Regulation of host cell survival by intracellular *Plasmodium* and *Theileria* parasites

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SUMMARY

Plasmodium and *Theileria* parasites are obligate intracellular protozoa of the phylum Apicomplexa. *Theileria* infection of bovine leukocytes induces transformation of host cells and infected leukocytes can be kept indefinitely in culture. *Theileria*-dependent host cell transformation has been the subject of interest for many years and the molecular basis of this unique phenomenon is quite well understood. The equivalent life cycle stage of *Plasmodium* is the infection of mammalian hepatocytes, where parasites reside for 2–7 days depending on the species. Some of the molecular details of parasite-host interactions in *P. berghei*-infected hepatocytes have emerged only very recently. Similar to what has been shown for *Theileria*-infected leukocytes these data suggest that malaria parasites within hepatocytes also protect their host cell from programmed cell death. However, the strategies employed to inhibit host cell apoptotic pathways appear to be different to those used by *Theileria*. This review discusses similarities and differences at the molecular level of *Plasmodium*- and *Theileria*-induced regulation of the host cell survival machinery.

Key words: *Plasmodium*, *Theileria*, apoptosis, signal transduction.

INTRODUCTION

Plasmodium and *Theileria* parasites are both transmitted by arthropod vectors. Mosquito-transmitted *Plasmodium* sporozoites enter blood vessels in the skin and are rapidly transported by the blood stream to the liver, where they invade hepatocytes and multiply, before being released into the blood stream to invade red blood cells (Amino, Menard and Frischknecht, 2005). Pathology associated with malaria is due to repeated infections of red blood cells by merozoites, whereas the liver stage is considered to be apathogenic due to the low infection rate. The vectors for *Theileria* parasites are ticks that inject sporozoites during a blood meal. *Theileria* sporozoites invade leukocytes, where they undergo schizogony and eventually merogony. Similar to the life cycle of *Plasmodium* parasites, *Theileria* merozoites are liberated from their host cells and invade red blood cells. Here however, they develop immediately into gamonts giving rise to the piroplasm stage. There are no repeated cycles of red blood cell invasion and consequently, the pathogenic stages for *Theileria* are the transformed schizont-infected leukocytes and not the piroplasm-infected erythrocytes.

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Because signalling pathways are rudimentary in anucleate erythrocytes, the most interesting stages concerning parasite-dependent host cell signalling events are schizont-infected hepatocytes for *Plasmodium* and schizont-infected leukocytes for *Theileria*, and this review will therefore concentrate on these stages although other parasite stages will be mentioned briefly. *Plasmodium* and *Theileria* parasites are quite closely related and have similar life cycles, but detailed analysis of their biology reveals fundamental differences. *Theileria* infection of leukocytes induces cell transformation resulting in clonal expansion of the infected cell (Dobbelaere and Kuenzi, 2004). Since the parasite is always distributed to both daughter cells, clonal expansion of leukocytes results in clonal expansion of the parasite population. Cellular transformation is a well-known phenomenon for virus-infected cells (O'Shea, 2005) but for eukaryotic parasites, *Theileria*-induced transformation is unique (Dobbelaere and Kuenzi, 2004).

Another remarkable difference is that *Plasmodium* schizonts reside within a parasitophorous vacuole, whereas *Theileria* schizonts live free in the cytoplasm of the host cell. The latter lifestyle is certainly more suitable for a fast exchange of information between the parasite and the host cell, because parasite molecules have to pass only a single membrane barrier to reach the signalling machinery within the host cell cytoplasm. *Plasmodium* molecules interacting with host cell proteins have to cross the parasite membrane, the parasitophorous vacuole (PV) and finally,

Parasitology (2006), **132**, S49–S60.
doi:10.1017/S0031182006000850 Printed in the United Kingdom

the parasitophorous vacuole membrane (PVM) before they reach the host cell cytoplasm. This makes parasite-host interactions certainly slower and less flexible than in the case of *Theileria* parasites. Because *Theileria*-infected cells are immortalised and the parasite is passed on to the daughter cells, the pathogen can almost be considered as an additional cell organelle. Since *Theileria* infection causes a deadly disease in cattle one is tempted to think that the parasite is not well adapted to its mammalian host, but it should be noted that some of the natural hosts of *Theileria* parasites (i.e. African Cape buffalo) are less severely affected by the infection.

Theileria-induced cell transformation has fascinated scientists for several decades and it is not surprising that the molecular details of this unique parasite-host relationship have been reasonably well characterized. In contrast, relatively little knowledge exists on parasite-dependent signalling in case of *Plasmodium* infection of hepatocytes. One reason why signalling events associated with the liver stage of *Plasmodium* have remained largely uncharacterized is that the pathogenic stage of the parasite's lifecyle is the blood stage, which consequently has attracted much more attention than the apathogenic liver stages. Another reason is the low infection rates *in vitro* and *in vivo* that results in the study of a relatively small number of parasite-infected hepatocytes against a large background of uninfected hepatocytes. This has made extensive biochemical studies of liver-stage development virtually impossible and restricted analysis to the single cell level. Lastly, infectious sporozoites are a prerequisite for the analysis of liver stages and consequently breeding infectious mosquitoes is a necessity. Despite these restrictions a number of recent observations have revealed that parasite-dependent signalling occurs also in *Plasmodium*-infected hepatocytes.

PARASITE-MEDIATED INHIBITION OF HOST CELL APOPTOSIS

Does infection trigger apoptosis?

If one assumes that intracellular parasites protect their host cells from stress-dependent apoptosis induced by infection, the logical consequence is that host cells should die if the pathogens are killed. For *Theileria* it is well established that drug-mediated elimination of the parasite results first in growth arrest of the host cell, followed by programmed cell death. The anti-apoptotic proteins c-FLIP, c-IAP, X chromosome-linked IAP and Mcl-1 are constitutively expressed in *T. parva*-transformed lymphocytes (Fig. 1), but they are rapidly down-regulated upon parasite elimination and this coincides with increased caspase activity and apoptosis (Guergnon *et al.* 2003*b*; Kuenzi, Schneider and Dobbelaere, 2003; Dessauge *et al.* 2005*a*). Although *T. parva*-infected lymphocytes express TNF receptors, TNF ligation does not induce apoptosis (Guergnon *et al.* 2003*a*). An explanation could be that NF-κB-dependent up-regulation of anti-apoptotic proteins also provides protection against TNF-induced apoptosis.

Similar experiments are missing for *Plasmodium*-infected hepatocytes, yet there is accumulating evidence for parasite-dependent survival of the host cell during liver stage development. Irradiated *Plasmodium* parasites are known to establish infection in hepatocytes, but the majority of these parasites disintegrate soon thereafter. A recent study suggests that dying parasites induce apoptosis of the host cell confirming that viable parasites are required to constantly stimulate host cell survival pathways (Leiriao *et al.* 2005*b*). Importantly, dentritic cells (DCs) were shown to phagocytose apoptotic cells containing the remains of intracellular parasites. Since it has been established earlier that DCs mediate the protective immune response induced by irradiated sporozoites (Hafalla *et al.* 2003) it can be concluded that apoptotic infected cells probably generate the protective immune response.

Another indirect piece of evidence that parasite death results in host cell apoptosis comes from studies of van de Sand and colleagues (van de Sand *et al.* 2005). Using the TUNEL assay, a proportion of *P. berghei* parasites were found to contain fragmented DNA. Interestingly, most of the nuclei of host cells harbouring dead parasites were also found to be TUNEL positive suggesting that host cell apoptosis had occurred upon death of the parasite. Dead cells cause a strong cellular infiltration that includes DCs. Whether this inflammatory reaction is the basis of acquired immunity against infected hepatocytes awaits further investigation, but there is reason to believe that this is the case. The work by Ocana-Morgner and colleagues has shown that liver infection can induce protective immunity if the blood-stage infection is eliminated by the use of artemisinin implying that blood stages suppress the immune response to the liver stages (Ocana-Morgner, Mota and Rodriguez, 2003).

SIGNALLING VIA SURFACE RECEPTORS

Extrinsic signalling – Theileria

T. parva-transformed B cells augment their proliferation via a GM-CSF autocrine loop that involves sustained PI3-K activation and AP-1 induction (Baumgartner *et al.* 2000; Dessauge *et al.* 2005*c*). Class I PI3-K enzymes are composed of a 110 kDa catalytic subunit that associates with an adapter molecule of 85 kDa. The regulatory p85 subunit is essential for p110 activation as it targets PI3-K to membranes, where its substrate is located.

https://doi.org/10.1017/S0031182006000850 Published online by Cambridge University Press

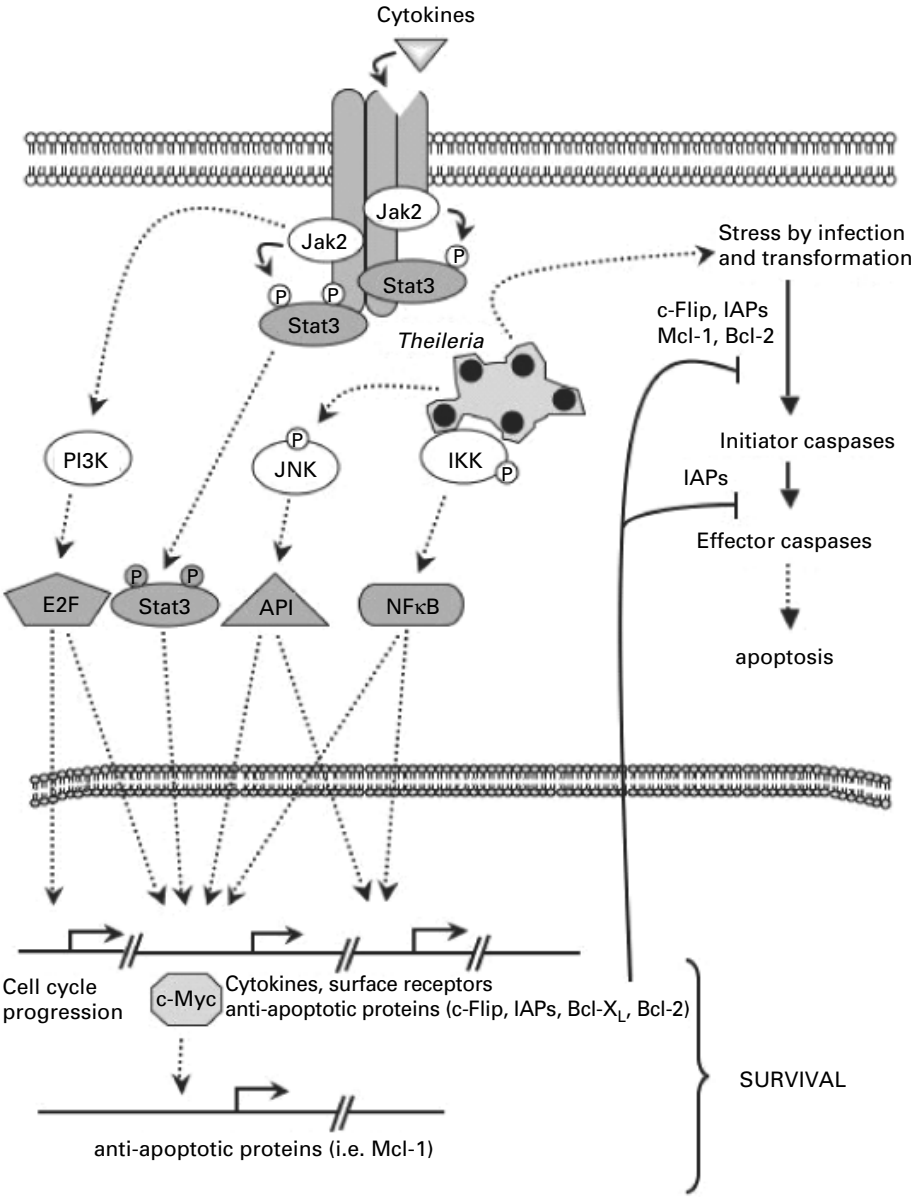

Fig. 1. *Theileria*-dependent signalling in bovine leukocytes. The presence of the parasite induces survival and apoptotic pathways simultaneously. As long as the parasite is viable, activation of NF-κB, JNK, JAK/STAT3 pathways results in the binding of the corresponding transcription factors (NF-κB, E2F, Stat3, AP1) to promotors of genes coding for anti-apoptotic molecules and c-Myc. Subsequent expression of the corresponding molecules inhibits apoptosis of the infected cell. Cytokines like IL-10 and GM-CSF are secreted by *Theileria*-infected cells and stimulate corresponding receptors in an autocrine manner. This, in turn, promotes proliferation via the PI3 kinase pathway and survival via the JAK/STAT3 pathway. Expression of c-Myc depends on the activation of these various pathways and has positive pleiotrophic effects itself on the survival of the host cell. Upon elimination of the parasite by theilericidal drugs, however, expression of anti-apoptotic proteins are down-regulated and apoptosis is initiated.

Interestingly, in *T. parva*-transformed B cells p85 remains permanently targeted to the membrane consistent with constitutive activation of the enzyme (Baumgartner *et al*. 2003).

Classically, constitutive or transient activation of PI3-K leads to activation of PKB (Akt) (Dhand *et al*. 1994) and provides an anti-apoptotic response via phosphorylation of several downstream targets. Together with other kinases, PKB can activate p70/S6 kinase (Stokoe *et al*. 1997), an enzyme critical for cell cycle progression (Lane *et al*. 1993; Alessi *et al*. 1997). However, we have shown that PKB is only minimally activated in *T. parva*-transformed B and T cells and PI3-K inhibition leads to reduced proliferation, but not apoptosis (Baumgartner *et al*. 2000; Heussler *et al*. 2001). Thus, reduced proliferation is probably due to loss of p70/S6 kinase activation and our recent results (Guergnon *et al*. 2006) indicate that low-level PKB activity does indeed mediate a survival response in *Theileria*-infected lymphocytes by phosphorylating Bad, but loss of PKB activity is not lethal, as Bad is phosphorylated

https://doi.org/10.1017/S0031182006000850 Published online by Cambridge University Press

by another kinase, PKA. Thus, host cell death only occurs upon inhibition of both kinases.

Extrinsic signalling – Plasmodium

It is known that binding of hepatocyte growth factor (HGF) to its receptor c-MET induces survival of uninfected hepatocytes and it has been suggested that HGF/c-MET signalling is induced by *P. berghei* infection to assure host cell survival (Leiriao *et al*. 2005*a*). Before a sporozoite finally settles in a hepatocyte it migrates through a number of other hepatocytes causing cell wounding and release of HGF (Carrolo *et al*. 2003). HGF binds to the c-MET receptor on *P. berghei*-infected hepatocytes and promotes cell survival in a PI3-K dependent manner (Leiriao *et al*. 2005*a*). Specific blockade of this pathway by treating cells at early time points after infection with the PI3-K inhibitor LY294002 resulted in significant apoptosis of infected cells, confirming the importance of active PI3-K for a successful establishment of infection. It is possible that irradiation of *Plasmodium* sporozoites blocks their ability to neutralize the apoptotic machinery of host cells allowing hepatocytes to resume their natural tendency to undergo apoptosis upon being infected. However, since it is expected that irradiated sporozoites behave initially like wild type sporozoites and traverse a number of hepatocytes before finally settling in one, HGF signalling via c-MET should also protect cells infected by irradiated sporozoites from dying. The data of Ocana-Morgner and coworkers suggest, however, that irradiated sporozoites induce apoptosis soon after entering the final hepatocyte (Ocana-Morgner *et al*. 2003). This obvious discrepancy was solved by a recent study, which clearly showed that the HGF effect is restricted to the first few hours of hepatocyte infection (van de Sand *et al*. 2005). As rapidly as 24 h post-infection host cell survival no longer depends on PI3-K signalling, but *P. berghei* parasites still interfere with the apoptotic machinery of the host cell. Treatment of infected cells 24 h post-infection with LY294002 did not affect parasite development, or the apoptotic state of the host cell. It is likely that at this stage the parasite has already started secreting molecules into the cytoplasm of the host cell activating pro-survival signalling directly without activation of PI3-K.

Different strategies for inhibiting apoptosis at early and late times of hepatocyte infection might be crucial for successful parasite development, because it could be difficult for the parasite to directly control the apoptotic machinery of the host cell during invasion. HGF signalling might be employed to bridge the time until parasite molecules are secreted into the host cell cytoplasm and activate survival signal transduction pathways. It is important for the parasite to control host cell survival at early times, because sporozoite invasion represents a bottleneck for *Plasmodium* (and *Theileria*) infection of the mammalian host. During a blood meal an infected mosquito normally injects less than 100 *Plasmodium* sporozoites and only a proportion of them finally reach the liver (Amino *et al*. 2005). To guarantee successful infection these few parasites need an optimal environment and migration through several hepatocytes might lead to the generation of a more favourable milieu by inducing the secretion of factors such as, but not exclusively, HGF.

As stated above, many receptor-mediated signalling events that promote cell survival induce PI3-K activation. Since in later phases of hepatocyte infection this pathway no longer plays a crucial role it is tempting to speculate that extrinsic signals are in general less important during schizogony of parasites. This view is supported by the fact that serum deprivation had no negative effect on the viability of *P. berghei*-infected cells (van de Sand *et al*. 2005). Generally, serum deprivation results in apoptosis of cultivated cells within a couple of days, because serum-derived growth factors that activate survival pathways are absent. In contrast to non-infected cells that undergo apoptosis under these conditions, infected hepatocytes survive serum withdrawal significantly better, confirming that parasitized cells have become largely independent of extrinsic signalling events. Similarly, *Theileria*-transformed leukocytes survive very well in only 0·5% serum and predominately suppress the intrinsic caspase 9-dependent apoptotic pathway (Guergnon *et al*. 2003*b*).

STRESS-SIGNALLING IN INFECTED CELLS

Intracellular parasites interact in many respects with their host cells. Very often, the pathogens grow to a considerable size, they use resources of the host cell, produce oxidative active substances which are exported into the host cell cytoplasm, interact with the host cell cytoskeleton and use host cell mitochondria as an additional source of nutrients. All these interactions are important stress signals and it can be expected that host cells react with the induction of stress-induced signalling events. Since stress signalling may result in anti-parasitic actions, or even apoptosis of the host cell, parasites need to control the corresponding host cell pathways by either supporting survival pathways, or by silencing pro-apoptotic signalling.

Theileria

The c-Jun N-terminal kinase JNK is a member of the mitogen-activated protein kinase (MAPK) family that are usually activated by multiple stress-related stimuli (Weston and Davis, 2002). However, it is known that JNK has a diversity of substrates and

https://doi.org/10.1017/S0031182006000850 Published online by Cambridge University Press

that probably explains why JNK activation can be either pro- or anti-apoptotic, depending on the stimuli given in a particular cellular context (Ameyar, Wisniewska and Weitzman, 2003; Kamata *et al*. 2005). It has previously been demonstrated that *Theileria*-dependent AP-1 induction and c-Jun phosphorylation is mediated exclusively by constitutive activation of JNK (Chaussepied *et al*. 1998). In addition, *Theileria*-induced JNK activity is also responsible for ATF-2 phosphorylation (Galley *et al*. 1997). Since JNK is constitutively activated and *Theileria*-infected lymphocytes proliferate in an uncontrolled manner it seems intuitive that JNK mediates a survival signal. To confirm this, a trans-dominant negative mutant of JNK was co-transfected together with GFP-tagged histone 2B and chromatin condensation was monitored (Lizundia *et al*. 2005, 2006). Inhibition of JNK clearly leads to infected B cell apoptosis, but given the multiplicity of potential JNK substrates it is difficult to ascertain the precise mechanism i.e. is it through transcriptional control, or phosphorylation of survival proteins, or both?

Plasmodium

Leiriao and colleagues investigated in *P. berghei*-infected hepatocytes whether signalling via MAP kinases conferred on the host cell protection from apoptosis (Leiriao *et al*. 2005*a*). Pre-treatment of hepatocytes with the MAP kinase inhibitor PD98059 induced a decrease in infection, but when apoptotic events were quantified no significant increase in the level of apoptosis was detected in treated cells. So, although both PI3-K and MAP kinase pathways play a role in infection, only the PI3-K pathway seems to protect *P. berghei*-infected hepatocytes from apoptosis during sporozoite invasion. Interestingly, treatment of *P. berghei*-infected hepatocytes with the MEK1 inhibitor UO126 at 24 h post-infection clearly inhibited parasite development, but did not induce significantly apoptosis of the host cell (S. Bolte and V. Heussler, unpublished observation) suggesting that signalling via host MAP kinase are not important during the schizont stage in infected hepatocytes. This notion is supported by the fact that activation of transcription factors like AP-1 and ATF that depend on MAP kinase signalling has not been detected in infected hepatocytes. Since typical MEK homologues do not exist in *Plasmodium* parasites (Dorin *et al*. 2005), an exciting explanation of the UO126-mediated effect would be that host cell MEK is involved in regulation of parasite development.

REGULATION OF ANTI-APOPTOTIC SIGNALLING PATHWAYS

Many infections are closely correlated with activation of the NF-κB (Nuclear Factor kappa B) pathway in infected, as well as in non-infected cells. Transient activation of NF-κB results in inflammatory anti-parasitic responses, but the NF-κB pathway is also known to mediate anti-apoptotic responses. It has been shown that a variety of intracellular pathogens (viruses, bacteria and parasites) manipulate this pathway to guarantee their own survival.

Theileria

Early work by Ivanov indicated an important role of the transcription factor NF-κB in the transformation process (Ivanov *et al*. 1989). Due to technical limitations in manipulating bovine leukocytes it was almost a decade before the role of NF-κB was verified. Palmer and co-workers demonstrated that constitutive NF-κB activation in *Theileria*-infected cells was dependent on continuous degradation of IκB, the specific inhibitor of NF-κB (Palmer *et al*. 1997). Using dominant-negative mutants of IκB and the p65 subunit of NF-κB, it was demonstrated that constitutive activation of NF-κB is essential for the survival of host cells (Heussler *et al*. 1999*a*, *b*). Drug-mediated inhibition of NF-κB signalling potently killed infected cells supporting a central role for this transcription factor in the survival of *Theileria*-infected cells. NF-κB is normally activated transiently by cytokines specifically binding to surface receptors, but in *Theileria*-infected cells it was shown that the parasite introduces a shortcut in this pathway (Heussler *et al*. 2002). Dominant-negative mutants of the different participants of the NF-κB pathway were used to investigate at which level the parasite interferes with signalling. This approach showed that the parasite directly activated the IKK kinase complex that phosphorylates IκB promoting its degradation and this results in translocation of NF-κB to the nucleus of the host cell (Heussler *et al*. 2002). Immunofluorescence imaging revealed superimposition of IKK signalosomes with the parasite surface. However, it is still not known how the accumulation of IKK molecules on the surface of the parasite results in activation, but the recently published genomes of *T. parva* and *T. annulata* (Pain *et al*. 2005) might help to identify parasite membrane molecules involved in this process (reviewed in Shiels *et al*. 2005). Finally, it was demonstrated that *Theileria* infection results in the expression of some well-known anti-apoptotic molecules including cIAP, cFLIP, X chromosome-linked IAP and Mcl-1 (Fig. 1) (Guergnon *et al*. 2003*b*; Kuenzi *et al*. 2003). Interestingly, the expression of these proteins is known to be regulated by NF-κB in other cell types and it is reasonable to assume that this is also the case in *Theileria*-infected leukocytes.

NF-κB pathway – Plasmodium

The NF-κB pathway is induced by a wide variety of stimuli including infection of NIH 3T3 cells by

https://doi.org/10.1017/S0031182006000850 Published online by Cambridge University Press

Toxoplasma gondii (Shapira *et al*. 2004). *Toxoplasma*, like *Theileria*, induces constitutive phosphorylation and subsequent degradation of IκB. For *Plasmodium*-infected cells however, there is no evidence for constitutive activation of this central mediator of survival. It has even been suggested that NF-κB activation in *P. berghei* infected cells correlates with apoptosis of host cells (Leiriao *et al*. 2005*b*). As has been pointed out earlier, infection of hepatocytes by irradiated *P. berghei* sporozoites results in apoptosis of the host cell. Staining of infected cells with an antibody against the p65 subunit of NF-κB indicated that in apoptotic infected cells NF-κB translocates to the nucleus, whereas in cells infected with viable parasites p65 remains in the cytoplasm of the host cell. It should be noted that NF-κB translocation to the nucleus does not necessarily mean that it induces apoptosis. Normally, NF-κB is activated to rescue cells in which apoptotic pathways are triggered (Kumar *et al*. 2004). If the pro-apoptotic signals outweigh the NF-κB-mediated survival effect, cells die even though this rescue pathway is activated. Thus, it remains to be shown whether hepatocyte infection by irradiated sporozoites activates pro-apoptotic signalling via NF-κB. However, an important take home message is that hepatocyte infection by viable parasites does not trigger constitutive NF-κB activation.

C-MYC AS A CENTRAL REGULATOR IN *THEILERIA*-INFECTED CELLS?

Theileria-infected lymphocytes use autocrine loops to augment their proliferation (Dobbelaere *et al*. 1991) and it has been demonstrated that inhibition of TNF did not provoke apoptosis (Guergnon *et al*. 2003*a*, *b*). In addition, we discussed above the role that a GM-CSF autocrine-loop plays in sustained PI3-K activation and AP-1 induction that characterizes *Theileria*-infected lymphocytes (Baumgartner *et al*. 2000; Dessauge *et al*. 2005*a*). Moreover, comparison of non-infected bovine B cells with *T. parva*-infected B cells indicated that the presence of *Theileria* also leads to c-Myc expression in infected cells (Dessauge *et al*. 2005*a*). Expression of c-Myc is directly due to *Theileria*, since treating infected B cells with a parasitocidal drug (BW 720c) resulted in a rapid decrease of c-Myc levels within 24 h. *Theileria* was shown to induce high c-Myc levels via JAK/STAT3-mediated activation of the *c-myc* promoter and moreover, addition of exogenous GM-CSF led to augmentation of *c-myc* transition (Dessauge *et al*. 2005*a*). Thus, one of the ways *Theileria* induces high levels of c-Myc involves a GM-CSF autocrine loop that activates a JAK/STAT3 pathway leading to transcriptional activation of the *c-myc* gene.

Parasite death provokes disequilibrium in the balance between pro- and anti-apoptotic proteins (Guergnon *et al*. 2003*a*; Kuenzi *et al*. 2003) due to the loss in GM-CSF, STAT3 phosphorylation and c-Myc expression (Dessauge *et al*. 2005*a*, *b*). Indeed, reduced Bcl-2 levels were observed when infected T cells died. In infected B cells, however, killing of parasites with buparvacone, or *c-myc* anti-sense treatment resulted in decreased Mcl-1 levels (Dessauge *et al*. 2005*a*). Mcl-1 is a Bcl-2 family member and has been described as a c-Myc target gene. Thus, one explanation for how c-Myc mediates its anti-apoptotic response in survival of *Theileria*-infected B cells is via induction of Mcl-1.

It was observed that parasite death provokes a rapid drop in c-Myc levels, but this occurs prior to any detectable loss in STAT3 phosphorylation (Dessauge *et al*. 2005*a*). This suggests that transcription of *c-myc* does not uniquely depend on the JAK2/STAT3 pathway and it is worth noting that E2F (Chaussepied and co-workers unpublished), AP-1 (Chaussepied *et al*. 1998) and NF-κB (Heussler *et al*. 2002) are all also constitutively active in *Theileria*-transformed lymphocytes. Transient transfection of dominant negative mutants of these transcription factors together with the *c-myc*-driven luciferase reporter construct showed that each transcription factor contributes to *c-myc* transactivation (Dessauge *et al*. 2005*b*). This suggests that *c-myc* transcription depends not only on STAT3, but also on E2F, AP-1 and NF-κB (see Fig. 1). Moreover, in *Theileria*-infected leukocytes constitutive AP-1 induction is exclusively JNK-dependent and inhibition of JNK also results in infected B cell apoptosis (Lizundia *et al*. 2005, 2006), again implying that the JNK-mediated survival signal involves *c-myc* activation. Thus, although multiple different survival pathways are activated upon *Theileria* infection they all seem to feed into c-Myc induction.

SPECULATIONS ON SIGNALLING IN *PLASMODIUM*-INFECTED HEPATOCYTES

Whereas a main feature of *Theileria*-infected cells is the constitutive activation of the host cell NF-κB, JNK kinase and PI3-K pathways, none of these pathways seem to play a role in *Plasmodium*-infected hepatocytes and it remains to be shown how the parasite promotes the survival of its host cell. One plausible explanation would be that *Plasmodium* parasites employ a similar strategy as bacteria of the genus *Chlamydia*, as it has recently been shown that these intracellular pathogens inhibit host cell death by degrading pro-apoptotic BH3-only proteins (Fischer *et al*. 2004). Another reason why inhibition, rather than stimulation, of host cell signalling pathways should be considered as an alternative survival strategy of *Plasmodium* parasites is that the closely related parasite *T. gondii* is known to block STAT signalling pathways of the host cell (Luder *et al*. 2001). More recently *T. gondii*-infected macrophages

https://doi.org/10.1017/S0031182006000850 Published online by Cambridge University Press

were shown to exhibit reduced expression of inducible nitric oxide synthase and it has been speculated that this inhibition depends on the down-regulation of JAK/STAT or the NF-κB signalling pathway (Luder *et al*. 2003). Since there is an ongoing debate on NF-κB signalling in *Toxoplasma*-infected cells, interested readers are referred to the article by Carsten Luder in this supplement for a more detailed discussion.

Effects of the circumsporozoite (*CS*) *protein*

Inhibition of host cell apoptosis during the invasion process might also be mediated by secreted CS protein. It has been shown that the CS protein blocks mRNA translation by physically binding to ribosomes of infected and neighbouring non-infected cells (Frevert *et al*. 1998). Whereas most non-infected cells did not survive, the infected cells re-activated protein synthesis and stayed viable. If the infection process induced stress-related signalling and the synthesis of pro-apoptotic proteins, blocking protein synthesis might initially kept the infected cell alive. However, prolonged CS-dependent inhibition of protein synthesis finally resulted in death of non-infected cells, whereas infected cells survived. It can be concluded therefore, that the presence of the parasite is important to keep the host cell alive. This goes well together with the notion that *Plasmodium* parasites interfere directly with apoptotic pathways of the host cell (van de Sand *et al*. 2005).

Signalling during merogony

Parasite-mediated protection from host cell apoptosis guarantees the survival of the pathogen during its intracellular development. In case of *Plasmodium* and *Theileria* this is most important during schizont development in hepatocytes or leukocytes, respectively. Very little is known about how the host cell reacts upon merozoite development. For *Theileria* it has been shown that merozoite formation correlates strictly with abrogation of the constitutive NF-κB activation (Shiels *et al*. 2004; Heussler *et al*. 2002). IKK signalosomes are no longer found at the surface of the parasite and NF-κB disappears from the nucleus. Whether this is followed by apoptosis of the host cell is an interesting question and will be one of the goals in future research on *Theileria*-dependent signalling.

For *Plasmodium* early work from Jacques Meis indicated that at the end of merogony the PVM is disrupted and merozoites mix with the host cell cytoplasm (Meis *et al*. 1985). Recently, the molecular details of these events have been investigated and it was observed that PVM disruption is mediated by cysteine proteases (V. Heussler, unpublished observations). Parasite cysteine proteases of the SERA family have been suggested to be responsible for the liberation of merozoites from *P. falciparum*-infected erythrocytes (Wickham, Culvenor and Cowman, 2003) and there exists evidence that liberation of sporozoites from oocysts in the midgut of *P. berghei*-infected mosquitoes are mediated by a SERA protease (Aly and Matuschewski, 2005). Our working hypothesis is that the continuous activity of SERA proteases in infected hepatocytes finally results in death of the host cell that resembles apoptosis. However, host cell caspases are not involved, because general inhibitors of caspases like zVAD-fmk do not inhibit host cell destruction. In order to distinguish this form of cell death from typical apoptosis it should be classified as parasite-dependent host cell death. Features of this cell death are condensation, but not fragmentation of host cell DNA, caspase-independent mitochondrial damage and cytochrome c release, detachment of infected cells *in vitro* and, most importantly, preservation of the cell membrane asymmetry. Obviously, parasite-induced cell death differs from apoptosis in several respects and should therefore be considered as different.

Erythrocytic stage of Plasmodium *parasites and protection from cell death*

For a long time apoptosis was not considered to be an important event in erythrocytes, because they have no organelles like mitochondria and nuclei. This view begins to change, since it has been shown that a kind of rudimentary cell death also occurs in aged erythrocytes including switching of phosphatidylserine (PS) residues to the outer leaflet of the membrane (Bosman, Willekens and Werre, 2005). Although there is some evidence that *P. falciparum*-infected erythrocytes induce PS exposition indicating erythrocyte death (Lang *et al*. 2004), recent data strongly suggest that *P. berghei*-infected red blood cells exhibit a certain resistance to treatment with death promoting nitric oxide (NO) (Sobolewski *et al*. 2005). This discrepancy might be due to the experimental setup of these studies since different *Plasmodium* species have been used. It has to be considered that *P. falciparum* and *P. berghei* infection of red blood cells might cause different effects. However, our own studies suggest that both parasites confer resistance to oxidative stress to the host cell (G. Mueller and V. Heussler, unpublished observations). Another reason for PS exposure in *P. falciparum in vitro* cultures might depend on the level of parasitaemia used. Cultures with high parasitemia have been shown to more readily expose PS (Huber *et al*. 2004) suggesting that PS exposure is more an *in vitro* phenomenon. On the other hand, it is possible that erythrocytes harbouring old schizonts might be more susceptible to PS exposure than cells with young parasites.

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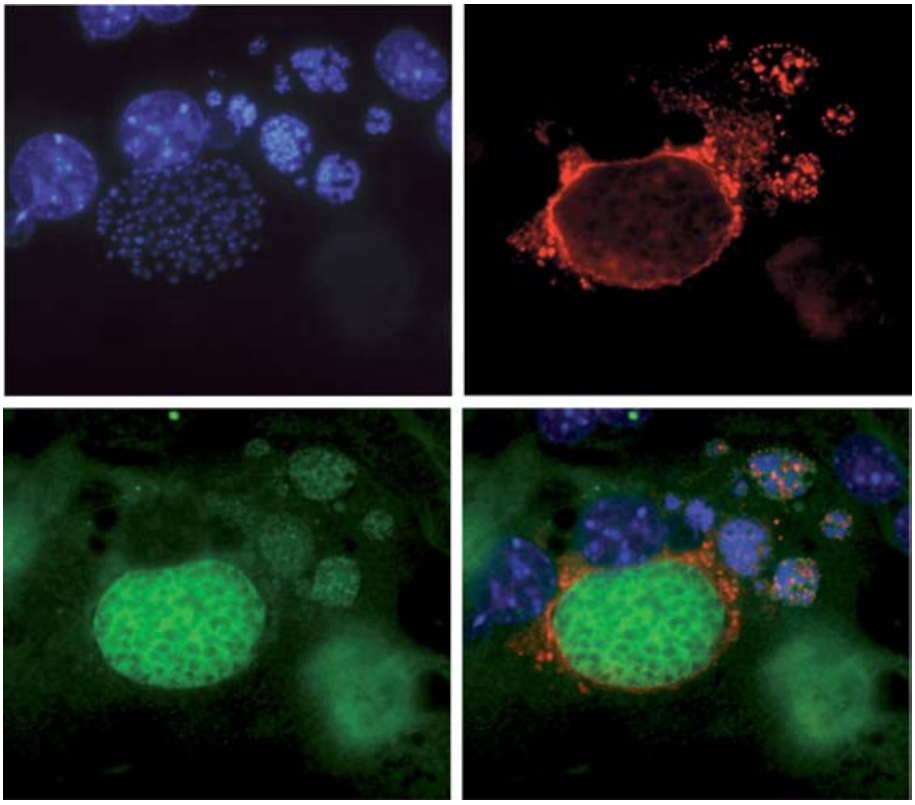

Fig. 2. Degenerating *P. berghei* parasites exhibit signs of programmed cell death. Rat hepatocytes were infected with *P. berghei* sporozoites and cultivated for 2 days. Cells were fixed and stained with an antiserum against *P. berghei* exported protein 1 (Exp1, red) and with a pan-hsp70 antiserum (green). DNA was stained with the Hoechst 33 258 dye. Next to a healthy parasite with an intact PVM incorporating Exp1, the remains of a degenerated parasite are visible. Signs of parasite degeneration are: parasite fragmentation, reduced hsp70 staining, lack of export of Exp1 and, most importantly, DNA condensation shown by bright Hoechst 33 258 staining in comparison to the DNA staining of the viable parasite.

Signs of programmed cell death in Plasmodium *parasites*

Ordered fragmentation of parasite DNA, as detected in infected hepatocytes by TUNEL staining suggests the presence of ancient apoptotic pathways in the parasite (van de Sand *et al*. 2005). Although caspases have not been found to be encoded in the genomes of *Plasmodium* parasites, it might well be that other parasite cysteine proteases are able to induce cell death including activation of protease sensitive DNAses, which results in the observed DNA fragmentation. TUNEL-positive *Plasmodium* parasites have also been described for the ookinete stage in the midgut of the mosquito (Al-Olayan *et al*. 2002). Altruistic cell death of parasites in the midgut is thought to protect the insect from super-infection by the parasite. When ookinetes pass through the midgut epithelium, apoptosis is induced in the traversed cells. If too many ookinetes are present in the midgut, midgut damage might be life-threatening for the insect and apoptosis of a proportion of the midgut ookinetes may not only guarantee the survival of the vector, but also of the rest of the parasite population. A remaining question is whether the observed cell death detected in various *Plasmodium* life cycle stages should be considered as apoptosis.

Activated caspases are the central molecules in apoptosis and the fact that caspase homologues do not exist in *Plasmodium* and *Theileria* parasites suggests an alternative cell death mechanism. However, it has been shown that apoptosis in higher eukaryotes including mammals can also be mediated by activation of other proteases than caspases (Johnson, 2000). Cathepsin B and calpain proteases can induce typical features of apoptosis in a variety of cells. The phenotype of the affected cell should therefore be considered as more important than the origin of the effector protease. If *Plasmodium* parasites with fragmented DNA show other features of apoptosis like membrane blebbing, DNA condensation, loss of the asymmetry of the cell membrane, cytochrome c release and oxidative stress, it is certainly justified to classify this cell death as apoptosis. However, some of the afore mentioned criteria are not easy to test, as long as the parasite resides within a host cell. Nonetheless, a combination of our knowledge about intracellular exoerythrocytic forms and extracellular merozoites suggests that *Plasmodium* parasites can indeed undergo a primitive form of apoptosis. Liver schizonts have been shown to disintegrate into many parasite filled vesicles within a host cell (Fig. 2). It should be noted that the DNA staining by the Hoechst 33 258 dye clearly indicates DNA condensation.

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Apoptotic markers like chromatin condensation, DNA fragmentation, and externalization of PS and induction of caspase-like activity have also been detected in mosquito midgut parasite stages suggesting the existence of programmed cell death (Al-Olayan *et al*. 2002). Although these observations support quite convincingly the hypothesis of apoptosis-like cell death in *Plasmodium* parasites more work is needed to characterize the accompanying destructive events. The most important goal will be the identification of parasite proteases that initiate the whole process. It will also be crucial to identify the molecules that lead to the activation of such initiator proteases, because they might represent the basis of potent new anti-malarial drugs.

What is the benefit for the parasite to undergo apoptosis during infection of the mammalian host? A clue might come from the observation that phagocytes differently remove apoptotic cells compared to removal of necrotic cells. Whereas necrotic cells and cell debris induce an inflammatory reaction, phagocytosed apoptotic cells inhibit inflammatory responses. It has be shown that Kupffer cells, the resident macrophages in the liver, can efficiently phagocytose merozoites (Terzakis *et al*. 1979) and since we have shown that liberated merozoites become PS positive (V. Heussler, unpublished observations) it is reasonable to assume that Kupffer cells recognise and phagocytose merozoites. This rather silent way of removing unsuccessful parasites might be beneficial for the parasite in that the subsequent immune response will be markedly different from a response induced by dead cells not exposing PS. This would result in strong inflammatory immune responses and a totally different cytokine pattern that could be deleterious for the parasite. Interaction of blood stage *P. berghei* parasites with DCs has been shown to suppress immune responses in the liver (Ocana-Morgner *et al*. 2003). It is plausible that PS-positive merozoites that did not infect red blood cells in time are phagocytosed by DCs and suppress protective cell-mediated immune responses against the liver stage. Considering the enormous number of infected red blood cells ($>10^{11}$ at a parasitemia of 1%) in course of a human *Plasmodium* infection, it can be calculated that a substantial number of merozoites are phagocytosed, even if only a very small percentage of them fail to invade red blood cells.

CONCLUDING REMARKS

This review on parasite-dependent regulation of host cell survival pathways clearly demonstrates how far advanced this field is for *Theileria*-infected leukocytes in comparison to *Plasmodium*-infected hepatocytes. On the other hand, we are still far from understanding the entire *Theileria*-host cell relationship and with the recent availability of the complete *Theileria* genome sequences (Pain *et al*. 2005) we can expect more exciting news from this fascinating and unique parasite. Since infected cells are proliferating in an uncontrolled manner it is not surprising that many signalling pathways are found activated. Some of them, such as those leading to NF-κB and c-Myc induction are obviously essential for the survival of infected cells and it has been suggested that NF-κB feeds into c-Myc expression (Dessauge *et al*. 2005*b*). Although this might be the case, constitutive c-Myc expression alone does not trigger transformation (Pelengaris, Khan and Evan, 2002). Additional signals are needed to induce immortalisation of cells and it is therefore important to investigate whether NF-κB and c-Myc act synergistically.

Although *Theileria* and *Plasmodium* parasites use different strategies to induce survival of their host cells it should be considered that they use common strategies to leave their host cells upon merogony. Liberation of *Plasmodium* parasites has been extensively studied for erythrocytic (Wickham *et al*. 2003) and very recently for the oocyst stage (Aly and Matuschewski, 2005). In both cases proteases of the SERA family are thought to play key roles and this might also to be true for the liver stage. Whether *Theileria* merozoite liberation is mediated by activation of parasite-derived proteases is not known, but it is an attractive hypothesis and certainly worth investigating.

Analysis of *Plasmodium*-dependent regulation of host cell signalling has just started, but it is safe to predict that this field will advance quickly and will deepen our understanding of this important parasite-host cell interaction. This, in turn, might open new avenues for the development of new anti-malarial therapies.

ACKNOWLEDGEMENTS

We thank Ulrike Froehlke for technical assistance and Tommy Regen for providing the images for figure 2. Dr. Robin DasGupta is thanked for critically reading the manuscript. This work was supported by a DFG grant to VH (HE 4497/1-1).

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