stability of c-Myc via phosphorylation of its C-terminal PEST sequence (Channavajhala and Seldin, 2002; Cavin *et al*, 2003). Therefore, we treated infected B cells for 6 h with increasing doses of the specific CK2 inhibitor Apigenin, and monitored both the phosphorylation status and stability of c-Myc (Figure 2b). One can see that 6 h treatment with as little as 50 μM of Apigenin leads to ablation of c-Myc phosphorylation and concomitant protein degradation, with actin levels remaining unchanged even at high inhibitor doses. Thus, the presence of live *Theileria* parasites leads to a CK2-mediated increase in the stability of c-Myc and this contributes to the high levels of c-Myc protein observed in Figure 1.

Theileria-*induced transcriptional activation of c*-myc *is mediated in part by GMC-SF via activation of STAT3*

To determine whether *Theileria* also induces high c-Myc levels via transcriptional regulation, we first monitored c-*myc* promoter activity in infected cells and, as shown in Figure 3a, the c-*myc* promoter is constitutively activated. Importantly, c-*myc* transactivation is dependent on live parasites, as luciferase levels diminish upon drug-induced parasite death (Figure 3b). One way the parasite induces c-*myc* transcription is via the GM-CSF autocrine loop that we have previously described (Baumgartner *et al*., 2000), as addition of 50 ng/ml of recombinant bovine GM-CSF to infected cells increases endogenous c-*myc* transactivation within 6 h and a three-fold increase is observed by 24 h (Figure 3c). In addition, we verified that elevated levels of endogenous c-Myc lead to transcriptional transactivation of c-Myc target genes. To this end, we stably introduced into *Theileria*-transformed B cells a minimal promoter containing 4 E-box elements upstream of the luciferase

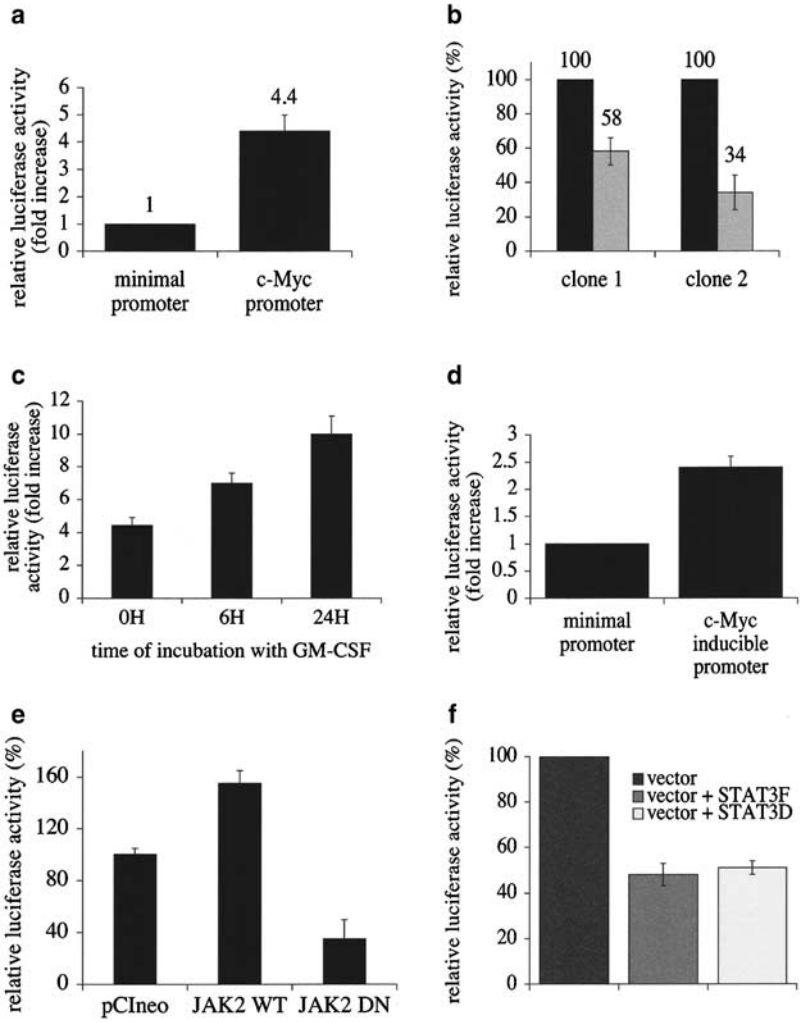

Figure 3 Transactivation of c-*myc* is STAT3-dependent. (**a**) A large (−2300 and + 500 bp relative to the transcription initiation site) promoter region of c-*myc* driving luciferase gene expression was transiently transfected into TpMB2 cells, together with a minimal promoter control. *Theileria* induces a fourfold increase in c-*myc*-driven luciferase activity. (**b**) Constitutive c-*myc* transcription is parasite-dependent. The same c-*myc*-luciferase construct was stably introduced into TpMB2 cells to generate TpMB2MP2 and the parasites killed in two independent clones. This led to a 58 and 34% drop in c-*myc*-driven luciferase activity. (**c**) Endogenous c-*myc* transactivation was stimulated by the addition of exogenous recombinant GM-CSF at 50 ng/ml. A stimulatory effect is seen as early as 6 h and is threefold higher at 24 h. (**d**) Parasite-induced transcription of c-*myc* target genes. A plasmid reporter containing four c-Myc (E-box) fixation sites driving the luciferase gene was stably introduced into TpMB2 cells. (**e**) JAK2 contributes to c-*myc* transcription in *Theileria*-transformed B cells. The c-*myc*-luciferase reporter plasmid was co-transfected with a vector control, or with wild-type JAK2, or dominant-negative, kinase-dead JAK2. Transient expression of wild-type JAK2 stimulates c-*myc*-driven luciferase, while kinase-dead JAK2 inhibited it. (**f**) STAT3 contributes to c-*myc* transcription in *Theileria*-transformed cells. The same c-*myc*-luciferase reporter plasmid was co-transfected with a vector control (black histogram), or with dominant-negative mutants of STAT3 (STAT3F, grey histogram; STAT3D, white histogram, see Materials and methods). All luciferase activities were assessed 48 h after transfection and transfection efficiences were standardized by β-gal expression. Means of four independent experiments and s.d. are shown for all transfections

gene and monitored the change in c-Myc target gene transcription (Figure 3d). It can be seen that *Theileria*-dependent c-*myc* activation results in enhanced activity of a c-Myc-regulated promoter construct and that elimination of the parasite leads to a 70% decrease in c-Myc activity (not shown). Finally, *Theileria*-dependent transcriptional induction of c-*myc* clearly involves JAK2 and STAT3, as in transient assays co-transfection of different trans-dominant-negative mutants markedly reduced c-*myc*-driven luciferase activity (Figure 3e and f).

Theileria-*transformed B cell survival is c*-myc-*dependent*

To examine directly the contribution of c-Myc to survival of *Theileria*-infected B cells, we ablated endogenous c-*myc* transcription with specific antisense oligonucleotides (Figure 4). Treatment with antisense c-*myc* oligomers for 48 h resulted in a significant degree of apoptosis compared to the sense oligonucleotide control (Figure 4a). Antisense-induced cell death correlated with loss of endogenous c-Myc levels, concomitant loss of Mcl-1 and poly(ADP-ribose) polymerase (PARP) cleavage, with no effect on STAT3 phosphorylation status (Figure 4b). Thus, the parasite-dependent induction of the antiapoptotic protein Mcl-1 (see Figure 1b) can be directly ascribed to c-Myc activation.

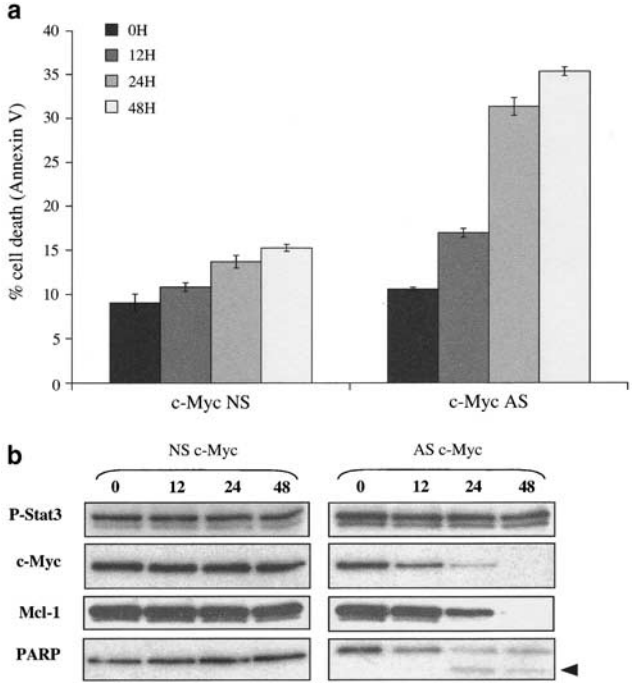

Figure 4 Inhibition of c-*myc* and Mcl-1 in TpMDB2 cells treated with antisense c-*myc* oligonucleotides. (**a**) TpMB2 cells are incubated with either antisense (AS) or nonsense (NS) oligonucleotides for 0, 12, 24 and 48 h, as indicated. Cell death is assessed by phosphatidyl serine exposure (Annexin-V binding). Within 24 h of treatment (grey columns), there is significant apoptosis. (**b**) TpMB2 cells are incubated with either antisense (AS) or nonsense (NS) oligonucleotides for 0, 24 and 48 h and total cell lysates analysed by Western blotting for phosphotyrosine 705-STAT3, c-Myc, Mcl-1 and PARP. From 24 h onwards, there is a decrease in c-Myc and Mcl-1 and PARP cleavage, with no change observed in STAT3 phosphorylation status

Inhibition of JAK2 results in caspase-dependent apoptosis of Theileria-*transformed B cells*

We have already noted that BW 720c-induced parasite death leads to caspase-dependent lymphocyte apoptosis (Guergnon *et al*., 2003b) and that this correlates with an ablation of STAT3 phosphorylation and a drop in c-Myc levels, an event paralleled by AG490 treatment (Figure 1). Therefore, we analysed in some detail the loss in B-cell viability upon AG490 treatment (Figure 5). Clearly, AG490-induced cell death also involves caspases, as it can be completely blocked by the pan-caspase inhibitor z-VAD (Figure 5a). Short-term (6 h) AG490 treatment led to a sixfold increase in Annexin-V positivity (5–35%), without any increase in membrane permeability as reflected by PI staining, whereas long-term (12 h) AG490 treatment generated strongly PI-positive cells (data not shown). In addition, similar to direct parasite death (Guergnon *et al*., 2003b), loss of cytokine-mediated induction of JAK2 results in activation of caspase 3 and 9, but not caspase 8 (Figure 5b), and concomitant with caspase 9 activation there is PARP cleavage (Figure 5c), Annexin-V positivity and nuclear fragmentation (Figure 5d). Thus, deprivation of JAK2-dependent cytokine signalling associated with *Theileria*-induced transformation mimics parasite death and leads to induction of an intrinsic pathway of programmed B-cell apoptosis.

Ectopic expression of c-myc *rescues* Theileria-*infected B cells from AG490-mediated apoptosis*

As apoptosis following JAK2 inhibition appears to be due to loss in the *Theileria*-dependent constitutive activation of c-Myc, it follows that forced ectopic expression of c-*myc* should counterbalance AG490-mediated apoptosis. This was demonstrated by transiently transfecting *Theileria*-infected B cells with c-*myc* under the control of a CMV promoter (Figure 6). Ectopic expression of c-*myc* resulted in a marked improvement in the survival of AG490-treated B cells compared to vector control, as cell death had been reduced by half (Figure 6a). As expected, enhanced expression increased c-Myc levels without altering the amount of STAT3, or its phosphorylation status (Figure 6b).

Discussion

Theileria infection leads to host cell transformation and we have shown that this is associated with a marked upregulation in endogenous levels of lymphocyte c-Myc (Figure 1). *Theileria* achieves this augmentation via constitutive activation of c-*myc* transcription (Figure 3), and parasite-dependent stabilization of the transcription factor (Figure 2), the combination of these two events generating a significant c-Myc-mediated antiapoptotic

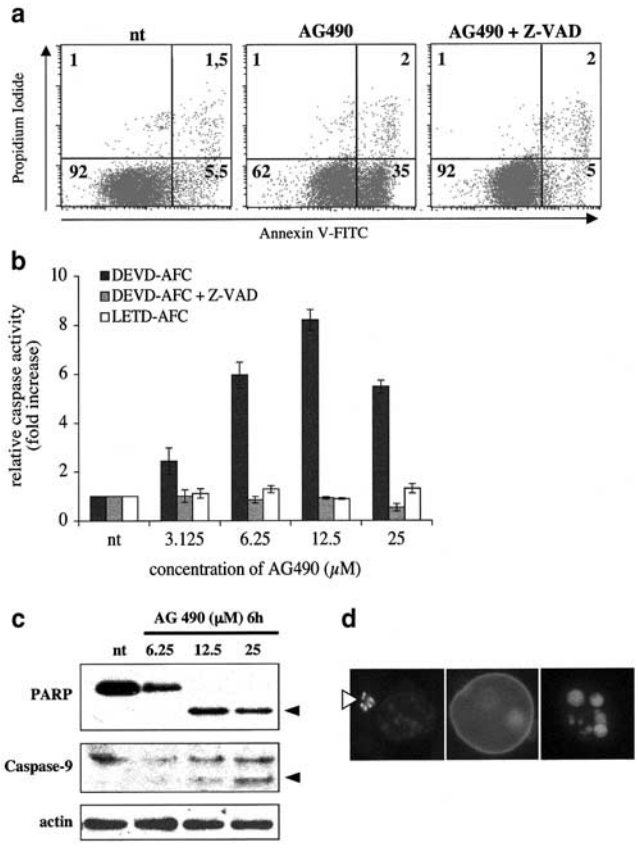

Figure 5 Inhibition of JAK2 leads to mitochondria-dependent B-cell apoptosis. (**a**) AG490-induced apoptosis is caspase-dependent. TpMB2 cells (left panel) were treated for 6 h with 12.5 μM of AG490 (middle panel), together with 50 μM of the general caspase inhibitor z-VAD-fmk (right panel). (**b**) Dose-dependent AG490 inhibition of JAK2 leads to activation of caspase 9, but not caspase 8. TpMB2 were treated with 0, 3.125, 6.25, 12.50 and 25.00 μM of AG490 for 6 h alone, or with z-VAD-fmk (50 μM). Following AG490 treatment, DEVD-like (black histogram) and LETD-like (grey histogram) activities were measured, as described in 'Materials and methods'. DEVD activity was also measured in the presence of z-VAD-fmk. (**c**) JAK2 inhibition and caspase activation lead to PARP cleavage. RIPA extracts from TpMB2 cells were analysed by Western blot with specific antibodies against PARP, caspase 9 and actin. (**d**) Left, Hoechst DNA stain of infected TpMB2 cell nucleus (large blue) and parasite multinucleated macroshizont (small blue dots, indicated with arrow). JAK2 inhibition leads to both Annexin-V positivity of B-cell plasma membrane (middle) and nuclear fragmentation (right)

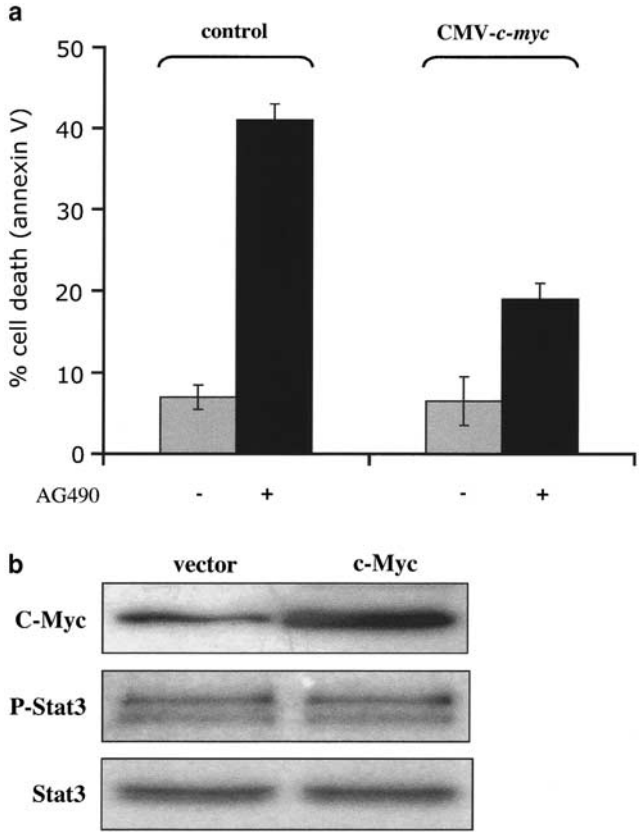

Figure 6 Ectopic expression of c-Myc rescues TpMB2 cells treated with AG490. (**a**) TpMB2 cells were transiently transfected with vector control (left), or CMV-c-*myc* (right) and 42 h later treated (grey columns) for 6 h with AG490 (25 μM) prior to Annexin-V staining and FACS acquisition at 48 h. The results are expressed as percentage viability compared to vector control and one can see that ectopic c-Myc expression lead to a 50% increase in survival following AG490 treatment (compare the left and right columns). (**b**) Extracts of transfected cells were examined for levels of c-Myc, phospho-STAT3 (P-STAT3) and STAT3. Ectopic expression of CMV-c-*myc* augments c-Myc levels, but does not effect STAT3 or its phosphorylation status

response that involves Mcl-1 and underlies infected B-cell survival (Figures 1, 4 and 6).

One of the ways that *Theileria* induces c-*myc* transcription is through activation of a GM-CSF autocrine loop (Baumgartner *et al*., 2000), since addition of exogenous recombinant GM-CSF to infected cells stimulated even further c-*myc* transactivation (Figure 3c). The GM-CSF receptor via the gp130 chain binds and activates JAK2 and we have shown that JAK2 inhibition by AG490 leads to loss of STAT3 phosphorylation and a reduction in the level of endogenous c-Myc (Figure 1c). In addition to AG490, transfection of a kinase-dead mutant of JAK2 leads to a reduction in c-Myc (Figure 3e) and transfection of two different dominant-negative mutants of STAT3 also decreased c-*myc*-driven luciferase activity, reinforcing the notion that this JAK/STAT3 signalling pathway contributes to c-*myc* activation (Figure 3e and f). Although we have clearly demonstrated that GM-CSF contributes to induction of c-*myc* transcription, we do not exclude that other gp130-dependent cytokines such as IL-6 potentially secreted by *Theileria*-transformed lymphocytes (McGuire *et al*., 2004) could also activate JAK2 and c-*myc*, via yet-to-be described autocrine loops. Indeed, we strongly suspected that other cytokines might be maintaining JAK2 and c-Myc activated, since specific inhibition of GM-CSF reduced proliferation, but did not generate apoptosis (Baumgartner *et al*., 2000). We reasoned that AG490 treatment that blocked all JAK2-signalling and ablated c-Myc activation might generate significant apoptosis and this indeed turned out to be the case (Figure 5), the conclusion being that our

previous failure to observe B-cell apoptosis upon GM-CSF inhibition is due to maintenance of JAK2 signalling by other cytokines, although the other cytokines involved have to be characterized. However, one should bear in mind that the cytokine profile probably depends on the type of leukocyte infected by *Theileria*, as several years ago when analysing macrophages we failed to detect STAT activity related to IFN-γ activation, drawing into question a role for this cytokine in these infected cells (Chaussepied and Langsley, 1996).

Does *Theileria* induce transcription of c-*myc* in ways more than one? Close inspection of panels b and c of Figure 1 reveals that multiple pathways may be involved, since parasite death provokes a rapid drop in c-Myc levels that occurs prior to any detectable loss in STAT3 phosphorylation. This suggests that transcription of c-*myc* does not uniquely depend on the JAK2/STAT3 pathway that we have described here. Indeed, E2F and AP-1 are constitutively active in *Theileria*-transformed lymphocytes (Chaussepied *et al*., 1998, unpublished) and transient transfection of dominant-negative mutants of both these transcription factors decreases c-*myc*-driven luciferase activity (data not shown). Therefore, BW 720c-induced parasite death would result not only in a reduction of JAK2/STAT3, but also E2F- and AP-1-driven transcription of c-*myc* (see the schema in Figure 7). Moreover, we have shown (Figure 2) that CK2-mediated phosphorylation increases c-Myc's half-life and BW 720c-induced parasite death leads to a more rapid turnover of c-Myc. Therefore, it is likely that losing all the three transcriptional activities, combined with reduced c-Myc stability, probably explains the very rapid drop in c-Myc levels observed upon parasite death in Figure 1b. Finally, the ablation of STAT3 phosphorylation by AG490 treatment that precedes reduction in c-Myc (Figure 1, panel c) might be due to continued JAK1 signalling to c-*myc* perhaps via STAT5, as this combination can be activated by a number of cytokines, such as IL-10 known to be secreted by *Theileria*-transformed lymphocytes (Mckeever *et al*., 1997).

Loss of c-Myc appears to be largely responsible for AG490-induced apoptosis and this conclusion is underscored by the fact that treatment with antisense c-*myc* oligonucleotides leads to a specific drop in endogenous c-Myc and Mcl-1 levels and apoptosis, as determined by Annexin-V staining and PARP cleavage (Figure 4). No change in STAT3 phosphorylation status was observed, nor did sense c-*myc* oligonucleotides have any effect on c-Myc and Mcl-1 levels. Furthermore, a significant degree of the apoptosis that arises following AG490 treatment can be abrogated by ectopic c-*myc* expression (Figure 6a). Again, this occurs without an alteration in STAT3 levels, or phosphorylation status (Figure 6b). Given that ectopic c-Myc expression does not rescue 100% of the cells from dying, we cannot exclude that loss of JAK2 signalling might provoke apoptosis via some other factor, but we consider it unlikely (see below).

The infected B-cell apoptosis that occurs upon AG490 treatment (Figure 5) has all of the hallmarks that we have observed to occur upon parasite death (Guergnon *et al*., 2003b). Both lead preferentially to activation of capase 9, rather than caspase 8, and subsequent caspase 3-mediated cleavage of PARP. This is compatible with activation of an intrinsic pathway of programmed cell death and is incompatible with death receptor signalling from TNF, or FAS receptors playing a role (Chen and Goeddel, 2002). In this context, we have previously described that *Theileria*-transformed B cells use a TNF-autocrine loop to augment their proliferation, but that inhibition of TNF signalling did not result in apoptosis (Guergnon *et al*., 2003a). The noninvolvement of TNF in survival signalling is consistent with the nonactivation of caspase 8 and nonactivation of an extrinsic pathway of apoptosis. Nonetheless, TNF does contribute to NF-κB activation in *Theileria*-transformed B cells (Guergnon *et al*., 2003a) and it is significant that the apoptosis generated upon inhibition of JAK2 can be ascribed to loss of c-Myc. Clearly, therefore, there are two major antiapoptotic responses activated by *Theileria*

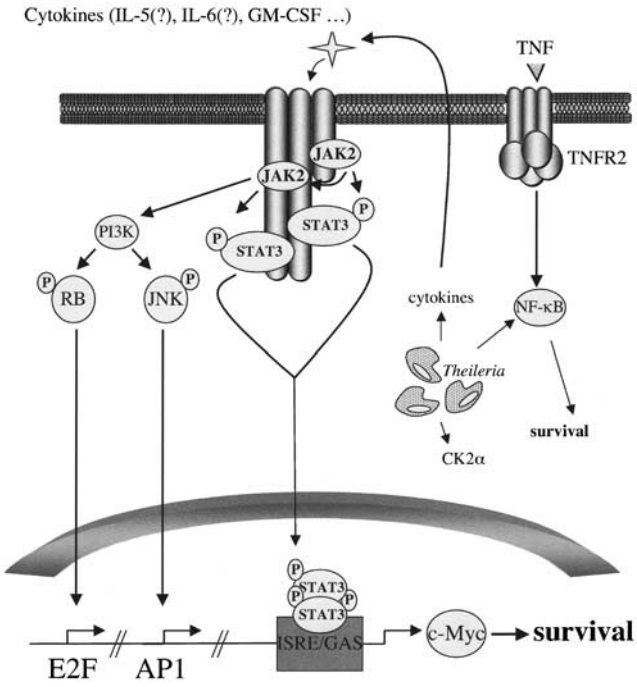

Figure 7 *Theileria* infection activates multiple pathways to induce c-Myc? *Theileria* infection induces transformed lymphocytes to secrete GM-CSF and potentially other cytokines such as IL-5 and Il-6 that signal through gp130/JAK2-specific receptors and activate STAT3, PI3-K, AP-1 and E2F, and all the three transcription factors contribute to c-*myc* transcription. Upon AG490 treatment one only loses STAT3 activation, but upon parasite death the transactivation capacity of all the three transcription factors decreases. In addition, upon parasite death one also loses *Theileria*-dependent prolongation of c-Myc's half-life due to loss of CK2 activation, and this, combined with reduced transcription, results in a rapid drop in c-Myc levels and B-cell apoptosis. *Theileria* activates c-Myc via pathways that do not lead to NF-κB induction, implying that the parasite induces two major anti-apoptotic responses to assure survival of its host cell. However, we do not exclude that some of apoptosis due to loss of NF-κB might also be due to a drop in c-Myc activation, as it is known that NF-κB can contribute c-*myc* transcription

and the pathways leading to their activation appear to be mutually exclusive. However, as NF-κB can regulate c-*myc* expression (Grumont *et al*., 2002), it is possible that some of the parasite-induced NF-κB-dependent survival signalling (Heussler *et al*., 2002) occurs via activation of c-*myc*.

Theileria infection also post-translationally induces increased stability of the c-Myc protein via activation of CK2 (Figure 2). Thus, the parasite exploits two mechanisms (increased transcription and stability) to augment c-Myc levels. Interestingly, transgenic mice overexpressing CK2α generate lymphomas, and when these transgenic mice co-express a c-*myc* transgene the propensity to develop lymphomas increased dramatically (Seldin and Leder, 1995). The similarity with *Theileria* infection is striking and argues that co-activation of both c-Myc and CK2α could significantly contribute to the parasite's ability to transform bovine lymphocytes. We have shown here that one way this could occur is by promoting survival of the infected cell.

Materials and methods

Cells and cell culture

TpMD409.B2 is a *T. parva Muguga*-infected B-cell clone (B2) described elsewhere (Dobbelaere *et al*., 1990) and is further referred to in this paper as simply TpMB2. The B-cell characteristics of this line grown in our laboratory have been confirmed (Moreau *et al*., 1999), and its cultivation has been described previously (Chaussepied *et al*., 1998). To eliminate the parasite, the hydroxynaphtoquinone derivative BW 720c (Hudson *et al*., 1985) was added at 30 ng/ml. BW 720c treatment was performed for 18–24–48–72 h, and cell viability was routinely tested by trypan blue exclusion. TpMB2MP2 is a TpMB2-derived cell line that stably expresses c-*myc* promoter-driven luciferase gene expression construct (pCG-362.5-myc-luc) containing the region from –2300 to +500 bp relative to the transcription initiation site of c-*myc* P1 promoter. TpM-M4EB1 is a TpMB2-derived cell line that stably expressed a plasmid reporter pGL2-Myc4-luciferase containing four sites for Myc fixation (E box: 4XTCCTGACGACCACGTGGTC TTACGGA). To obtain these two lines, 40 μg of pCG-362. 5-myc-luc, or pGL2-Myc4-luc plasmid, was co-transfected with 1 μg of neomycin expression vector. Transfected cells were seeded at 5×10^4 cells/ml in 96-well plates before selection in 500 μg/ml G418 (Gibco BRL) for 2 weeks. CD21+ non-infected B cells were obtained by flow cytometric (FACS) sorting as described in Baumgartner *et al*. (2003) and were estimated to be between 98.3 and 98.5% pure.

Plasmids expressing wild-type JAK2 and kinase-dead JAK2 have been described (Saharinen *et al*., 2000). Plasmids that express dominant-negative STAT3 were cloned in pCAGGS-Neo driven by chicken *actin b* promoter. Construction STAT3F encodes a protein mutated at Tyrosine 705 and construction STAT3D encodes a protein mutated in its DNA-binding domain (Nakajima *et al*., 1996).

Transient transfection experiments

Exponentially growing cells were harvested at 3.5×10^5 cells/ml and washed once in RPMI 1640. Approximately 5×10^6 cells were collected in 500 μl of RPMI 1640 containing 1 mM dithiothreitol (DTT) and 1 mM sucrose. Cells were transfected by electroporation performed in 4 mm gap cuvettes (500 μF, 280 V, two pulses at room temperature), followed by 15 min incubation at room temperature. After that, cells were resuspended in 5 ml of prewarmed cell culture medium and grown for 48 h. For transient transfection experiments, transfections were carried out using 15 μg of pCG-362.5-luc, pGL2-Myc4-luc and 25 μg of pCAGGS-Neo HA STAT3F, pCAGGS-Neo HA STAT3D. CMV-driven β-galactosidase-expressing vector (5 μg) for the standardization of transfection efficiency was included in each sample. CMV-c-*myc*, consisting of the coding exons 2 and 3 for human c-*myc*, was obtained from Dr G Sonenshein, Boston University School of Medicine. For transient transfections with this plasmid, TpMB2 cells were transfected with DuoFect Transfection System (Q-BIOgene).

Inhibitors and reagents

The drug buparvaquone (BW 720c) was a gift from Mallinchrodt Vet GmbH. Z-VAD.FMK, the caspase 8 substrate LETD-AFC.TCA and the general caspase substrate DEVD-AFC were from Enzyme System Products. AG490, NS (5′-CACGTTGAGGGGCAT-3′) and AS (5′-AGTGGCGGA GACTCT-3′) c-*myc* were purchased from Calbiochem and Apigenin from Sigma. All reagents were diluted directly in culture medium. For assessment of c-Myc protein, cells were incubated with 10 μM c-*myc* AS, or NS, for 0, 12, 24 and 48 h. CHX was dissolved in ethanol and used at a concentration of 10 μg/ml to inhibit protein synthesis.

Caspase activities

Cells were cultivated in 25 cm^2 flask at 2×10^5 cells/ml and treated with different concentrations of AG490 in the presence or absence of Z-VAD-fmk. Cells were washed twice in PBS and lysed with Ac-DEVD-afc lysis luffer without the Ac-DEVD-afc, as described previously (Yuste *et al*., 2002). The supernatant was clarified by centrifuging at 13 000 *g* in a microcentrifuge at 4°C, and proteins were quantified using the Bio-Rad protein assay Bradford-based method. In all, 25 μg of protein was added to 100 μl of the same lysis buffer containing 20 μM of Ac-DEVD-afc. The samples were incubated at 37°C for 6 h and read with the fluorimeter. The same protocol was used to measure LETD activity.

Cell death (annexin-V binding) assay and FACS analysis

For detection of apoptotic cells, the annexin-V-fluorescent binding assay (Roche) was used, and experiments were performed according to the manufacturer's protocol. Cells (20×10^4 per sample) were acquired in a Becton Dickinson flow cytometer. PI staining excluded dead cells, and only single annexin-V-positive cells were considered as apoptotic.

Immunoblotting

Total protein extracts of TpMB2 cells were prepared by lysing the cells in RIPA lysis buffer. Insoluble debris was removed by centrifugation (13 000 r.p.m. in a microfuge), and 25 μg of protein was separated by denaturating SDS–PAGE and electrotransferred onto a nitrocellulose membrane (Scheicher and Schuell). Incubation with antibodies was performed in 5% nonfat milk in PBS, 0.1% Tween 20. Proteins were detected by horseradish peroxidase-conjugated antibodies, and immunoblots were developed using the ECL detection kit (Amersham). Antibodies used in immunoblotting were as follows: anti-STAT3 (H-190: sc-7179, Santa Cruz), anti-Phospho-STAT3 (Tyr705) (#9131, Cell Signaling), anti-c-Myc (#06-340, Upstate), anti-phospho-c-Myc (#9401,

Cell Signalling), anti-PARP (Enzyme Systems Products) and anti-caspase 9 (sc-8355, Santa Cruz).

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