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Intracellular survival of apicomplexan parasites and host cell modification

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Abstract

The intracellular stages of apicomplexan parasites are known to extensively modify their host cells to ensure their own survival. Recently, considerable progress has been made in understanding the molecular details of these parasite-dependent effects for *Plasmodium*-, *Toxoplasma*- and *Theileria*-infected cells. We have begun to understand how *Plasmodium* liver stage parasites protect their host hepatocytes from apoptosis during parasite development and how they induce an ordered cell death at the end of the liver stage. *Toxoplasma* parasites are also known to regulate host cell survival pathways and, additionally, it has been convincingly demonstrated that they block host cell MHC-dependent antigen presentation of parasite epitopes to avoid cell-mediated immune responses. *Theileria* parasites are the masters of host cell modulation because their presence completely immortalizes the infected cell. It is now accepted that multiple pathways are activated to induce *Theileria*-dependent host cell transformation. Although it has turned out that similar host cell pathways are affected by the different parasites, the outcome for the infected cell varies considerably. Improved imaging techniques and new methods to control expression of parasite and host cell proteins will now help us to analyze the molecular details of parasite-dependent host cell modifications.

Keywords: *Plasmodium*; *Toxoplasma*; *Theileria*; host cell modification; apoptosis; signalling; parasite-host interaction

Introduction

This review concentrates on the post-genomic era of apicomplexan research. In this period, considerable progress has been made in the field of parasite-dependent host cell modification for *Toxoplasma*, *Theileria* and *Plasmodium* parasites and we therefore focus on these organisms and their effects on their respective host cells. However, it should be mentioned that other Apicomplexa, such as *Eimeria*, *Neospora* and *Cryptosporidium* also have profound effects on their respective host cells and the reader is kindly referred to related literature (Herman et al., 2007) (Liu et al., 2008) (del Cacho et al., 2004).

The COST action 857 "Apicomplexan biology in the post genomic era" represented an ideal platform for European scientists to exchange ideas and to combine forces to make large steps forward in the field of parasite-host interactions. A number of very fruitful collaborations have been started during this COST action, resulting in some major discoveries.

The very dynamic European malaria research community has provided us with important insights into the migration of *Plasmodium* sporozoites in the skin and liver (Amino et al., 2007) (Mota and Rodriguez, 2004). Although it is still a matter of debate, why sporozoites transmigrate through cells, interesting concepts have been suggested by participants of this COST action and will be discussed in this review (Leiriao et al., 2005a) (Amino et al., 2008).

A collaboration, which was initiated at the COST meeting in Lisbon in 2004 and is still ongoing resulted in the first description of merosomes as vehicles for the transport of hepatocyte-derived merozoites to the blood vessels of the liver (Sturm et al., 2006). This finding now completes our knowledge about the life cycle of *Plasmodium* parasites. Merosome development and other host cell modifications by *Plasmodium* parasites will be summarized by Rebecca Stanway.

Toxoplasma gondii-dependent signalling in host cells is a long-standing interest of Carsten Lüder. He describes here how intracellular *T. gondii* parasites interfere with several host signalling pathways to avoid production of NO, MHC-dependent antigen presentation and

induction of host cell apoptosis and he discusses future directions in this field. We will hopefully learn more about *T. gondii*-induced effects on host cell signalling by the large-scale siRNA knockdown approach targeting signalling proteins of the host cell, started by the laboratory of Markus Meissner in Heidelberg.

Theileria-induced reversible host cell transformation is probably the most extreme example of how intracellular parasites influence the phenotype of their host cells. This phenomenon has fascinated researchers for decades and scientists involved in the COST action 857 are worldwide leaders in this field. It is now accepted that the influence of *Theileria* parasites on their host cells is manifold and not restricted to one central pathway (Heussler et al., 2002) (Dessauge et al., 2005b). Many of the new discoveries came from Gordon Langsley's laboratory in Paris and he, together with Marie Chaussepied, summarizes here the recent publications in the *Theileria* field and raises some challenging hypotheses. A very exciting talk at the 2007 COST meeting in Porticcio was given by Dirk Dobbelaere, who reported on kinases involved in both host cell cytokinesis and in the equal distribution of the *Theileria* schizonts to both daughter cells and we hope to see these data published in the near future. The organizers of the COST 857 action always endeavoured to and succeeded in integrating young parasitologists and the selection of authors for this review followed this philosophy. Apart from the principal investigators, two young and enthusiastic postdocs, Rebecca Stanway and Marie Chaussepied, joined the team and the review certainly benefited from their input.

Plasmodium - the parasite dictates live and death of the host cell

The *Plasmodium* parasite must invade and traverse a vast range of different cell types during its life cycle, but has only two true host cells, within which it grows and divides, these being the hepatocyte and the erythrocyte. The parasite must modify both of these host cells to allow its own development and at the same time must prevent host cell death. This section of the

review will focus primarily on the modification, by the *Plasmodium* parasite, of the host hepatocyte. In the past four years of COST action 857, our understanding of *Plasmodium* development within the liver has advanced greatly, but much remains to be understood, particularly on a molecular level.

Early stage development of the Plasmodium *parasite within the hepatocyte*

In 2001, it was published that sporozoites, on entering the liver, transmigrate through a number of hepatocytes before invading a final one by the formation of a parasitophorous vacuole (Mota et al, 2001). This process of hepatocyte transmigration has been shown to involve the perforin-like proteins spect (sporozoite protein esential for cell traversal), (Ishino et al., 2004) and spect 2 (also known as *Plasmodium* perforin-like protein 1, (pplp1) (Ishino et al., 2005)). Additionally, the protein TLP (TRAP-like protein) has recently been identified, which appears to play a role in hepatocyte traversal (Moreira et al., 2008), potentially in anchoring the sporozoite to the hepatocyte prior to and during movement into the cell. It has been hypothesized that traversal of hepatocytes is necessary to activate sporozoites for invasion of a final hepatocyte by formation of a parasitophorous vacuole (PV). Sporozoite activation occurs by contact with the host cell cytoplasm and results in regulated exocytosis by the sporozoite of proteins contained within apical secretory organelles (Mota et al., 2002). In support of this hypothesis, it has been shown that stimulation of regulated exocytosis leads to a significantly increased level of *P. yoelii* sporozoite infectivity to hepatocytes (Ono et al., 2008). However, in *in vitro* culture, spect2-deficient sporozoites, that are unable to traverse hepatocytes, infect HepG2 cells at the same level as wild-type sporozoites (Ishino et al., 2005), showing that activation of parasites by cell traversal is not essential for establishment of infection. Another role and the one most relevant to this review is that transmigration of hepatocytes and the cell wounding this involves, leads to protection of infected cells. Hepatocyte wounding by sporozoites releases hepatocyte growth factor (HGF) (Carrolo et al.,

2003) and this binds to c-MET on the surface of hepatocytes, signalling via PI3-K to inhibit apoptosis of infected and presumably non-infected hepatocytes (Leiriao et al., 2005a). Supporting this, inhibition of PI3 kinase at early stages of infection leads to apoptosis of infected cells (Leiriao et al., 2005a) (Leiriao et al., 2005b). Such HGF-c-MET signalling was therefore thought to be responsible for preventing apoptosis in the initial phase of infection. However, when spect2 is disrupted, sporozoites are incapable of cell wounding and thus HGF release, but *in vitro* do not show a reduced survival following infection, despite a presumed absence or great reduction in HGF-c-MET signalling (Ishino et al., 2005). It is currently therefore not possible to conclude completely the role of HGF-c-MET signalling in prevention of host cell apoptosis. We know, however, that prevention of host cell apoptosis initially appears to involve signalling via PI3-K(Leiriao et al., 2005a), but what signals upstream of PI3-K remains to be identified.

Later stage inhibition of apoptosis by Plasmodium *in the hepatocyte - lessons from the erythrocyte stage*

Following early stages of infection, inhibition of apoptosis becomes independent of PI3 kinase activity (van de Sand et al., 2005) and it is thought that once the parasite is established in the hepatocyte, it actively inhibits apoptosis of the host cell, most probably via secretion of parasite molecules into the host cell cytoplasm. Already at 18 hours post infection, infected cells are more resistant to external stimuli of apoptosis than non-infected cells (Leiriao et al., 2005a), showing that the presence of the parasite confers resistance to apoptosis. Host cell survival certainly requires the parasite to stay alive, as mutant parasites that invade hepatocytes but do not develop, such as *P. berghei* parasites lacking the P36p protein, fail to protect the host cell from undergoing apoptosis after the initial phase of infection (van Dijk et al., 2005). Additionally, it has been shown that host cells invaded by irradiated sporozoites die by apoptosis shortly after invasion (Leiriao et al., 2005b).

158 Secretion of *Plasmodium* proteins into the host cell cytoplasm and onto the host cell surface
159 has best been characterized in the *P. falciparum* blood stage, where the parasite similarly
160 resides within a parasitophorous vacuole and must ensure survival of the host erythrocyte,
161 until maturity of daughter parasites. Here secretion into the host cell occurs primarily by use
162 of a *Plasmodium* export element (Pexel) (Hiller et al., 2004; Marti et al., 2004), which appears
163 to be processed during export (Chang et al., 2008). Bioinformatic prediction of such motifs
164 has allowed the generation of a predicted secretome for the *P. falciparum* blood stage, with up
165 to 150 potentially exported proteins (van Ooij et al., 2008). Liver stage parasites must also
166 secrete molecules into the hepatocyte cytoplasm, including those presumably involved in
167 inhibition of host cell death, but which proteins are secreted and which signalling pathways
168 and events they interfere with is not clear. One might predict that secreted proteins include
169 remodelers of the host cell cytoskeleton, required to allow the extensive growth of the
170 parasite. Several proteins secreted into the erythrocyte by the *P. falciparum* parasite bind to
171 elements of the red blood cell cytoskeleton (reviewed in (van Ooij and Haldar, 2007)). The
172 protein RESA, for example, binds to spectrin beneath the plasma membrane and is thought to
173 stabilise the membrane of the host cell, preventing subsequent merozoite invasions and thus
174 ensuring proper parasite development and also protecting the erythrocyte from heat damage
175 during malarial fever (Mills et al., 2007). *Plasmodium* parasites express a homolog of the
176 secreted *Theileria* protein TaSE (Schneider et al., 2007), which binds to host cell
177 microtubules and it will be interesting to investigate the localization and function of the
178 *Plasmodium* homolog in infected hepatocytes and erythrocytes. In *P. berghei*, the predicted
179 secretome contains many fewer proteins than that of *P. falciparum*, but the machinery for
180 Pexel-based secretion appears not to be *P. falciparum*-specific as the *P. berghei* parasite has
181 been shown to successful secrete a protein containing a *P. falciparum* Pexel sequence into the
182 erythrocyte cytoplasm (MacKenzie et al., 2008). The relatively small predicted secretome of
183 *P. berghei* compared to *P. falciparum* seems to be consistent with the biology of *P. berghei*

blood stages, where remodelling of the red blood cell is much less extreme than seen in *P. falciparum* infection, with the parasite failing to produce the visible adhesive knob structures on the red blood cell surface. Erythrocytes containing *P. berghei* early schizont stages are sequestered from peripheral circulation, implying parasite modification of the red blood cell leading to cytoadhesion, but thus far no parasite proteins have been conclusively localised to the surface of the *P. berghei*-infected red blood cell. In other apicomplexan parasites, very few Pexel sequences are predicted and *Toxoplasma* proteins that have been modified to contain a *P. falciparum* Pexel sequence do not show targeting to the host cell (van Ooij and Haldar, 2007). It has been suggested that *P. falciparum* blood stages show an expansion of proteins secreted to the host cell due to the inability of its host erythrocyte, being enucleated, to modify itself to accommodate the growing parasite, and that other Apicomplexa do not require such a large exportome, due to their host cells being nucleated and thus able to modify themselves (van Ooij and Haldar, 2007). It seems more likely, however, that other Apicomplexa have developed different mechanisms to allow secretion of proteins into the host cell and that *Plasmodium* also secretes proteins into the host cell via a Pexel-independent mechanism. In *Plasmodium* liver stages, the only protein thus far shown to be secreted into the host cell, prior to parasitophorous vacuole membrane (PVM) disruption, is the circumsporozoite protein (CSP). This sporozoite and early liver stage-expressed protein contains two predicted Pexel motifs, both of which appear to be functional (Singh et al., 2007). The protein is also predicted to contain a nuclear targeting sequence and has been shown to be present in the host cell nucleus at early stages on liver stage infection (Singh et al., 2007). The targeting of CSP to the hepatocyte nucleus has been suggested to lead to both the up- and down-regulation in expression of many genes responsible for processes including immune responses, cell growth, attachment and apoptosis (Singh et al., 2007). The timing of the presence of CSP in the nucleus is, however, at odds with the modification of host cell gene expression for genes involved in certain processes. The need for suppression of pro-

inflammatory responses of the host cell would be predicted to be greatest at late liver stages, but CSP protein is detected within the nucleus only at early liver stages (Singh et al., 2007). The authors also claim that CSP has no effect on protein synthesis although earlier studies, from the same institute, suggested CSP to block mRNA translation in infected and non-infected hepatocytes (Frevert et al., 1998). Considering the conflicting results on the role of CSP in hepatocytes and its minimal expression during liver stages, more work is needed to ascertain whether CSP is indeed involved in regulation of gene expression of infected hepatocytes. Another major goal of liver stage *Plasmodium* research must be to further establish which parasite proteins are secreted into the host cell cytoplasm and how these proteins modify the host cell.

Plasmodium-*induced hepatocyte death, as a means of safe daughter cell release*

At the late liver stage, upon maturity of merozoites, the parasites actively induces host cell death. The parasitophorous vacuole membrane that surrounds the newly formed daughter merozoites breaks down and merozoites are released into the host cell cytoplasm (Meis et al., 1985). This process requires the action of cysteine proteases, as inhibition of this class of proteases by E64 causes a block in PVM disruption and subsequent events (Sturm et al., 2006). As in apoptotic death, the host cell plasma membrane remains intact and the host cell detaches from surrounding cells, nuclear condensation is seen and cytochrome c is released from mitochondria (Sturm et al., 2006). However, other key features of apoptosis are not detected. DNA fragmentation is not observed and phosphatidyl serine residues are not switched to the outer leaflet of the plasma membrane, which would normally mark the cell for destruction by the immune system. This form of host cell death most closely resembles autophagy, a process characterized by self-digestion. One might expect that host cell death at the end of *Plasmodium* liver stage development results from the parasite ceasing to inhibit the default process of host cell death. However, caspases that execute apoptosis are only weakly

activated in dying host cells and thus cell death appears to be controlled by the parasite rather than the host. Several members of the *P. berghei* serine repeat antigen (SERA) family of cysteine proteases are upregulated at late liver stages (Schmidt-Christensen et al., 2008) and members of the family have been shown to be responsible for merozoite liberation from red blood cells (Hodder et al., 2003) and sporozoite liberation from oocysts (Aly and Matuschewski, 2005). It is therefore hypothesized that these proteases may play a role in disruption of the PVM and then in later events leading to host cell death. Once infected hepatocytes have detached from neighbouring cells, spheres of host cell membrane containing merozoites bud from these detached cells and are called merosomes. Merosomes occur both *in vitro* and *in vivo*, where they are extruded into the liver sinusoids and released into the blood stream. The discovery of merosome formation and transport into the blood stream answered one of the long-standing questions concerning the *Plasmodium* life cycle, as to how merozoites reached the blood stream. Merosomes have now been tracked by intravital microscopy, as travelling to the lungs, sometimes subdividing into smaller merosomes in the circulation (Baer et al., 2007). Thus, the parasite shows extensive modification of its host hepatocyte, first inhibiting cell death during parasite development and later actively triggering it, allowing safe release of daughter parasites. Determining the molecular players, belonging to both the parasite and the host, involved in these processes is a critical next step in *Plasmodium* liver stage research.

***Toxoplasma gondii* - calming down the host cell**

Strategies to facilitate long-term survival within the host

A hallmark of the life cycle of *T. gondii* is the parasite's ability to induce long-lasting chronic infections in virtually any mammalian or avian host. This is facilitated by specific parasite-host adaptations including differentiation processes of the parasite, i.e. conversion from the replicative and lytic tachyzoite stage to the quiescent bradyzoite stage, formation and

remodelling of a PV as a safe intracellular compartment, and modulation of host cell responses to either external signals or the intracellular infection. Whether preferential localization of *T. gondii* in immune privileged sites like the brain during the chronic phase of infection also represents a parasite adaptation remains to be elucidated.

Genome sequencing efforts and the broad availability of DNA microarrays that cover the genomes of several major host species have allowed high-throughput approaches for the analyses of host cell modifications by *Toxoplasma* (Blader et al., 2001; Saeij et al., 2007). Furthermore, significant technical improvements in proteome analyses have recently enabled investigations into how intracellular *T. gondii* manipulates its host cell at a post-transcriptional level (Nelson et al., 2008). These studies have yielded valuable insights into the regulation of glycolysis, lipid and sterol metabolism, the cell cycle, apoptosis and the cytoskeleton in the host cell (Blader et al., 2001; Saeij et al., 2007). Our knowledge about the level of complexity of such processes will certainly increase further in the near future, when detailed analyses of post-translational protein modifications, i.e. phosphorylation, methylation, acetylation, sumoylation and others have been performed. Furthermore it remains to be resolved whether all mRNA and/or protein levels altered due to infection by *T. gondii* have a functional relevance. Functionally important *T. gondii*-induced modifications that have already been investigated to a considerable extent during recent years are the inhibition of inflammatory host responses, the evasion of anti-parasitic effector mechanisms, and the prevention of host cell apoptosis by *T. gondii*.

Parasite-dependent anti-inflammatory effects

Immunity against *T. gondii* predominantly relies on Th1-type responses. However, it is now evident that the parasite partially antagonizes or even subverts inflammatory host responses. Besides the well-known upregulation of host anti-inflammatory molecules such as interleukin (IL)-10, transforming growth factor (TGF)-β and lipoxin A4, direct modulations of different

288 pro-inflammatory signalling cascades by intracellular *T. gondii* have been characterized
289 (Butcher et al., 2001; Kim et al., 2004; Butcher et al., 2005; Shapira et al., 2005). Together,
290 anti-inflammatory effects during *Toxoplasma* infection restrict the production of IL-12,
291 interferon (IFN)-γ and tumour necrosis factor (TNF)-α by dendritic cells, macrophages and/or
292 T cells thereby down-regulating both host immunopathology and anti-parasitic effector
293 mechanisms.
294 The nuclear factor κB (NF-κB) and mitogen activated protein kinase (MAPK) pathways are
295 critical for inducing expression of pro-inflammatory TNF-α and IL-12. Infection of
296 macrophages or dendritic cells with *T. gondii* rapidly induces phosphorylation and subsequent
297 inactivation of the cellular NF-κB inhibitor IκB (Butcher et al., 2001), possibly by a kinase
298 activity at the *T. gondii* parasitophorous vacuole membrane (Molestina and Sinai, 2005).
299 Despite inactivation of IκB, however, several studies using both murine macrophages and
300 human fibroblasts have indicated that NF-κB fails to translocate into the nucleus of *T. gondii*-
301 infected cells (Butcher et al., 2001), possibly because phosphorylation of NF-κB itself is
302 modulated (Shapira et al., 2005). Contrary to that, Molestina et al. (2003) described the
303 translocation of NF-κB following infection of immortalized murine embryonic fibroblasts and
304 Vero cells with *T. gondii*. The reason for this discrepancy has not yet been fully resolved but
305 may involve host cell- and parasite strain-dependent differences. Importantly,
306 lipopolysaccaride (LPS)-induced activation of the NF-κB pathway is temporarily also
307 inhibited (Butcher et al., 2001; Shapira et al., 2005) thereby diminishing pro-inflammatory
308 cytokine production in response to inflammatory stimuli. Likewise, early activation of
309 specific MAPKs by *T. gondii* renders infected macrophages refractory to subsequent LPS-
310 induced IL-12 and TNF-α production (Kim et al., 2004). Recently, parasite-driven activation
311 of signal transducers and activators of transcription 3 (STAT3) has been shown to suppress
312 LPS-induced TNF-α and IL-12 production in infected host cells (Butcher et al., 2005).

Whereas in non-infected cells STAT3 is activated by IL-10 receptor ligation, in infected cells, *T. gondii* activates STAT3 independently of IL-10 to establish an environment in which anti-inflammatory effects counterbalance pro-inflammatory responses of the host. Remarkably, the ability to modulate STAT3 signalling significantly differs between distinct genotypic lineages of *T. gondii* and depends on the polymorphic rhoptry kinase ROP16 (Saeij et al., 2007). It is evident that host cell modification by *T. gondii* is an important pathogenic factor that may also critically influence disease outcome.

Interference with regulatory and effector functions of immune cells

In addition to anti-inflammatory effects, *T. gondii* has a major impact on regulatory and effector functions of its host cells. Expression of major histocompatibility complex (MHC) class I and II molecules is strongly diminished in *T. gondii*-infected cells including macrophages and dendritic cells (DCs) (Luder et al., 2001; McKee et al., 2004). Importantly, *T. gondii* not only fails to activate macrophages and DCs during host cell entry and subsequent intracellular development, but also blocks the activation-induced MHC expression. Consequently, the antigen presentation to CD4+ T cells by infected host cells is also inhibited (Luder et al., 2001; McKee et al., 2004) and it can be hypothesized that this subverts CD4+ T cell expansion and effector functions against the parasite.

Expression of MHC class II molecules in macrophages is regulated by IFN-γ-triggered activation of the JAK/STAT pathway. It appears that intracellular *T. gondii* parasites do not interfere with initial activation and nuclear translocation of the transcription factor STAT1, but nevertheless block the activity of STAT1-dependent promoters (Lang et al., 2006; Kim et al., 2007). Although the exact mechanism remains to be established, it can be speculated that the parasite interferes with the assembly or activity of the basal transcriptional machinery of STAT1-responsive promoters.

The finding that *Toxoplasma* blocks STAT1-dependent promoter activity raised the intriguing possibility that transcription of other IFN-γ-regulated genes may also be inhibited in the presence of the parasite. Since IFN-γ is the major mediator of resistance against *T. gondii*, such general interference with IFN-γ-regulated gene expression may be critical for the parasite's intracellular survival by evasion of anti-parasitic effector mechanisms. This view is supported by the finding that *T. gondii* partially abrogates the IFN-γ-induced expression of the inducible nitric oxide synthase (iNOS) and the production of NO (Luder et al., 2003). Most importantly, such host cell modification is crucial for allowing parasite replication in moderately activated macrophages. Interestingly, microarray analyses have shown that *T. gondii* generally deregulates IFN-γ-induced gene expression in human fibroblasts (Kim et al., 2007) indicating that *T. gondii* infection has a deep impact on signalling events of its host cells.

Modulation of host cell apoptosis

Modulation of host cell apoptosis is a prominent feature of *T. gondii* infections and may be critical for the course of infection. *T. gondii* can exert opposite effects on the cell death program of the host, being able to both trigger (Khan et al., 1996; Gavrilescu and Denkers, 2001; Mordue et al., 2001) and inhibit apoptosis (Nash et al., 1998; Goebel et al., 2001; Payne et al., 2003). Obviously, induction of apoptosis in T and B lymphocytes, natural killer (NK) cells, macrophages and granulocytes contributes to evasion of the host’s immune attack during the acute phase of infection (Gavrilescu and Denkers, 2001; Mordue et al., 2001). Whereas moderate levels of leukocyte apoptosis may both assist intracellular survival of *T. gondii* and restrict immunopathology, exaggerated leukocyte apoptosis leads to unrestricted parasite multiplication and the host’s death. High levels of apoptosis result from an overwhelming inflammatory response after infection and may be mediated by both Fas/FasL-

363 dependent and TNF-α/TNF-R1-dependent mechanisms (Gavrilescu and Denkers, 2001;
364 Mordue et al., 2001).
365 Besides triggering apoptosis in certain cell populations during acute infection, *T. gondii* exerts
366 prominent anti-apoptotic effects. As with other intracellular pathogens, inhibition of host cell
367 apoptosis by intracellular *T. gondii* may be required to counteract the intrinsic cellular suicide
368 program of infected cells as well as the extrinsic FasL or granzyme/perforin-mediated killing
369 of infected host cells by T and NK lymphocytes. During recent years, considerable progress
370 has been made in understanding the molecular mechanisms of the anti-apoptotic effects of *T.*
371 *gondii*. It has been established that modification of multiple host cell targets at both
372 transcriptional and post-transcriptional levels play an important role. Such redundancy may
373 explain why the parasite can protect a variety of different cell types against apoptosis induced
374 by a broad spectrum of extrinsic and intrinsic pro-apoptotic stimuli (Nash et al., 1998; Goebel
375 et al., 2001; Payne et al., 2003; Keller et al., 2006; Kim et al., 2006; Vutova et al., 2007;
376 Hippe et al., 2008). In contrast to *Plasmodium* or *Theileria,* with their restricted host cell
377 range, *T. gondii* may indeed require multiple mechanisms in order to efficiently block host
378 cell apoptosis in different host cell populations. For example, *T. gondii* inhibits Fas/CD95-
379 mediated apoptosis in cells that directly activate downstream effector caspase 3 via the death
380 receptor pathway, by aberrant cleavage and degradation of the furthest upstream initiator,
381 caspase 8 (Vutova et al., 2007). In contrast, in cells in which Fas/CD95-triggered apoptosis
382 relies on the mitochondrial amplification loop, parasite-dependent inhibition of apoptosis is
383 primarily achieved by protection of the mitochondria (Hippe et al., 2008). A critical step in
384 transducing upstream triggers into the mitochondrial apoptotic pathway is normally the
385 Bak/Bax-assisted release of cytochrome c into the cytosol. *T. gondii* has been shown to inhibit
386 cytochrome c release and this correlates with an altered balance of pro- and anti-apoptotic
387 Bcl-2 family members (Goebel et al., 2001; Molestina et al., 2003; Carmen et al., 2006).
388 Transcriptional up-regulation of anti-apoptotic Bfl-1 (Molestina et al., 2003), increased levels

of anti-apoptotic Mcl-1 protein (Goebel et al., 2001), and decreased levels of pro-apoptotic Bax and Bad proteins (Carmen et al., 2006) have been described. However, the contributions of each of these different mechanisms in inhibiting cytochrome c release remain to be elucidated. Furthermore, activation or inhibition of PI3 kinase (Kim and Denkers, 2006) and c-Jun N-terminal kinase (JNK) (Carmen et al., 2006) signalling pathways, respectively, may also account for some of the anti-apoptotic effects of *T. gondii*. Finally, using an in vitro reconstitution system, activation of caspase 3 by cytochrome c but not granzyme B was shown to be directly abolished by viable parasites or excretory-secretory molecules of *T. gondii* (Keller et al., 2006). This raises the interesting possibility that *Toxoplasma* is able to interfere with activation of caspase 3 independently of an altered cytochrome c release via the translocation of a still uncharacterized parasite effector molecule into the host cell cytosol. The above-mentioned parasite-dependent survival mechanisms all rely on an inhibition of apoptotic signalling pathways. Recently, an alternative explanation has been put forward. It has been shown that death receptor ligation or perforin treatment results in a Ca^{2+}-dependent egress of *T. gondii* leading to necrotic host cell death (Persson et al., 2007). At first, these results are difficult to reconcile with previous morphological data, which has clearly established that cells containing intracellular parasites are protected from undergoing death receptor-triggered apoptosis (Payne et al., 2003; Hippe et al., 2008). However, it might well be that besides active interference of intracellular *T. gondii* with an ordered cell death, parasite egress and necrotic host cell death can be induced. More experiments are needed to unravel the significance of these different mechanisms used by *T. gondii* parasites to modify the host cell to ensure its own survival.

***Theileria*-infected leukocytes – a unique example of one eukaryote cell transforming another eukaryote cell.**

How *Theileria* parasites transform their host leukocytes is a fascinating example of a host-parasite interaction that has a wider relevance to cancer biology. As with tumour cells, *Theileria*-induced pathology can be broken down into 3 phases: 1) inducing the infected host cell to survive, or in other words anti-apoptosis signalling; 2) inducing the infected host cell to divide *i.e.* uncontrolled proliferation; 3) inducing illicit leukocyte metastasis – for example, the massive invasion of the lungs by *T. parva*-infected T cells in East Coast Fever.

In contrast to *Plasmodium* and *Toxoplasma* parasites, *Theileria* macroschizonts are not enclosed within a parasitophorous vacuole, but are associated with host cell microtubules (Shaw et al., 1991). This means that any protein located on the surface of *Theileria,* or secreted by the parasite is immediately in contact with the host cell cytosol and can become associated with the host cell cytoskeleton (Schneider et al., 2007). Moreover, secreted proteins, such as the AT-hook DNA-binding proteins of the TashAT family, can even end up in the host cell nucleus (Shiels et al., 2004), where they may influence host cell gene expression. However, specific host cell genes regulated by TashAT family members have yet to be identified.

Anti-apoptotic signalling in Theileria-infected leukocytes

Activation of the transcription factor NF-κB was the first signalling event shown to be associated with parasite-mediated leukocyte survival. After the seminal paper in 2002 that described the hijacking of the IKK signalosome by the parasite (Heussler et al., 2002), a follow-up paper illustrated how NF-κB mediates the *Theileria*-dependent anti-apoptotic response (Kuenzi et al., 2003). However, how the parasite actually recruits the IKK signalosome remains obscure. In the last four years, it became clear that additional host cell pathways are activated by the presence of the parasite and contribute to survival and proliferation of infected leukocytes (Figure 1).

First, came the description that JNK mediates an anti-apoptotic response, since inhibition of JNK led to chromatin condensation and host cell death (Lizundia et al., 2005). It had been known for some time that infection by *Theileria* activates host leukocyte JNK (Galley et al., 1997), but this was believed to be part of a signal transduction pathway that leads to induction of the AP-1 transcription factor (Chaussepied et al., 1998). Subsequently it was shown that inhibition of AP-1 by expression of a dominant negative c-Jun mutant rendered *Theileria*-infected B cells more likely to die of apoptosis (Lizundia et al., 2006). Since the levels of the anti-apoptotic proteins cIAP and Mcl1 were diminished following forced AP-1 down-regulation, it seemed reasonable to suppose that cIAP and Mcl1 mediate, at least in part, the *Theileria*-dependent anti-apoptotic response. Although these data provided the first mechanistic insight as to how *Theileria* might promote host cell survival, additional JNK substrates that could also contribute to the anti-apoptotic response should be considered. JNK can also mediate an anti-apoptotic response by direct phosphorylation of Mcl1 (Inoshita et al., 2002) and Bcl2 (Deng et al., 2001). In addition to Mcl1, Bcl2 and its related family members are upregulated in *Theileria*-infected leukocytes (Kuenzi et al., 2003) (Guergnon et al., 2003), but whether they are indeed phosphorylated by JNK has not yet been addressed.

Theileria-infected leukocytes secrete a number of different cytokines, so it was natural to examine cytokine-mediated signalling and host cell survival. *T. parva*-infected B cells were known to produce granulocyte macrophage colony-stimulating factor (GM-CSF) and use it, via an autocrine loop, to augment infected lymphocyte proliferation (Baumgartner et al., 2000). In a subsequent study, GM-CSF was shown to signal via JAK2 to STAT3 and to contribute to the induction of the transcription factor c-Myc (Dessauge et al., 2005a). Anti-sense knockdown of c-Myc expression generated infected B cell apoptosis, demonstrating that c-Myc induction is yet another way *Theileria* parasites assure host cell survival (Dessauge et al., 2005a). Many different cytokines can signal through JAK2, so it was significant that ectopic expression of c-Myc rescued *T. parva*-infected B cells from death induced by JAK2

inhibition (Dessauge et al., 2005a). This implies that in *Theileria*-infected leukocytes, cytokine signalling results in JAK2 activation and this, in turn activates c-Myc (Dessauge et al., 2005a). Moreover and consistently, c-Myc activation leads to an increase in Mcl1 levels (Dessauge et al., 2005a). Finally, as both AP-1 and NF-κB contribute to *c-myc* transcription in *Theileria*-infected leukocytes and up-regulated casein kinase CK2 (ole-MoiYoi et al., 1993) phosphorylates c-Myc and thus increases its half-life (Dessauge et al., 2005c), it led to the notion that all pathways activated by *Theileria* infection might feed into c-Myc activation (Dessauge et al., 2005c). In support of this assumption, it has been shown that a forced inactivation of AP-1 in *Theileria*-infected B cells results in decreased levels of Mcl1 and it is well possible that this is mediated indirectly by reduced c-Myc expression.

Theileria-induced host cell proliferation

One of the noteworthy cancer-like features of *Theileria*-infected leukocytes is their ability to grow continuously *in vitro* without the necessity of adding exogenous growth factors. As stated above, using GM-CSF as an example, one way that *Theileria* achieves this growth factor independence is by inducing its host leukocyte to secrete cytokines that then feed back to re-stimulate infected host cell proliferation via autocrine loops (Baumgartner et al., 2000). Although an elegant explanation, one is rapidly confronted with a "chicken and the egg" situation when it comes to GM-CSF (and perhaps other autocrine and paracrine signals), as its production is PI3-K dependent yet it also stimulates PI-3K (Baumgartner et al., 2000). So, does *Theileria* induce PI3-K (Baumgartner et al., 2000) (Heussler et al., 2001) to produce GM-CSF (and other cytokines), or does it produce GM-CSF (and other cytokines) to stimulate PI3-K?

Cytokines are not the only growth factors required by proliferating leukocytes, as they also need transferrin (Tf) and as a consequence *Theileria*-infected leukocytes up-regulate transferrin receptor (TfR; (Naessens et al., 1996)). *Theileria* achieves accelerated recycling of

host cell TfR and increased Tf uptake via induction of both the Rab5A and Rab11A endocytic compartments (Baumgartner et al., 2005). Infected leukocyte proliferation had previously been shown to be PI3-K dependent by pharmacological blockage with wortmannin and LY292004 (Baumgartner et al., 2000) (Heussler et al., 2001), but these drugs also block TfR recycling due to their inhibition of type III PI3-K (Vps34), suggesting that drug-inhibition of infected leukocyte proliferation could be due in part to ablated TfR recycling.

How *Theileria* induces increased amounts of Rab11A to assure the accelerated recycling of TfR has been studied in some detail and found to be JNK/AP-1 dependent (Lizundia et al., 2007). In the host cell survival section we discussed how *Theileria* infection activates the JNK/AP-1 pathway to promote transcription of anti-apoptotic genes, but AP-1 transactivation also leads to transcription of the gene coding for Rab11A (Lizundia et al., 2007). In eukaryotes, not only does AP-1 mediate *rab11a* transcription, but it also activates that of *cyclin D1* and *TfR* and if this occurs in *Theileria*-infected leukocytes, the parasite could then coordinate the induction of several genes required for the G1 to S transition of its host leukocyte's cell cycle (Lizundia et al., 2007). The composition of AP-1 complexes binding to the promoter of the matrix metallo-proteinase 9 (*mmp9*) gene changes during attenuation (loss of metastatic potential) of *Theileria*-infected macrophages (Adamson et al., 2000). This implies that different AP-1 subsets are induced by *Theileria*-mediated host cell transformation and that specific AP-1 complexes might regulate host cell survival, proliferation and invasiveness of *Theileria*-infected leukocytes.

Alterations in host cell cytoskeleton and illicit invasiveness of Theileria-infected leukocytes

Theileria-infected leukocytes behave as tumours and invade multiple different organs, causing leukaemia-like pathologies (Lizundia et al., 2006). Upon *Theileria*-induced transformation, infected macrophages loose their ability to spread on fibronectin, as can be readily detected by immunofluorescence analysis using anti-CD44 and anti-Paxillin

517 antibodies to stain infected and drug-cured macrophages, as shown in Figure 2A. Podosomes
518 are actin- and fimbrin-containing adhesions in macrophages and efficient spreading of
519 macrophages on fibronectin requires strong and stable focal adhesions that appear to be
520 diminished upon *Theileria* infection. Clearly, just how *Theileria* regulates host cell spreading
521 on fibronectin merits investigation, but regulation of host cell microtubule polymerisation
522 could be the underlying mechanism (Evans et al., 2003). Interestingly, a secreted *Theileria*
523 protein (TaSE) has recently been observed to be associated with host cell alpha-tubulin
524 (Schneider et al., 2007).
525 Another way that *Theileria* might regulate the cytoskeleton, promote host leukocyte motility
526 and illicit invasiveness is via its induction of STAT3 (Gao and Bromberg, 2006). We have
527 previous mentioned *Theileria*'s ability to induce STAT3, as part of a GM-CSF mediated
528 autocrine loop that leads to c-Myc activation (Dessauge et al., 2005a). Now, we are proposing
529 that cytosolic STAT3, present in *Theileria*-infected leukocytes, could regulate host cell
530 microtubules by antagonizing the depolymerization activity of stathmin (also known as
531 oncoprotein 18, OP18) (Ng et al., 2006). Stathmin's microtubule destabilizing activity can
532 also be downregulated by phosphorylation by PKA (Gradin et al., 1998) and we have
533 previously shown that *Theileria* infection leads to augmented PKA activity of host leukocytes
534 (Guergnon et al., 2006). PKA clearly plays an important role in negatively regulating
535 stathmin, arguing that PKA phosphorylation of stathmin might be relevant in *Theileria*-
536 infected leukocytes.
537 Finally, a third potential way for *Theileria* to alter leukocyte motility is via PKA-mediated
538 phosphorylation of integrin alpha-4. This possibility is suggested by the work of Mark
539 Ginsberg's laboratory, that recently showed that integrin alpha-4 is both a PKA anchor
540 protein (AKAP), as well as a substrate for the kinase (Lim et al., 2007). As *Theileria*-infected
541 B cells express integrin alpha-4 and have augmented PKA activity, it is not surprising that
542 alpha-4 is indeed phosphorylated (Figure 2B). Phosphorylated integrin alpha-4 appears to be

543 at the basal side of *Theileria*-infected B cells, where the lymphocyte has contact with
544 substratum. It follows that *Theileria*-infected lymphocytes might be capable of signalling
545 through activated integrins, either due to contact with substratum, or via cell-cell contact
546 between infected lymphocytes. It has been known for a long time that cell-cell contact of
547 *Theileria*-infected T cells increases their proliferation (Dobbelaere et al., 1991) (Chaussepied
548 et al., 2006). Clustering of integrins in *Theileria*-infected leukocytes may be sufficient to
549 recruit PKA and consequently to direct bound PKA to phosphorylate additional substrates at
550 podosomes. In this context, metallo matrix protease 9 (MMP-9) up-regulation in B cell
551 chronic lymphocytic leukaemia is mediated by integrin alpha-4 engagement in podosomes
552 (Redondo-Munoz et al., 2006) and *Theileria* infection is known to induce MMP-9 (Baylis et
553 al., 1995) (Adamson et al., 2000). This strongly suggests that *Theileria*-dependent PKA
554 activation and its phosphorylation of integrin alpha-4 combined with PKA's potential to
555 phosphorylate stathmin might lead to altered microtubule dynamics at podosomes of infected
556 cells and this could be one of the major mechanisms used by the parasite to increase leukocyte
557 invasiveness.
558
559 **Concluding remarks and future directions**
560 It is generally accepted that *Plasmodium*, *Toxoplasma* and *Theileria* parasites modulate
561 signalling of their respective host cells and we are now beginning to understand the molecular
562 details of this phenomenon. The emerging picture reveals similarities and differences in
563 parasite-dependent signalling events in host cells (Figure 3). All three parasites seem to
564 activate the host cell PI3 kinase pathway, although for *Plasmodium* liver stage parasites, this
565 activation appears to be restricted to the early stages developing in hepatocytes. Interestingly,
566 the effects of PI3 kinase activation on the respective host cells are entirely different. PI3
567 kinase activation in *Theileria*-infected leukocytes is clearly linked to host cell proliferation,
568 whereas in *Plasmodium*- and *Toxoplasma*-infected cells it is rather a cell survival signal.

Interestingly cell survival of *Theileria*-infected cells is ensured by NF-κB-dependent gene transcription. In *Plasmodium*-infected cells, however, NF-κB-dependent expression of host cell genes is even blocked, most likely to avoid inflammatory immune responses.

Cells infected by *Toxoplasma* and *Theileria* show activation of STAT3 and exhibit elevated levels of anti-apoptotic members of the Bcl-2 family (see Figure 3). The obvious question is now why the phenotypes of *Theileria* and *Toxoplasma*-infected cells are so different despite similar pathways being activated. Whereas *Theileria*-infected leukocytes acquire a transformed phenotype, the effects of a *Toxoplasma* infection are restricted to short term survival signals and suppression of inflammatory signals in the infected cell. Although it is possible that in *Theileria*-infected cells, the additional activation of c-Myc and NF-κB is responsible for the transformed status of the cell, it is more likely that a combination of several pathways is necessary to immortalize the infected cell. Since it has been shown that the level of NF-κB activation decides whether a cell survives or undergoes apoptosis, the parasite needs to tightly control the state of activation, probably by a kind of feedback mechanism.

An important challenge for the near future is to identify secreted parasite molecules that decide the fate of the host cell. Although a number of secreted proteins have already been identified for *Theileria*, *Toxoplasma* and *Plasmodium*, the function of these proteins remains in most cases somewhat enigmatic. Most likely, it is not a single secreted parasite molecule but a combination of molecules that dictates the phenotype of their respective host cells. Since a viable host cell is so essential for the developing parasite, a certain level of redundancy of secreted parasite molecules in inducing host cell survival would not be surprising. In any case, it will be difficult to decipher the molecular details of parasite-dependant host cell modulation and new technologies allowing simultaneous targeting of several genes or proteins are needed.

A potentially very powerful technique for controlling protein expression, that uses a destabilization domain (DD) fused to the protein of interest was recently reported for *P. falciparum* (Armstrong and Goldberg, 2007) and *T. gondii* (Herm-Gotz et al., 2007) and the future will show whether this technique can be applied generally or is only useful for the control of selected proteins. This or similar approaches are very important for malaria research as siRNA knockdown approaches do not work in *Plasmodium*. Since the generation of conditional KO parasites is a very time consuming process, using proteins containing DD domains would significantly simplify the analysis of protein function in various life cycle stages.

The COST action 857 has provided a very important forum for discussing parasite-host cell interactions and many productive collaborations have been initiated and strengthened at the annual COST meetings.

What can we expect in the coming years? Improved imaging techniques combined with the use of fluorescent parasite strains and host cells will help to investigate in detail parasite entry and intracellular development of apicomplexan parasites. For example, it is still an unsolved issue how *Plasmodium* liver stage parasites develop from one single sporozoite entering a cell to more than 30 000 merozoites in less than two days. This is a unique feature of the parasite and it is safe to predict that corresponding molecular pathways will be identified, which might serve as potent targets for new anti-parasitic approaches. So far relatively little is known about parasite-dependent signalling events in *Plasmodium*-infected hepatocytes but we have evidence that tyrosine phosphorylation events occur at the PVM (Figure 4), which is the interface between parasite and host cell cytoplasm and thus ideal for transmitting information in both directions. Since no typical tyrosine kinases have been found in the *Plasmodium* kinome (Ward et al., 2004), it is likely that host cell kinases are responsible for the observed tyrosine phosphorylation in the PVM. However, the relevance of these signalling events and the involved kinase(s) remain to be identified.

Other dynamic processes like migration of *Theileria*-infected cells, which are known for their metastatic potential, are an open field of research with a great potential. Unfortunately, the inability to routinely transfect *Theileria* parasites remains an unsolved issue despite the recent efforts in this area. However, the host cell can be manipulated and we can expect exciting news on host cell signalling events in *Theileria*-infected cells and how the parasites manage to stimulate host cell division and connect to the spindle apparatus of the dividing host cell to ensure distribution to both daughter cells.

Since *T. gondii* is relatively easy to manipulate and because inducible systems are well established for this parasite, it can be expected that research on *Toxoplasma*-host cell interaction advances rapidly in the future. Additionally, the DD technique appears to work well in *Toxoplasma* (Herm-Gotz et al., 2007) and thus will be a useful tool to analyze the function of proteins of interest.

Hopefully the growing community in the field of apicomplexan research in Europe will further combine forces, perhaps in frame of another COST action, to overcome technical hurdles and decipher more fascinating details of parasite-dependent host cell modifications.

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646 **Figure legends**

647 Figure 1. *Theileria* parasites invade bovine leukocytes and activate a number of signal
648 transduction pathways. This results in the translocation of transcription factors to the host cell
649 nucleus, leading to the transcription of specific genes. *Theileria* parasites are associated with
650 microtubules, in close proximity to microtubule organising centre (green spot). Cytokines
651 (CK) secreted by transformed leukocytes can sometimes bind to their cognate receptors,
652 stimulating them to further activate signal transduction pathways via autocrine loops. We
653 propose that STAT3 and JNK may be associated with and regulate microtubules in a parasite-
654 dependent manner.

655

656 Figure 2. A) *Theileria annulata* infects and transforms host macrophages that then loose their
657 ability to spread on fibronectin. When infected macrophages are treated with the *Theileria*-
658 specific drug Buparvacone (B+), the parasite dies and the host macrophage recovers its
659 phenotype of spreading on fibronectin, forming podosomes enriched in CD44 (green) and
660 Paxillin (red). B) *Theileria parva*-infected B cells express phosphorylated integrin alpha-4
661 (red staining) that localises at the basal side of the infected B cell, where the lymphocyte has
662 contact with the substratum. DNA is stained with DAPI (blue).

663

664 Figure 3. Parasite-dependent host cell signalling pathways and molecules.

665

666 Figure 4. Tyrosine phosphorylation events in the PVM of *P. berghei*-infected hepatocytes 48
667 h post infection. PVM is stained with an anti-Exp1 chicken antiserum (green) and tyrosine
668 phosphorylation is detected by staining with a anti-phosotyrosine mouse monoclonal antibody
669 (red). DNA is stained with Dapi (blue).

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*** Response to Reviewers**

Reviewers' comments:

Reviewer #1: The review article by Luder et al. is a quite good summary of the main findings of the last few years concerning the host cell modifications induced by 3 different Apicomplexan parasites. The review becomes particularly interesting because the 3 parasites, although similar in certain aspects, have developed quite distinct approaches to survive and become successful inside their hosts.

There are only a few points that I think will be worth changing in order for the review to become more coherent.

Page 6 - line 114 - The cited reference "Mota and Rodrigues, 2001" is not the original paper describing transmigration through cells. The original reference (Mota et al., 2001. Science 291(5501):141-4) should be included.

done

Page 6 - line 114 - Why only mention spect 2? Spect 1 was published before spect 2. In addition besides spect 1 and 2 at least 2 other Plasmodium proteins have been shown to be involved in transmigration as well: PbPL (Bhanot et al., 2005. JBC 280(8):6752-60) and TLP (Moreira et al. 2008. Cell. Microb. 10(7):1505-16).
At least the original spect 1 paper should be cited.

We have added the TLP story (and ref) but since we discuss hepatocyte transmigration and not transmigration in the mosquito, we did not add the PbPl.

Page 6 - line 117 - When the authors state that "Two major roles have been hypothesized." this is not entirely correct. Several possible roles have been hypothesized, including traversal of cells from the skin to reach the liver in a review that should be mentioned here (Mota and Rodrigues, 2001. Microbes and Infection 3(13):1123-8).

We agree and have changed the text accordingly.

Page 6 - line 124 - Here the authors should mention that regulated exocytosis seems to contribute by increasing the efficiency of infection by 50% (Ono et al., 2008. PloS Pathogens 4(2):e1000008).

done

Page 7 - lines 147-151 - Here the authors should mention that irradiated sporozoites also seem to inhibit apoptosis to a lesser extent than WT parasites do (Leiriao et al., 2005. Cell. Microb. 191(10):1576-81).

done

Page 9 - line 208 - Change "affect" to "effect".

done

Figures - It is a little bit strange that the first figures are on Theileria and the last one on Plasmodium while the text flows in the opposite order. Also, the labels should be consistent and similar in all the figures. For example the 2 figures with immunofluorescence are labelled in distinct ways.

The order of the figures is strictly following their appearence in the text, so we don't see a point to change it. Labelling of the figures are now consistent - thanks for spotting this mistake!

Reviewer #2: The manuscript titled , "Intracellular survival by apicomplexan parasites and host cell modification" represents a combination of a review article and a report on a conference. The level of detail is appropriate given the broad intended audience. Importantly some unpublished data presented at the conference is stated or alluded to as is a section on broad future directions in what is a rapidly evolving field. Minor and easily addressable concerns regarding this manuscript are presented below.
1. To investigators outside Europe the term COST action 857 has meaning. A sentence in the introduction for informational processes would be useful.

I guess that you (Dominique) will address this issue in a general preface of the special IJP issue and I didn't want to be too redundant.

2. Given that this work is a hybrid between a conference report and review article mention of the specific conference should be made in the title or at the very least in the abstract. Something like, "Intracellular survival by apicomplexan parasites and host cell modification-Proceedings of the Corsica 2007 COST meeting"

It is not only the Corsica meeting covered but the entire COST action and therefore this suggestion is not applicable.

There is one issue on line 290-291 regarding the translocation of NFkB in toxoplasma infection that requires a more precise description. The work presented in Butcher et al as well as other papers from the Denkers and Hunter groups indicate that the inhibition of NFkB nuclear translocation is in fact transient and seen in the early stages of LPS stimulation of infected cells. With established infections nuclear NFkB (p65) is detected. Although a nuanced effect its impact is significant when examining the temporal expression of target genes.

We agree that conflicting results have been published concerning the NF-kB activation following Toxoplasma infection. We have now included these reports and have also included a statement on possible reasons for the discrepancy of the different studies.

On line 469 text should be modified to"(and perhaps other autocrine and paracrine signals)"

done

Line 514: rhoptry is misspelled

Thanks for spotting

Section starting at line 522: This section could be greatly abbreviated as the roles of the stathmin domains and STAT3 signaling in the context of Theileria while exciting and provocative still lack proof. The section invoking this potential pathway reads like a series of self fulfilling circular arguments. Thus these 2 pages can be greatly condensed

We fully agree on this comment and shortened the chapter accordingly.

Line 568: Change heading of Conclusive remarks... to Concluding remarks

done

Lines 579-81: In context of the statement above regarding the timing of NFkB activation this strong statement cannot be made without clarification of the specific cell types examined where differences for toxoplasma infection of macrophages (transient block of NFkB) and fibroblasts/ epithelial cells (early and sustained activation) are supported by both the biochemical data and temporal gene expression studies. The statement as written is misleading and should presented as spectrum of responses that is host cell type specific.

The statement has now been weakened in order to account for the conflicting results as outlined above.

Figure(s)
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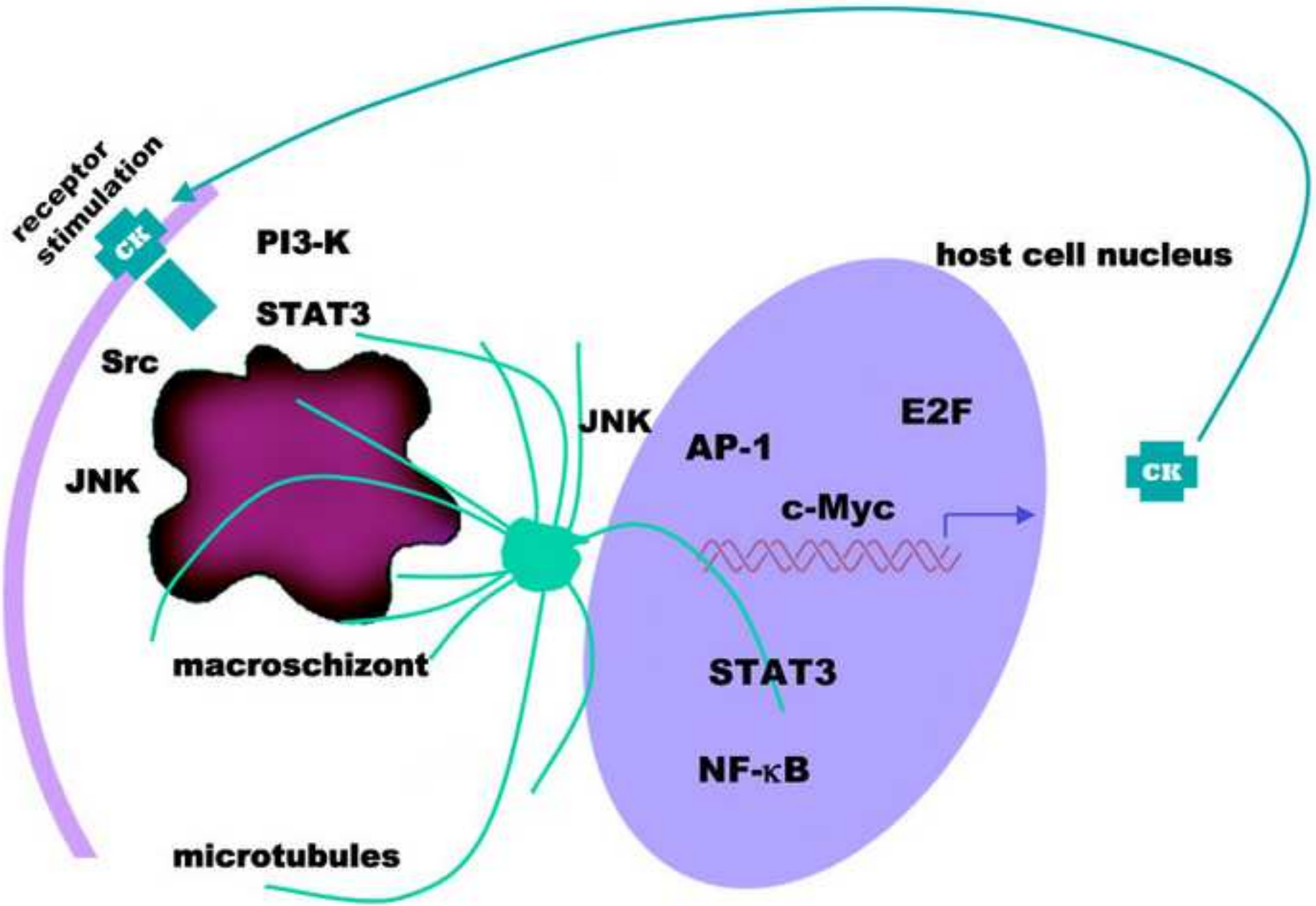
Figure 1, Lüder et al.



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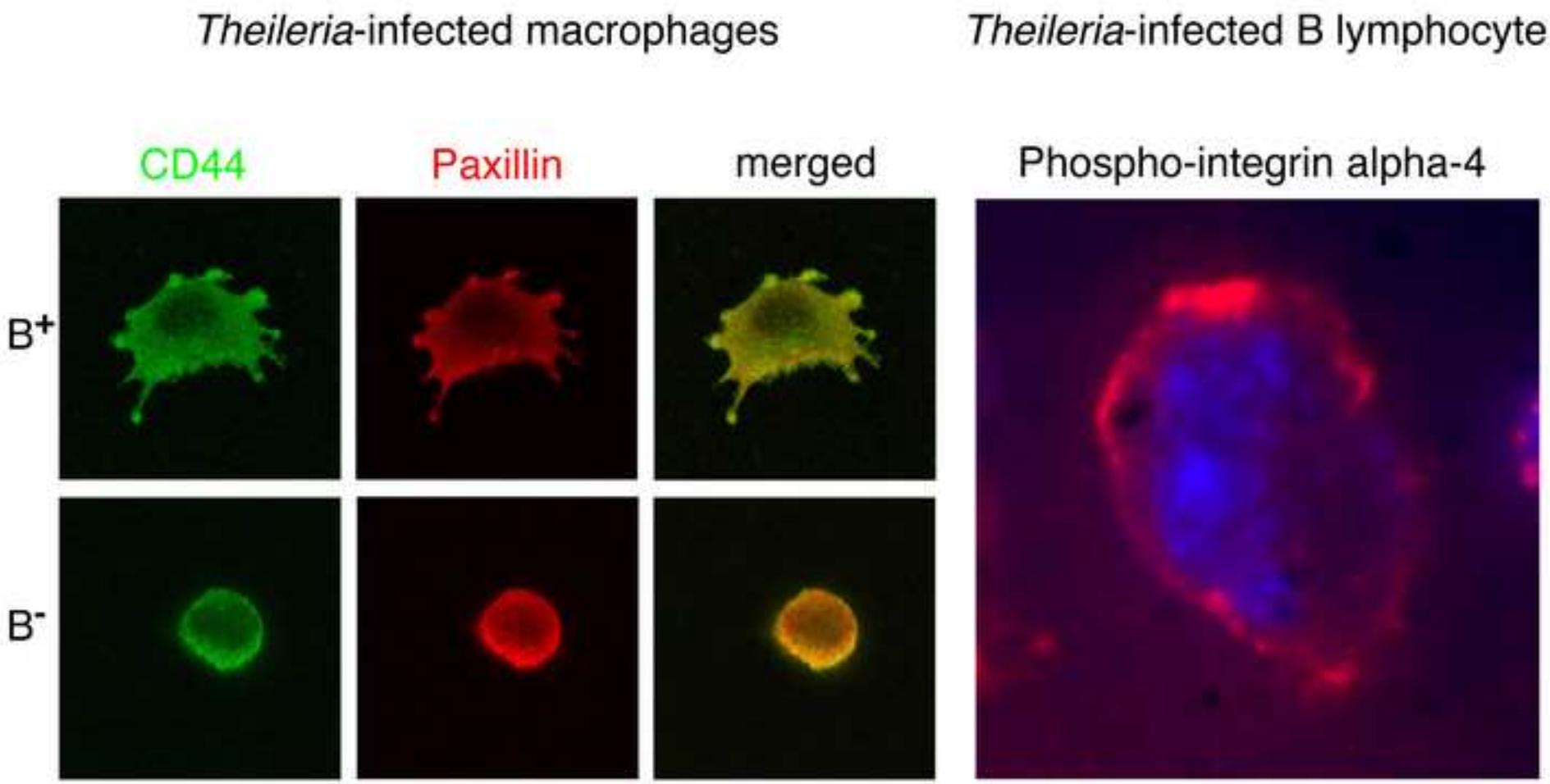

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Figure 3, Lüder et al.

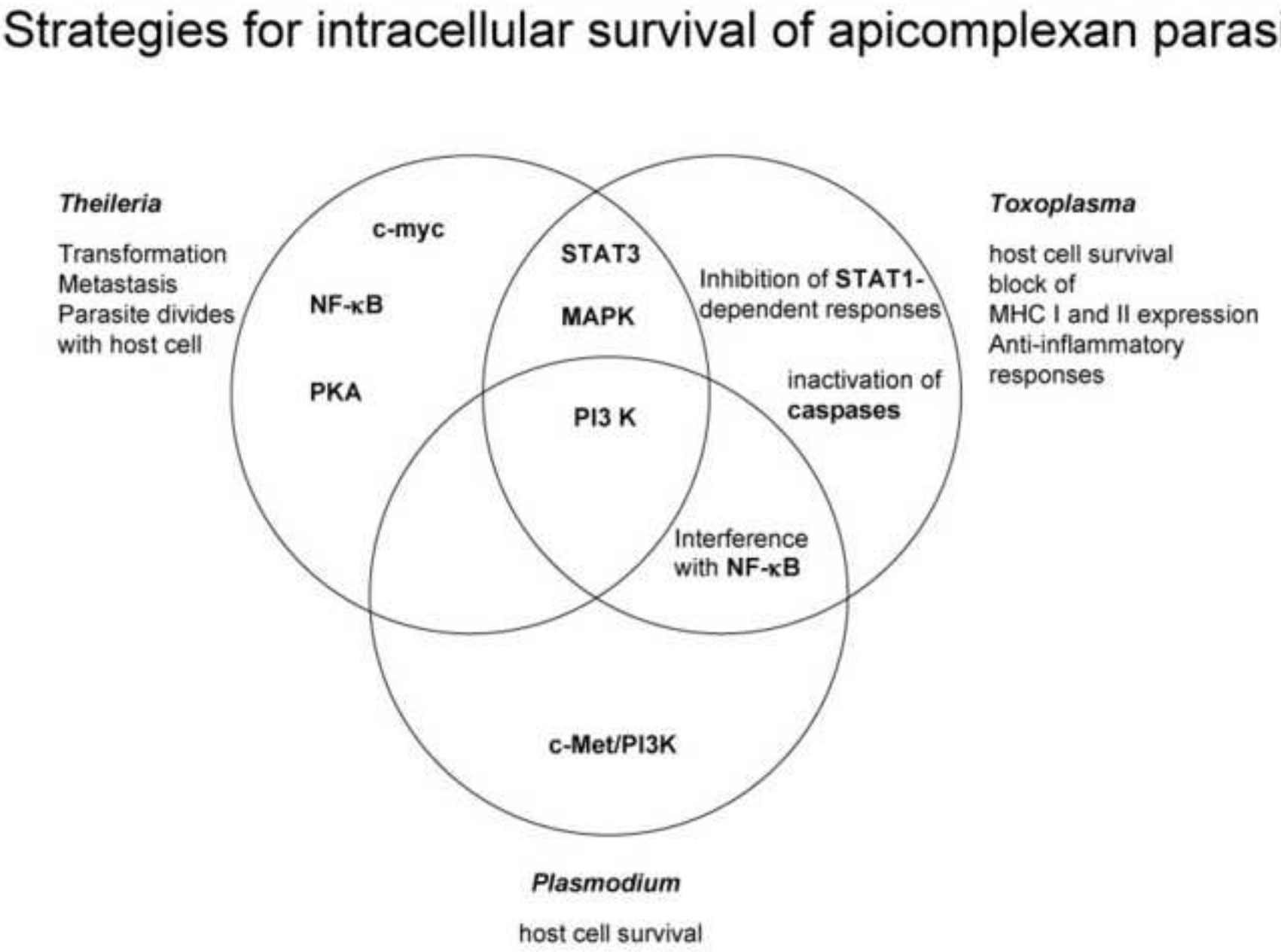

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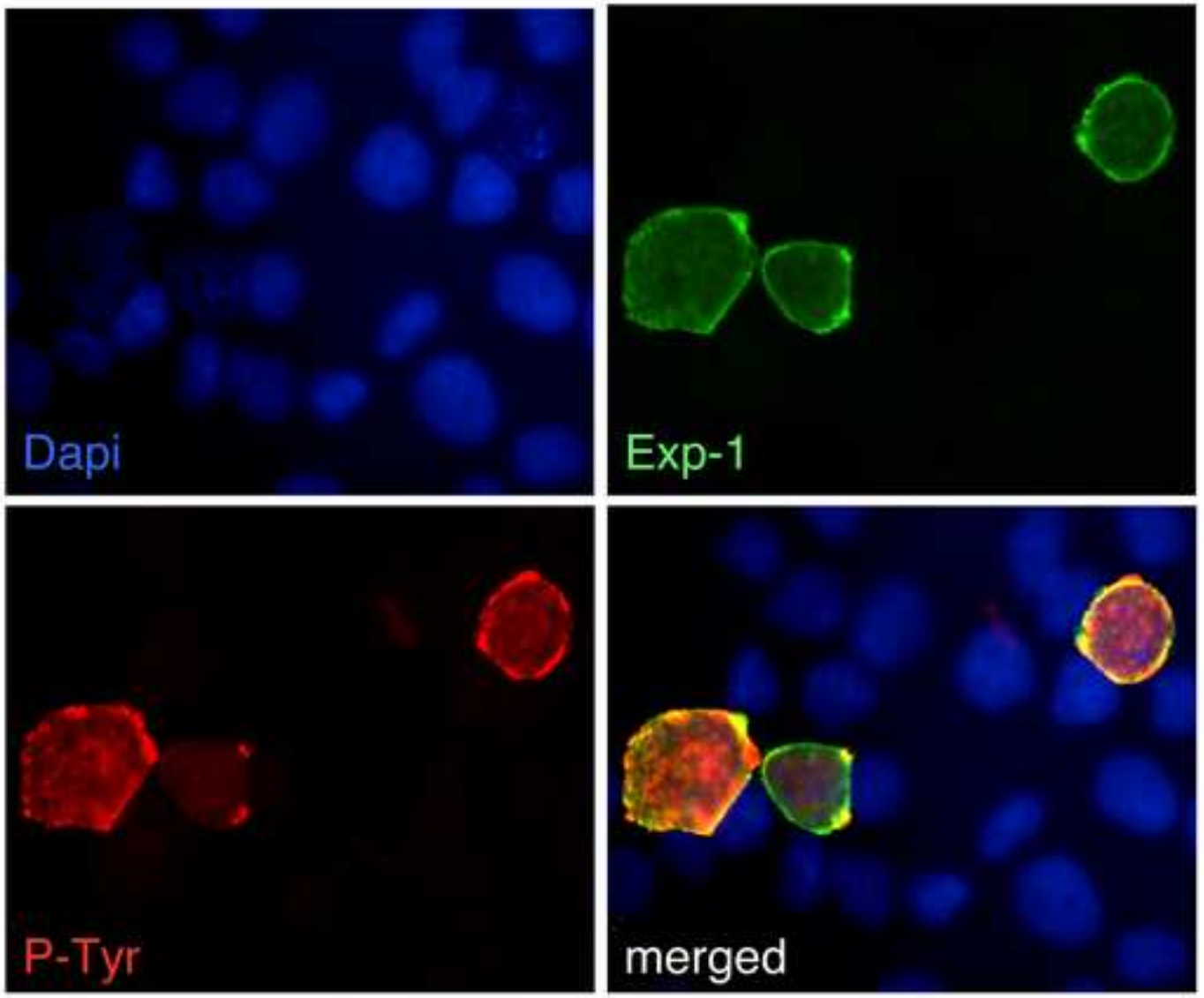
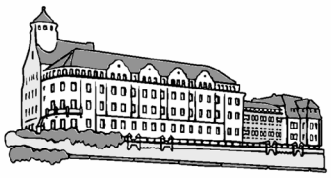
Figure 4, Lüder et al.



*** Cover Letter**



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Dominique Soldati
Editor IJP

Dear Dominique,

Enclosed please find the revised manuscript "Intracellular survival of apicomplexan parasites and host cell modification" by Lüder et al.
The criticism by the reviewers was very fair and we have changed the manuscript accordingly. I have also included a point-to-point response to the comments of the reviewers.
If you need any further information, please don't hesitate to contact me.

Warm regards,

Volker Heussler