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The strategies of the *Theileria* parasite: a new twist in host–pathogen interactions

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Theileria parasites infect and transform cells of the ruminant immune system. Continuous proliferation and survival of *Theileria*-transformed cells involves the well-orchestrated activation of several host-cell signalling pathways. Constitutive NF- κ B (nuclear factor kappaB) activation is accomplished by recruiting the IKK (I κ B kinase) complex, a central regulator of NF- κ B pathways, to the surface of the transforming schizont, where it becomes permanently activated. Constitutive activation of the PI-3K–PKB [phosphoinositide 3-kinase–(Akt) protein kinase B] pathway is likely to be indirect and is essential for continuous proliferation. *Theileria*-transformed T cells express a range of anti-apoptotic proteins that can be expected to provide protection against apoptosis induced by death receptors, as well as cellular control mechanisms that are mobilised to eliminate cells that entered a cycle of uncontrolled proliferation.

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Abbreviations

FLIP	FLICE (Fas-associated death domain protein-like interleukin-1 β -converting enzyme) inhibitory protein
IAP	inhibitor of apoptosis protein
IKK	I κ B Kinase
IκB	inhibitory NF- κ B
IL	interleukin
JNK	c-Jun NH2-terminal kinase
MKK	mitogen-activated protein kinase kinase
NF-κB	nuclear factor kappaB
PI-3K	phosphoinositide 3-kinase
PKB	(Akt) protein kinase B
PTEN	phosphatase and tensin homolog deleted on chromosome 10
TNF	tumour necrosis factor
TRAF	TNF-receptor-associated factor

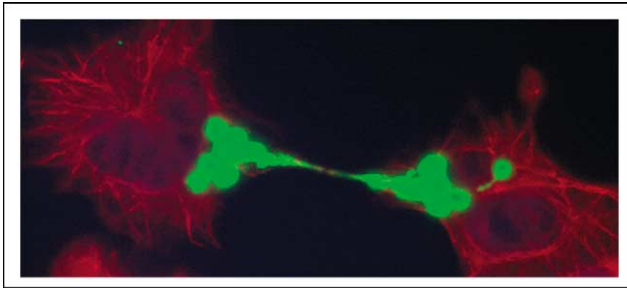
Introduction

Protozoan *Theileria* parasites are transmitted by ticks and cause severe diseases in ruminants (predominantly cattle,

but also sheep and goat), inflicting heavy economic losses to livestock producers in the affected regions. *Theileria* sporozoites, introduced with the tick saliva as the tick takes a blood meal, rapidly invade leukocytes via receptors that have not yet been identified [1]. Shortly after entry, the enveloping host cell membrane is eliminated and the parasite develops into a schizont, which, by contrast to most apicomplexan parasites, resides freely in the host cell cytoplasm rather than in a parasitophorous vacuole. This way, the parasite escapes lysosomal destruction and, from its cytoplasmic location, the schizont is perfectly poised to interfere with host cell signalling pathways that regulate host cell proliferation and survival. The capacity of the schizont to trigger the uncontrolled proliferation of the infected cell is closely associated with the pronounced pathology and mortality that is caused by *Theileria* infections. In many ways, *Theileria*-transformed cells resemble tumour cells; they become independent of exogenous growth factors, can be cloned by limiting dilution, form tumours in immunocompromised mice and acquire a metastatic phenotype that allows parasitized cells to invade and multiply in non-lymphoid as well as lymphoid tissues (reviewed in [2]). One striking difference between these cells and tumour cells is that transformation is entirely reversible. Upon killing of the parasite by treatment with the naphthoquinone derivative BW720c, cell proliferation is halted and, in the case of T cells, exogenous growth factors need to be added to prevent apoptosis of the cured cells [3]. Parasite multiplication appears to be semi-synchronized with the host cell cycle [4] and the tight association of the schizont with the host cell mitotic apparatus ensures the distribution of the schizont over the daughter cells (Figure 1). *Theileria parva*, *Theileria annulata*, *Theileria lestoquardi* and *Theileria taurotragi* infect and transform cells of the immune system. *T. parva* transforms T cells and B cells, whereas *T. annulata* mainly transforms B cells and cells of monocyte or macrophage lineage. The cells targeted by *T. lestoquardi* (a parasite of small ruminants) and *T. taurotragi* (a parasite of water-buck) have not yet been characterised in detail.

At first sight, seeking refuge in those cells that are normally recruited to destroy invading organisms might appear cunning. Inducing uncontrolled host cell proliferation, however, is nothing less than foolhardy, particularly when we consider the fact that immune cells are subject to stringent control mechanisms, with apoptosis frequently used as a lethal device to prevent uncontrolled expansion. This review focuses on how recent findings could help to explain the complex phenotypic changes

Figure 1



Dividing *T. annulata*-transformed cells. During host cell division, the parasite uses the host cell mitotic apparatus to become distributed over the two daughter cells. The *T. annulata* schizont is visualized using an antibody directed against the surface proteins PIM (green; kind gift from J Ahmed, Borstel). Host cell microtubules are stained using an anti- α -tubulin antibody (red); nuclei are stained blue with Hoechst-dye.

that accompany *Theileria*-induced transformation and some models are presented on how parasite-transformed cells escape apoptosis.

Signalling pathways that are activated in *Theileria*-transformed cells

With the exception of interleukin (IL)-10, which is expressed in all *Theileria*-transformed cell lines tested, there is considerable variation in the pattern of cytokine expression [5]. This would suggest that different signalling pathways are activated, depending on the cell type that is infected. An *in vitro* analysis of different cell lines, however, reveals a remarkably similar pattern of signal pathway activation. Thus, members of the src family of protein kinases appear to be activated in all cell lines [6,7,8^{*}], and so is casein kinase 2 [9], a kinase with a broad spectrum of substrates. Three additional pathways will be discussed in more detail in the following sections.

Mitogen activated protein kinases (MAPKs)

MAPKs form part of some of the most ancient signalling pathways and are involved in virtually all aspects of immune responses [10]. It was therefore surprising that, of the three major groups, the extracellular signal-regulated protein kinases (ERKs), the p38 MAPKs and the c-Jun NH₂-terminal kinases (JNKs), only the JNKs were found to be activated [11,12]. This was even more unexpected, because ERK activation has been linked to cell proliferation, whereas JNK (and also p38) is generally associated with the induction of apoptosis. JNK and its cellular substrate c-Jun play an active role in apoptosis; in particular, in Fas-mediated cell death. They regulate FasL expression, repress anti-apoptotic Bcl-2-family proteins, and induce the upregulation of the proapoptotic Bcl-2-family protein BIM (reviewed in [10,13]); however, JNK and c-Jun have also been linked to proliferation. C-Jun is essential for embryonic development and also for

normal cell proliferation — mouse embryo fibroblasts that are deficient for c-Jun show a senescence-like growth arrest *in vitro*. Linking c-Jun to transformation, MEFs (mouse embryo fibroblasts) are resistant to transformation by the Ras oncogene in the absence of c-Jun, and the virally encoded v-Jun is involved in tumourigenesis [13]. It is therefore conceivable that JNK, by inducing permanent activation of the transcription factors AP1 and ATF-2 in *Theileria*-transformed cells [12,14], also contributes to their uncontrolled proliferation.

Constitutive NF- κ B activation

The fact that a potentially lethal pair such as JNK and c-Jun is functional in *Theileria*-transformed cells implies that the cell must have erected a solid line of defence against apoptosis. NF- κ B is constitutively activated in *Theileria*-transformed cells [15,16] and protects *T. parva*-transformed T cells from apoptosis [17]. NF- κ B activation is normally triggered through a range of different surface receptors or intracellular events, including cellular stress. These pathways all converge onto a multi-subunit kinase complex, called IKK [IkB (inhibitory NF- κ B) kinase] [18]. This complex typically consists of two catalytic subunits, IKK1 (IKK α) and IKK2 (IKK β), and a modulating unit NEMO (NF- κ B essential modulator; IKK γ). NF- κ B is sequestered in the cytoplasm by inhibitors (IkBs) that are phosphorylated by activated IKK, tagging them for polyubiquitination and rapid proteasomal degradation. This allows the released NF- κ B to translocate to the nucleus, where it participates in regulating the expression of a range of genes that are involved in immune and inflammatory responses [19], proliferation [20] and survival [21^{**}].

Electrophoretic mobility shift and UV-crosslinking assays that have been performed on *T. parva*-transformed T cells have revealed the abundant presence of p50, p65 and also RelB; p52 and c-Rel, however, were not apparent [22]. The absence of p52 DNA-binding activity indicates that the non-canonical NIK (NF- κ B-inducing kinase)/IKK1-dependent NF- κ B pathway is not active in *T. parva*-transformed T cells. C-Rel is crucial for IL-2 expression in T cells and the absence of both c-Rel and ERK activation in *T. parva*-transformed T cells helps to explain the low level of IL-2 gene transcription [2].

Using digital confocal microscopy, it has been demonstrated that the parasite induces constitutive NF- κ B activation by a novel mechanism, involving the recruitment of large aggregates of IKK signalosomes to its surface [23^{**}]. IKK activation, triggered by pro-inflammatory mediators such as TNF (tumour necrosis factor)- α and IL-1, requires the concerted action of upstream adaptor proteins such as TRAFs [TNF-receptor (TNF-R)-associated factors], as well as a range of kinases and other regulatory molecules, but the involvement of such upstream components in parasite-induced NF- κ B

activation appears to be excluded. Interestingly, this also included RIP2, a kinase with an important function in innate and adaptive immunity and NF- κ B activation in response to intracellular bacteria [24]. Direct interference with the IKK complex or the phosphorylation of its substrate I κ B α , however, blocked parasite-induced NF- κ B activation, and functional IKK2 rather than IKK1 proved to be important. The lack of IKK1 involvement is in agreement with the absence of p52 DNA-binding activity in *T. parva*-transformed T cells. The NF- κ B activation pathway that emanates from the T cell antigen receptor has recently been elucidated in more detail, revealing an important role for CARMA1 (CARD-MAGUK; Caspase recruitment domain-containing membrane-associated guanylate kinase), MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1), Bcl10 and PKC θ (reviewed in [25]). Interestingly, overexpression of dominant-negative mutant forms of CARMA1, Bcl10 and PKC θ , all failed to interfere with parasite-induced NF- κ B activation in *T. parva*-transformed T cells. Likewise, the tumour suppressor cylindromatosis protein, CYLD, which functions as a deubiquitinating enzyme and downregulates TRAF-dependent IKK activation [26], also failed to block parasite-induced NF- κ B activation when overexpressed in *T. parva*-transformed T cells (C Casanova, S Rottenberg and DD, in preparation). Taken together, these findings strongly indicate that *Theileria* bypasses the common upstream regulatory components that link surface receptors to IKK activation. The enforced oligomerisation of IKK components at the parasite surface probably triggers trans-autophosphorylation and activation of IKK in a process known as ‘proximity-induced activation’ [19,27].

It is worth noting that the direct, parasite-induced IKK activation that is observed in the cell lines investigated by Heussler and colleagues [23**] does not necessarily exclude a contribution of classical NF- κ B activation mechanism to the permanently elevated NF- κ B activity in other *Theileria*-transformed cell lines. In this context, Guernon *et al.* [28] recently reported that a TNF α auto-crine loop might contribute, at least in part, to NF- κ B activation in a *T. parva*-transformed B cell line. Compatible with the presence of a parasite-induced direct mode of activation, however, blocking TNF α binding, or TNF-R signalling by overexpression of dn-TRAF2, failed to block NF- κ B activity by more than 50%.

The PI-3K pathway

Of the multiple pathways that control cell proliferation and survival, phosphoinositide kinases occupy a central position. PI-3Ks are recruited to the plasma membrane in response to triggers that are delivered by a wide range of stimuli, including growth factors, immune receptors and extracellular matrix components. PI-3K stimulation results in activation of the serine/threonine kinase Akt (also called PKB), which, in addition to regulating glucose

metabolism, also contributes to cell-cycle regulation and protection against apoptosis (reviewed in [29*]). PI-3K is continuously activated in *Theileria*-transformed cells [30–32], inducing PKB activation [32]. Blocking the PI-3K–PKB pathway resulted in proliferation arrest without inducing immediate apoptosis, indicating a role in the support of proliferation rather than cell survival. In several systems, evidence has been presented for PKB-dependent IKK activation (summarized in [33]). In *T. parva*-transformed T cells, a role for the PI-3K–PKB pathway in parasite-induced constitutive NF- κ B activation has been excluded [23**]. Also, upon parasite elimination, the PI-3K–PKB pathway is downregulated with much slower kinetics than the NF- κ B pathway, arguing against a direct control by the parasite. The hypothesis we presently favour is that PI-3K is activated indirectly through one or more surface receptors or adhesion molecules that are upregulated in *Theileria*-transformed cells. Signals emanating from such surface receptors might persist for some time after the parasite has been eliminated and it cannot be excluded that some of these surface molecules — or their ligands — are expressed in a NF- κ B-dependent manner, as proposed by Baumgartner and co-workers [31]. IL-10, which is expressed in all *T. parva*-transformed cell lines, could qualify as a candidate and, in this context, it is interesting that IL-10 stimulation of PI-3K and p70^{S6K} has been shown to account for the proliferative, but not the anti-inflammatory effects of IL-10 [34].

Theileria-transformed cells: a decided preference for staying alive

Stimulation of FasL gene transcription by JNK via c-Jun in response to a range of stress signals can contribute to the induction of cell death in many different cell types (reviewed in [13]). In addition to c-Jun, NF- κ B also participates in regulating the expression of both FasL [35–37] and Fas [38,39]. Not unexpectedly, both Fas and FasL are expressed in *T. parva*-transformed T cells [40*], but pronounced susceptibility to Fas/FasL-induced apoptosis only appears upon drug-induced elimination of the parasite. In normal T cells, the sensitivity to death-receptor killing is regulated by specific inhibitor proteins, several of which are regulated by NF- κ B. The anti-apoptotic protein c-FLIP [FLICE (Fas-associated death domain protein-like interleukin-1 β -converting enzyme) inhibitory protein] functions as a catalytically inactive form of caspase-8 and blocks Fas-mediated activation-induced cell death (AICD) [41]. In addition to c-FLIP, c-IAP and X chromosome-linked IAP (which block downstream executioner caspases) are constitutively expressed in *T. parva*-transformed T cells. They are rapidly downregulated upon parasite elimination and this coincides with increased caspase activity and apoptosis. Interestingly, in contrast to findings in tumour cell lines, a functional PI-3K–Akt pathway is not essential for c-FLIP expression [40*]. To what extent Fas/FasL

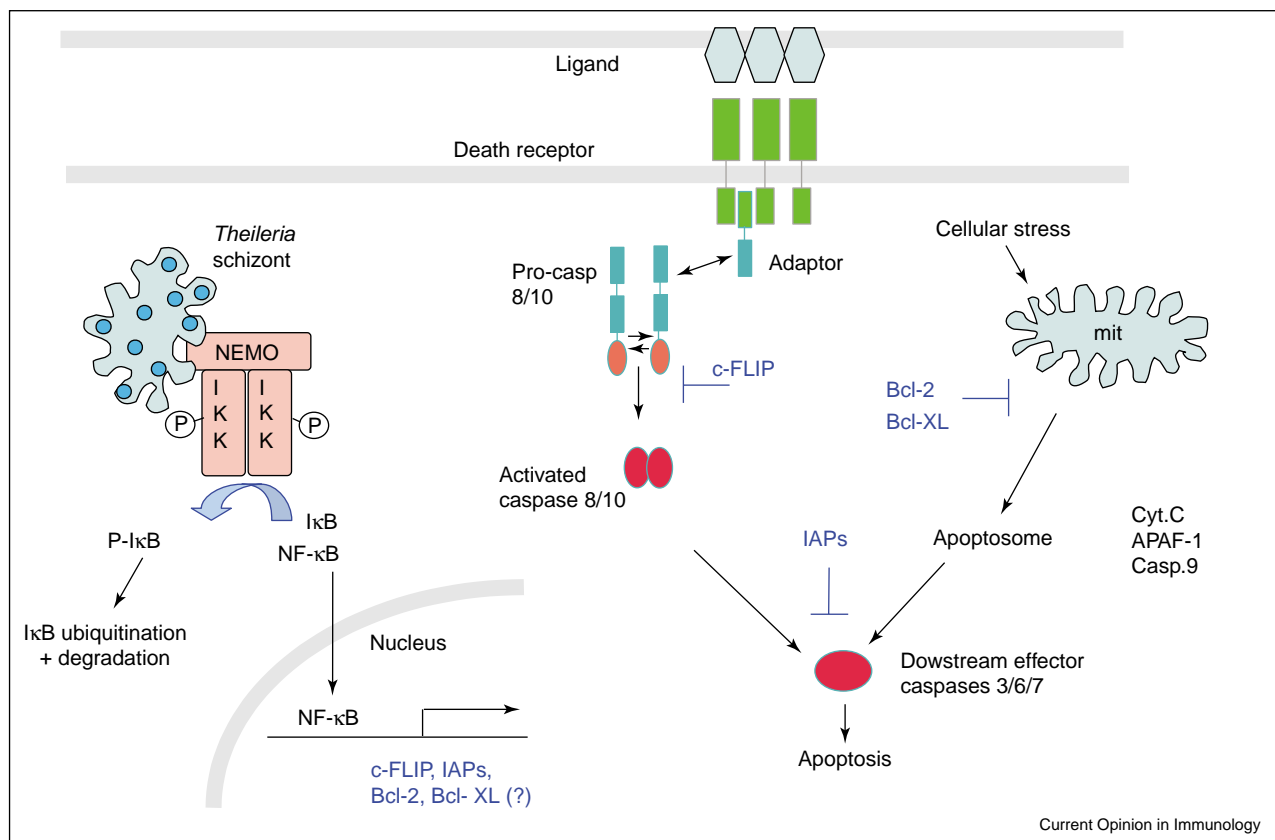
expression plays a role in the extensive lytic processes that accompany Theileriosis is not known. Although *T. parva*-infected B and T cells express TNF receptors, TNF α does not induce apoptosis [28,42]. A logical explanation could be that NF- κ B-dependent upregulation of anti-apoptotic proteins also provides protection against TNF-induced apoptosis. A model for how *Theileria*-transformed cells achieve protection against apoptosis is summarized in Figure 2.

Additional mechanisms of defence against apoptosis may be functional in *Theileria*-transformed cells. NF- κ B has been shown to induce the expression of Gadd45 β (also called MyD118) [43], which blocks MKK (mitogen-activated protein kinase kinase)/JNKK2, an essential JNK activator, preventing TNF α -induced apoptosis [44]. Likewise, NF- κ B-dependent Gadd45 β expression, induced through CD40, protects B cells against Fas-mediated apoptosis [45]. The exact role of Gadd45 β in the crosstalk between NF- κ B and JNK has

not yet been resolved (discussed in [21]). In *Theileria*-transformed cells, JNK is clearly induced in a parasite-dependent manner, despite constitutive NF- κ B activation [11]. JNK activity is not maximal, however, and can be induced further by phorphol ester stimulation. It will be interesting to ascertain whether the function of Gadd45 β in *T. parva*-transformed cells is to keep levels of JNK under control, allowing promotion of proliferation without endangering survival, and to determine how this balance is maintained. Whatever the case, although JNK may have the reputation of ‘a killer on a transcriptional leash’ [46], it appears to be less of a threat in *Theileria*-transformed cells.

The exact role of NF- κ B in regulating the expression of the anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, is still under debate [47]. Both are expressed in *T. parva*-transformed cells, but do not appear to be tightly regulated by the presence of the parasite [40,48]. Bcl-2 was reported to protect T cells from ‘activated T cell

Figure 2



Simplified model, depicting how *Theileria*-transformed cells are protected against apoptosis. The *Theileria* schizont induces constitutive NF- κ B activation by recruiting the IKK complex to its surface. At the schizont surface, IKK is activated and induces the phosphorylation and subsequent proteasomal degradation of I κ B α , the cytoplasmic inhibitor of NF- κ B. In the nucleus, NF- κ B, together with other transcription factors, induces the expression of anti-apoptotic proteins, such as c-FLIP, XIAP, c-IAP1/2 and potentially also Bcl-2 and Bcl-XL. These can provide protection against apoptosis at different levels. For instance, c-FLIP could block the processing of procaspase 8/10, triggered upon ligand-induced activation of death receptors (e.g. Fas). IAPs counter effector caspases, and pro-survival proteins of the Bcl-2 family prevent mitochondria-initiated apoptosis, by regulating cytochrome C release. APAF-1, apoptotic protease activating factor 1.

autonomous death' (ACAD), a form of T-cell death that is distinguishable from that driven by death receptors (reviewed in [49]). Bcl-2 also counters apoptotic pathways induced by cellular stress that involve mitochondria and caspase-9 activation. Caspase-9 and caspase-3 are activated upon elimination of the parasite from *Theileria*-transformed T and B cell lines [40*,48], but to what extent Bcl-2 family members contribute to protection has not yet been established.

Finally, NF- κ B also interferes with pro-apoptotic pathways that are linked to the tumour suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10). PTEN possesses lipid and protein phosphatase activity and acts as a negative regulator of the PI-3K-PKB pathway [29*]. Suppression of PTEN expression by NF- κ B [50*] relies on the sequestration of limiting pools of the transcriptional coactivator CBP (cAMP response element binding protein, binding protein)/p300 by p65 rather than p65 transcriptional activity. Perhaps, in *Theileria*-transformed cells, constitutive I κ B degradation [16] and p65 release helps to facilitate PI-3K activation.

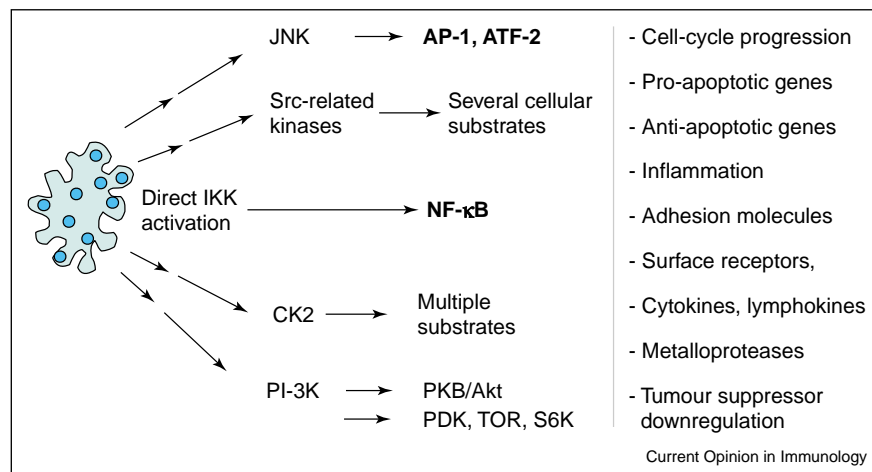
Conclusions

Theileria-transformed cells provide a fascinating example of how pathogen interference with host-cell signal transduction results in the reprogramming of the host cell phenotype. A summary of the main features is presented

in Figure 3. The capacity of pathogens to transform eukaryotic cells is largely restricted to viruses. The similarities between the strategies used by *Theileria* and HTLV-1 are striking [51]. These include constitutive JNK and ATF-2 activity, as well as permanent NF- κ B activation via the direct stimulation of the IKK complex. The exact mechanism by which the parasite recruits IKK complexes to its surface is still unknown, but the failure to identify parasite proteins that interact directly with IKK components indicates that additional host cell proteins may be involved. The 'patchy' accumulation of IKK complexes on the parasite surface argues against an interaction with a parasite surface molecule that is evenly expressed on the schizont surface, and could reflect clustering in the parasite plasma membrane, induced by the multimeric IKK complexes. Alternatively, IKK transport could rely on host-cell cytoskeletal components and accumulation only occurs at sites where the cytoskeleton interacts with the parasite surface.

Many open questions remain. The advent of RNAi technology, in particular in combination with inducible systems, should enable us to determine the precise contribution of individual host cell proteins to the transformation process and protection against apoptosis. The genomes of *T. annulata* and *T. parva* have now been sequenced and annotated, opening up the search for candidate transforming-parasite genes. In this context, a comparison with the genome of a non-transforming

Figure 3



Host-cell signal transduction pathways that are constitutively activated in *Theileria*-transformed cells. JNK is the only MAPK that is permanently activated and appears to be responsible for AP-1 and ATF-2 activation. Different members of the Src-related kinase family are active, depending on the cell type that is transformed, and can participate in signalling pathways emanating from different surface receptors. Casein kinase 2, which has a multitude of different substrates, is strongly upregulated. The NF- κ B pathway is activated directly by the parasite through the recruitment and activation of IKK complexes at the schizont surface. Input from other pathways, however, might also contribute to NF- κ B activity, depending on the cell type. Although the NF- κ B pathway might be important for stimulating entry into S-phase, it also regulates the expression of anti-apoptotic genes that contribute to cell survival. The PI-3K-PKB pathway is constitutively active and is essential for proliferation of *Theileria*-transformed cells. A direct role in protection against apoptosis, however, is not obvious. Taken together, the activation of these signalling pathways could help to explain many of the phenotypic changes that have been observed in *Theileria*-transformed cells (listed on the right of the figure) that may also contribute to the pathogenesis of Theileriosis.

Theileria could be highly informative. Transforming parasite proteins might be expressed on the schizont surface or secreted into the host cell cytoplasm. It has recently been reported that *Theileria* proteins containing AT hook DNA-binding domains are released from the parasite and localise to the host cell nucleus [52*,53], potentially modulating the host cell phenotype [54*]. Although promising, their role in transformation is still to be confirmed. For this purpose, the technology to knock out parasite genes or silence gene expression in *Theileria* needs to be developed urgently.

Acknowledgements

Unfortunately, the work of many colleagues could not be cited, due to space restraints; we hope they will show understanding. We thank the Swiss National Science Foundation, Oncosuisse, the Roche Research Foundation and the European Union for financial support.

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