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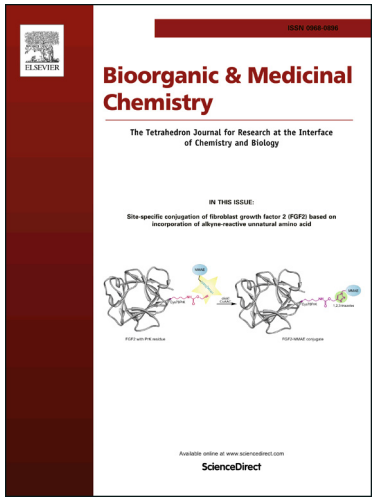

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Cell penetrating peptides to dissect host-pathogen protein-protein interactions in *Theileria*-transformed leukocytes

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Abstract

One powerful application of cell penetrating peptides is the delivery into cells of molecules that function as specific competitors or inhibitors of protein-protein interactions. Ablating defined protein-protein interactions is a refined way to explore their contribution to a particular cellular phenotype in a given disease context. Cell-penetrating peptides can be synthetically constrained through various chemical modifications that stabilize a given structural fold with the potential to improve competitive binding to specific targets. *Theileria*-transformed leukocytes display high PKA activity, but PKA is an enzyme that plays key roles in multiple cellular processes; consequently genetic ablation of kinase activity gives rise to a myriad of confounding phenotypes. By contrast, ablation of a specific kinase-substrate interaction has the potential to give more refined information and we illustrate this here by describing how surgically ablating PKA interactions with BAD gives precise information on the type of glycolysis performed by *Theileria*-transformed leukocytes. In addition, we provide two other examples of how ablating specific protein-protein interactions in *Theileria*-infected leukocytes leads to precise phenotypes and argue that constrained penetrating peptides have great therapeutic potential to combat infectious diseases in general.

1. Introduction

General Introduction to bovine leukocytes infected and transformed by *Theileria* parasites

Theileria is an intracellular protozoan transmitted by ticks, which affects ruminants. Tropical theileriosis exhibits many features of human leukemia and is caused by *Theileria annulata*, whereas *Theileria parva* causes a lymphoma-like disease called East Coast Fever. *T. annulata* infects bovine B cells and macrophages/monocytes, while *T. parva* infects bovine B and T lymphocytes (Chaussepied and Langsley, 1996, Dobbelaere and Heussler, 1999). When infected, the leukocytes become fully transformed and disseminate throughout the animal infiltrating both lymphoid and non-lymphoid tissues (lungs and gastrointestinal tract). Moreover, when *Theileria*-transformed macrophages are injected into immune-compromised mice they form disseminating tumors (Echebli et al., 2014, Lizundia et al., 2006, Somerville et al., 1998). Another cancer-like phenotype is that *in vitro Theileria*-transformed leukocytes proliferate in an uncontrolled manner and do not require exogenous growth factors (Dobbelaere and Heussler, 1999). Importantly, *Theileria*-dependent transformation is entirely reversible upon theilericidal drug treatment and so does not involve irreversible changes (mutations) to the host genome. The theilericidal drug buparvaquone specifically kills the parasite and the previously transformed leukocytes stop proliferating and regain their dependence on exogenous growth factors (Dobbelaere et al., 1988).

Importantly, live attenuated vaccines exist to tropical theileriosis (Darghouth, 2008) that are generated by multiple passages of infected macrophages, which with time become attenuated for virulence i.e. they lose their hyper-disseminating virulence trait (Baylis et al., 1995, Echebli et al., 2014, Hall et al., 1999). A number of studies have provided evidence on how infection manipulates leukocyte signal transduction pathways, contributing to different

aspects cellular transformation (Chaussepied and Langsley, 1996, Cheeseman and Weitzman, 2015, Dessauge et al., 2005, Dobbelaere and Heussler, 1999). Thus, *Theileria*-provoked leukocyte transformation provides a unique, reversible model with the potential to provide key insights relevant to leukemia. Here, we discuss the use of penetrating peptides to competitively ablate specific protein-protein interactions in infected cells and the consequences this has on tumor phenotypes of *Theileria*-transformed leukocytes.

Cell penetrating peptides (CPPs)

CPPs have gained much attention due to their ability to enter cells via a receptor-independent mechanism and deliver biologically active cargo molecules into the cell interior (Huang et al., 2013). The protein transduction domain (PTD) of the CPPs is made up of 3-30 amino acids and possesses an overall positive charge, but has no specific consensus sequence (De Coupade et al., 2005). Many CPPs have been successfully given to different mammalian cell populations to deliver different types of 'cargo' molecules, including peptides, oligonucleotides, proteins, and drug-loaded nanoparticles (Heitz et al., 2009). Moreover, CPPs have been employed to deliver a variety of therapeutics to different types of cancer cells (Selivanova et al., 1997, Tseng et al., 2002), including breast cancer (Lindgren et al., 2006), carcinoma (Gusarova et al., 2007) and melanoma (Harbour et al., 2002). One powerful application of CPPs is the delivery of molecules that function as specific competitors or blockers of protein-protein interactions into the intracellular environment. Ablating defined protein-protein interactions is a refined way to explore their contribution to a particular cellular phenotype in a given disease context.

Many studies have shown the therapeutic application of CPPs for the treatment of a variety of hyper-proliferating cells (Kardinal et al., 2001) (Cussac et al., 1999). For example, a CPP-based chimeric protein bound to the CrkL-SH3N interface caused a significant decrease in the

formation of p210Bcr-Abl-CrkL complexes in Chronic Myeloid Leukemia (Kardinal et al., 2001). Furthermore, it also caused a significant decrease in the *in vitro* proliferation index of primary CML cells and cell lines derived from BCR-ABL-positive patients (Kardinal et al., 2000). CPP-mediated disruption of SH3 domain-dependent complexes, as a strategy for inhibition of specific signaling pathways has been tested in several studies (Cussac et al., 1999{Posern, 1998 #55, Nguyen et al., 1998)}.

Unconstrained peptides may have numerous shortcomings including susceptibility to proteolysis, loss of secondary structural fold and inability to permeate cell membranes. By synthetically constraining a peptide sequence through various chemical modifications that stabilize a structural fold such as an alpha-helix, beta-turn or loop structure, the peptide may have entropically favorable properties for binding (Kutchukian et al., 2009). Further, constrained peptides can offer many additional attributes including retention of secondary structural folds, cell permeability, improved target affinity and resistance to proteolytic degradation(Verdine and Hilinski, 2012, Wang et al., 2014, Hanold et al., 2015). Given these properties, constrained peptides have been used to disrupt a variety of protein-protein interactions (Hanold et al., 2017, Watkins and Arora, 2015, Pelay-Gimeno et al., 2015).

2. Results and Discussion

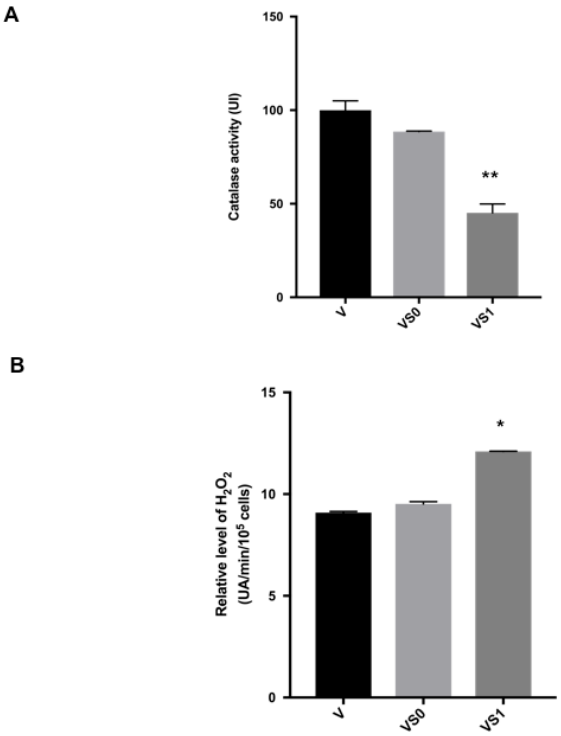
Inhibition of *Theileria*-induced leukocyte transformation by cell-penetrating peptides: Grb2 penetrating SH3 domain competing peptide

Grb2 (Growth factor receptor-bound protein 2) serves as a potentially interesting adaptor to target in the context of host-pathogen interactions and was identified from examination of the Ode vaccine line against tropical theileriosis. Previous microarray analyses indicated a significant decrease in macrophage transcription of *Grb2* in the live attenuated vaccine line,

as opposed to its virulent transformed progenitor macrophage cell line (Chaussepied et al., 2010). Grb2 is an adaptor protein that participates in signal transduction pathways (Vidal et al., 1998). Grb2 contains three domains; one Src Homology 2 (SH2) and two Src Homology 3 (SH3) domains located at the N- and C-termini (Lowenstein et al., 1992), (Chardin et al., 1995). The two SH3 domains form a direct complex with the proline-rich regions of the other partner proteins, while the SH2 domain binds to tyrosine phosphorylated peptides in specific receptors (Ye et al., 2008).

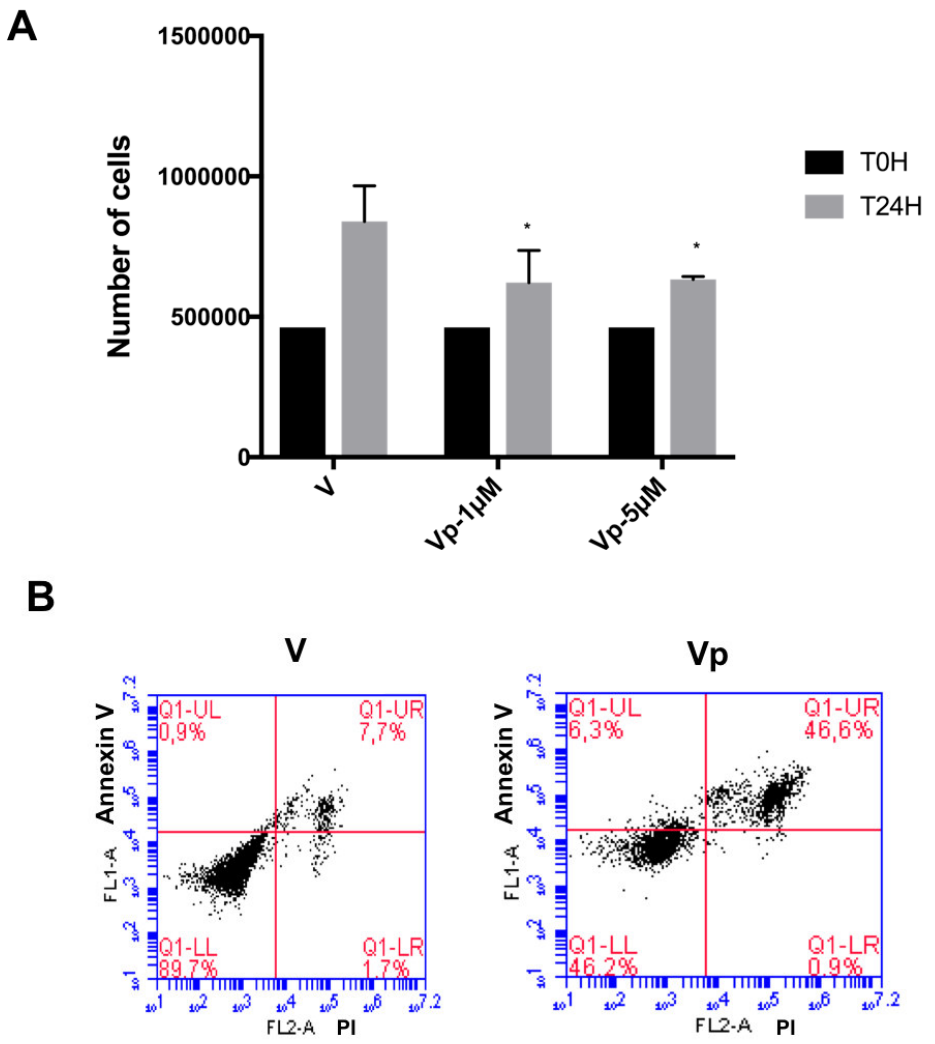
We have previously shown that *Theileria*-infected macrophages produce many pro-inflammatory cytokines such as transforming growth factor beta (TGF-β) (Chaussepied et al., 2010) that have been shown to active Grb2-signaling in certain cell lines (Galliher-Beckley and Schiemann, 2008). Moreover, both we and others have also demonstrated that *T. parva* and *T. annulata*-transformed B-lymphocytes rely on constitutively activated phosphoinositide 3-kinase (PI3-K) for their continued proliferation (Baumgartner et al., 2000), (Heussler et al., 2001), (Guergnon et al., 2006). We ablated SH3-domain interactions of Grb2 by adding the non-constrained competing peptide to virulent *Theileria*-transformed macrophages, and this blocked PI3-K recruitment to TGF-β receptor II and diminished the hyper-disseminating virulence phenotype (Haidar et al., 2015a). Another described Grb2-SH3 domain binding partner is catalase (Yano et al., 2004), an enzyme that converts H_2O_2 into H_2O, and thus enables *Theileria*-transformed leukocytes to better resist oxidative stress stemming from their uncontrolled proliferation (Metheni et al., 2015). Ablation of the Grb2-SH3 interaction using the same CPP as described in (Haidar et al., 2015a) disrupted its binding of catalase, reduced catalase activity and consequently increased H_2O_2 output (Figure 1) consistent with a role for Grb-SH3 domain interactions in regulating the redox status of *Theileria*-transformed macrophages (Metheni et al., 2015). The ensemble indicates that

perhaps constrained penetrating peptides that abolish Grb2 SH3-domain interactions could eventually become a treatment for tropical theileriosis and perhaps even have applications in treatment for a wide range of cancers.



Non-constrained CPP-provoked disruption of p104/JNK2 complex and diminished matrigel traversal of *T. annulata*-transformed macrophages: JNK (c-Jun N-terminal Kinase) is constitutively activated in *Theileria*-transformed macrophages (Chaussepied et al., 1998, Galley et al., 1997, Haidar et al., 2015b). JNK activation occurs in response to stress and pro-inflammatory cytokine production, leading to different biological processes such as survival, proliferation and migration. The virulence of *Theileria*-infected macrophages has been attributed to the ability of the parasite to constitutively activate infected leukocyte JNK kinase, leading to a permanent induction of Matrix Metallo-Proteinase 9 (MMP9), which

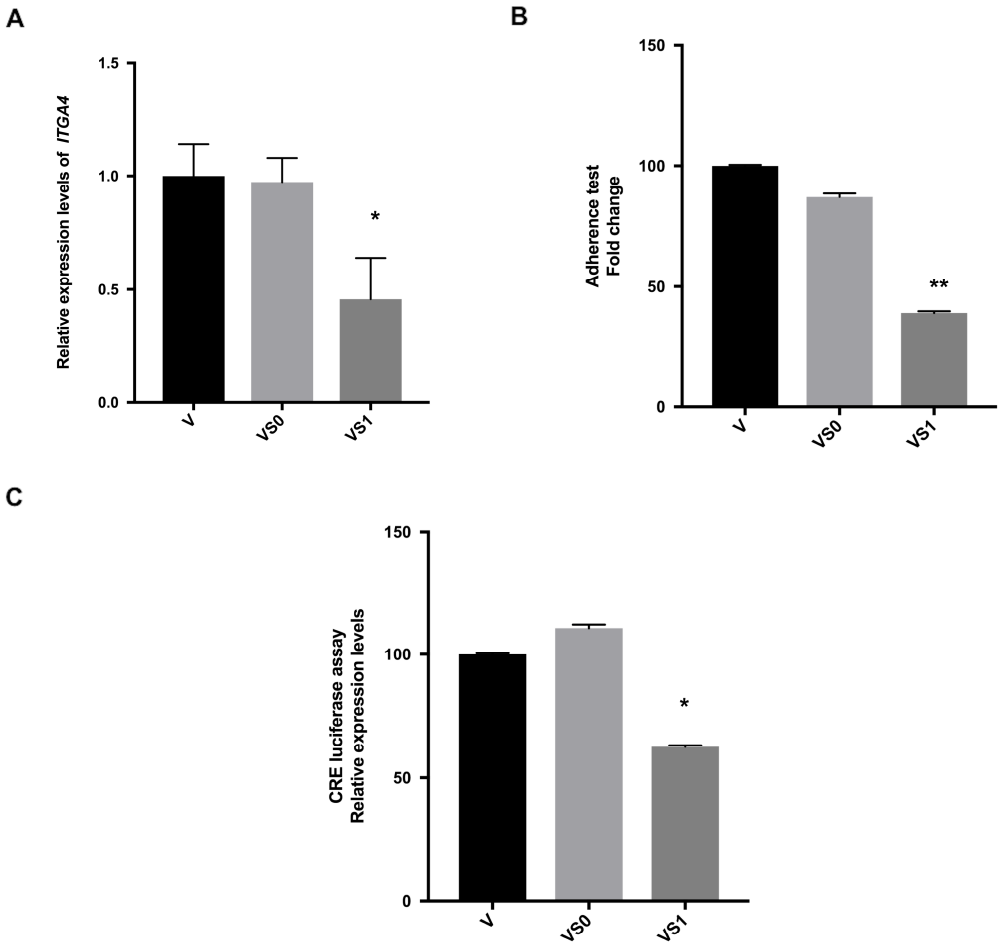
promotes the dissemination of transformed macrophages (Echebli et al., 2014, Lizundia et al., 2006). Bioinformatic analyses identified JNK-binding motifs in a parasite surface protein called p104. P104 (gene ID: TA08425) is a GPI anchored major surface protein of *T. annulata* that co-localizes with leukocyte EB1 (End-Binding protein 1 which promotes microtubule growth) at the microtubule plus ends (Woods et al., 2013). Leukocytes express both JNK1 and JNK2 and we recently examined the subcellular distribution of both isoforms in *Theileria*-infected macrophages. As expected, constitutively activated JNK1 was found in the nucleus where it phosphorylates c-Jun (Lizundia et al., 2006). Remarkably however, JNK2 was found in the cytosol and was closely associated with the parasite. Therefore, *Theileria*-transformed macrophages were treated with a non-constrained peptide harboring a predicted JNK-binding motif that competes for JNK2 binding to p104. The penetrating peptide (HVKKKKIKREIKITGKIVKL**KRSKSFDDLT**TK-FITC) harboring the JNK-binding motif (shown in bold) was applied at 1 µM and 5 µM for 2 h and appeared to cause disruption of JNK2/p104 complexes. Conversely, the recruitment of JNK2 to p104 located on the parasite surface sustained JNK2 levels, allowing the cytosol-localized kinase to participate in dissemination of *Theileria*-transformed macrophages (Latré de Laté et al submitted). In addition, treatment of virulent *Theileria*-infected macrophages with the same peptide also triggered infected leukocyte apoptosis (Figure 2). Clearly, the CCP technique is a powerful tool to manipulate the host-parasite protein-protein interactions, as illustrated here for the interaction between JNK2 and p104.



Manipulation of PKA-mediated signaling using constrained STAD-2 in *Theileria*-infected macrophages: PKA is an enzyme that plays key roles in a number of cellular processes such as DNA replication (Costanzo et al., 1999, Costanzo et al., 2000), cell growth and metabolism (Smith et al., 1998), cell division and rearrangement of the actin cytoskeleton (Gerits et al., 2007, Liu et al., 2001). It also regulates gene transcription by phosphorylating

the cAMP Response Element-Binding Protein (CREB) (Mayr and Montminy, 2001) to induce a transcriptional cascade involved in immune response, cellular metabolism and mitochondrial biogenesis (Wu et al., 2006, Chowanadisai et al., 2010, Shaywitz and Greenberg, 1999). PKA-mediated phosphorylation regulates many other signaling proteins, such as phospholipase C (PLC)(Liu and Simon, 1996), protein kinase C (PKC) (Yoon et al., 2000)and PI3-K (Cosentino et al., 2007, Ciullo et al., 2001). PKA is a tetrameric complex composed of two catalytic subunits (either Cα, Cβ or Cγ) and two regulatory subunits (either RIα, RIβ, RIIα or RIIβ). In mammalian cells, the PKA tetrameric holoenzyme is made up of two regulatory and two catalytic subunits, contrasting with apicomplexan parasites where the holoenzyme is believed to dimeric and composed of just one regulatory and one catalytic subunit. The subcellular localization of PKA depends on its regulatory subunit interactions with a class of scaffolding proteins termed A Kinase Anchoring Proteins (AKAPs) (Pidoux and Tasken, 2010, Wong and Scott, 2004). The constrained peptide termed Stapled AKAP Disruptor 2 (STAD-2) was designed to inhibit the protein-protein interface between PKA-RII and AKAPs (Wang et al., 2014, Flaherty et al., 2015, Kennedy and Scott, 2015).

Theileria-transformed leukocytes display high levels of PKA activity that contribute both to uncontrolled proliferation and dissemination of transformed leukocytes (Guergnon et al., 2006, Haidar et al., 2015a). Treatment of *Theileria*-infected macrophages with STAD-2 to disrupt PKA-RII binding to AKAPs diminished both the capacity of transformed macrophages to adhere to fibronectin and the degree of CREB-driven transcription (Figure 3).



Theileria is an apicomplexan parasite, like the better known *Plasmodia* and *Toxoplasma* parasites, where both host cell- and parasite-derived cAMP-dependent PKA activity plays keys roles in the described processes. In higher eukaryotes, PKA is composed of two regulatory subunits bound to two catalytic subunits in its inactive form (Francis and Corbin, 1994). The general structural features of mammalian PKA are conserved in all the four regulatory isoforms, with moderate changes in their biochemical properties (Taylor et al., 2013). Expression of each of the isoforms of PKA varies by tissue- and cell-type, and thus the catalytic subunit isoforms (Cα, Cβ, Cγ) may be combined to a homodimer of regulatory

subunits to generate tetrameric holoenzymes with different properties. During both physiological and pathological conditions, the composition of the PKA holoenzymes, as well as their intracellular localization may change, inducing different effects (Kardinal et al., 2001). The specificity of PKA signaling is determined by the cell type-specific expression of the different regulatory and catalytic PKA isoforms, the wide range of PKA substrates and also by the subcellular localization of PKA. Targeting of PKA to specific sites within the cell is largely achieved by binding of R subunits to AKAPs (Pidoux and Tasken, 2010, Wong and Scott, 2004). The isoform diversity of PKA complexes, however, is notably reduced in medically important *Apicomplexa* parasites like *Plasmodium*, *Theileria* and *Toxoplasma* that have just single R and C subunit isoforms, only form a dimeric complex rather than a tetrameric complex and have no gene encoding PKI (Merckx et al., 2008). This suggests that the use of constrained peptides such as STAD-2, which ablates interactions between R subunits and AKAPs, holds much promise in dissecting apicomplexan parasite pathogenesis, although higher doses of STAD-2 appears to kill malaria causing *Plasmodium falciparum* though a PKA-independent mechanism (Flaherty et al., 2015).

Constrained CPP-mediated disruption of Hexokinase 2 (HK2) recruitment to phospho-BAD promotes HK2 degradation and forces *Theileria*-transformed macrophages to switch from Warburg to oxidative phosphorylation: *Theileria*-transformed leukocytes acquire many of the characteristics of cancer cells such as producing the energy required for uncontrolled proliferation via the Warburg effect (Medjkane et al., 2014, Metheni et al., 2014, Metheni et al., 2015). The anti-apoptotic protein Bcl-2 family member BAD also plays an important regulatory role in glycolysis, where its activity is regulated by phosphorylation in response to growth factors and survival (Gimenez-Cassina et al., 2014, Ljubicic et al., 2015). *T. annulata* infection of B cells induces PKA-mediated phosphorylation of S155 on BAD

(Guergnon et al., 2006). Further, transformed leukocytes express Hexokinase 2 (HK2), which is a key glycolytic enzyme that phosphorylates glucose to yield glucose-6-phosphate and augmented HK2 expression typifies Warburg glycolysis (Wang et al., 2014). Based on these findings, we reasoned that PKA-mediated phosphorylation of BAD on residue S155 might recruit HK2 to the mitochondria and may lead to the preferential use of Warburg glycolysis (Metheni et al., 2014, Medjkane et al., 2014). Therefore, a constrained cell penetrating, a non-phosphorylatable substrate peptide (BAD substrate sequence where S155 was modified to Alanine) was given to *Theileria*-transformed macrophages (Haidar et al., 2017). The S155A peptide was found to function as a non-phosphorylatable substrate but not as a catalytic inhibitor of PKA, since overall constitutive PKA activity was unchanged in treated macrophages (Haidar et al., 2017). Since the S155A peptide inhibited phosphorylation of BAD on S155, this peptide likely serves as a non-phosphorylatable pseudosubstrate occupying the substrate-binding sites for kinases (PKA, RSK etc.) that target phosphorylation of S155 on BAD. By contrast, the S155D peptide did not act as a competitive inhibitor for BAD S155 phosphorylation, since it mimics the phosphorylation product and likely has a faster kinase off-rate. We also used a S155D penetrating peptide that mimics the phosphorylation product, however this is predicted to have a fast off-rate from the kinase and therefore should not act as a competitive inhibitor for BAD S155 phosphorylation. HK2 therefore remains associated with BAD, where it's protected from degradation and can mediate Warburg glycolysis to promote proliferation of *Theileria*-transformed leukocytes (Haidar et al., 2017). As the S155A peptide substrate cannot be phosphorylated, it may act as a pseudosubstrate where its off-rate from the active site of PKA may be slow and ultimately lead to dampening of kinase activity, whereupon S155 phosphorylation is inhibited and the association between HK2 and BAD is lost. Upon disruption of the HK2/BAD complex, HK2 is ubiquitylated and degraded, and in the absence of HK2 *Theileria*-transformed leukocytes

can no longer perform Warburg glycolysis (Haidar et al., 2017). With loss of HK2, *Theileria*-transformed macrophages and B cells consumed more oxygen and produced less lactate which is indicative of oxidative glycolysis (Haidar et al., 2017).

Proliferation was not as robust when *Theileria*-transformed macrophages were grown with galactose as compared to glucose. However, galactose is converted into galactose-1 phosphate by galactokinase and the Leloir pathway is independent of hexokinase activity and, as a consequence, growth of *Theileria*-transformed macrophages in the presence of galactose became insensitive to treatment with the S155A peptide, as loss of HK2 was not detrimental on this sugar source. By contrast, when grown on glucose, dissociation of HK2 from BAD and its subsequent degradation dampens proliferation, as infected leukocytes are forced to use HK1-mediated oxidative glycolysis (Haidar et al., 2017). This demonstrates the target specificity of the S155A peptide, as its effects are no longer observed when HK2 activity is bypassed and clearly shows the selective targeting of the HK2/BAD association by the penetrating inhibitor peptide. Importantly, these findings revealed a novel non-transcriptional way to regulate HK2 levels via Hypoxia Inducible Factor 1-alpha (HIF-1α). HIF-1α is a major regulator of the Warburg effect by mediating the switch of energy (Adenosine triphosphate, ATP) production from oxidative phosphorylation (OXPHOS, ATP produced via the mitochondrial respiratory chain) to aerobic glycolysis (ATP produced through the glycolysis). This demonstrates that *Theileria*-transformed leukocytes proliferate faster on glucose and gives an additional implication for the use of Warburg glycolysis by tumors over and above as a response to hypoxic conditions that are typical of solid tumors but are less characteristic with circulating leukemias. Further, the cell penetrating, non-phosphorylatable S155A substrate peptide contrasts with classical chemical inhibition of kinase activity (ATP analogues such as H89 or KT5720) which broadly ablates PKA-mediated phosphorylation of

a myriad of substrates and thus the complexity clouds assignment of the physiological consequences of a site-specific phosphorylation for a given substrate (i.e. S155 phosphorylation of BAD).

3. Conclusions

Intracellular pathogens have multiple interactions with their host cells, largely with the goal of dissemination of the infectious agent. Although these interactions are beneficial to the pathogen, some are detrimental to the host and underpin disease pathogenesis. Discerning the contribution of a given protein-protein interaction to pathogenesis is one of the great challenges in infectious disease research. By their precise competitive ablation of given protein-protein interactions, cell penetrating peptides are powerful tools to dissect the contribution of a particular interaction to the disease process. For example, treating the human malaria parasite *Plasmodium falciparum*-infected red blood cells with a 15-mer C-terminal Myosin A-tail peptide inhibited parasite growth *in vitro* with an IC_{50} of 84 μM illustrating how CPP has the potential to have widespread applications to other apicomplexan parasites (Bosch et al., 2006). We have illustrated this by describing how ablating three different protein-protein interactions in *Theileria*-infected leukocytes gave rise to precise insights into how this remarkable apicomplexan parasite transforms its host leukocyte into a disseminating tumor.

4. Methods

4.1 Peptide synthesis

GRB2 peptide synthesis: Assembly of the protected peptide chains was carried out using the stepwise solid-phase method of Merrifield (Merrifield, 1963) on a CEM systems Liberty1 automated peptide synthesizer with CEM standard-scale Fmoc (N-(9-fluorenyl)

methoxycarbonyl) chemistry on a NovaSyn TGA resin and DIC/Oxymapure coupling method. Fmoc groups were removed by piperidine (20% in dimethylformamide).

Peptidimer-C was synthesized by coupling Fmoc-Lys(Fmoc)-OH to the resin; after Fmoc deprotection, the two VPPPVPPRRR motifs were coupled simultaneously to both the side chain and back bone amino acids and the backbone of the amino groups of the lysine. Peptidimer-C was obtained after total amino acid deprotection and cleaveage from the resin with TFA.

Antennapedia peptidimer (Antp) conjugate was synthesized in a similar way, each amino acid of Antennapedia was first coupled using as Fmoc protected amino groups and with non-basic sensitive protections of the lateral chains (Fmoc-Trp(Boc)-OH, FmocArg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH) on the NovaTGA resin; Ahx and Lys with Fmoc protection were then coupled. After deprotection of both NH_2 groups of the latter lysine residue, dimeric coupling of each amino acid of the proline-rich sequence was performed as for peptidimer-C. Finally, peptide-C and antennapedia were purified by high-performance liquid chromatography on a C18 column (250X10 mm0 Vydac) and a linear gradient of B (where A is trifluoroacetic acid 0.1% and B is CH_3CN 70%, trifluoroacetic acid 0.09%) at a flow rate of 2 ml/min, with detection at 220 nm. The identity of the peptides was checked by electrospray mass spectroscopy (peptidimer-C: MSÅ2451.6; antennapedia: MSÅ4792.7).

BAD peptide synthesis: The amino acid sequence of BAD is based on the bovine sequence (<u>VKKKKIKREIKI</u>AAQRYGRELRRMSDEFHV), where S155 (S119 in bovine BAD) in wild type BAD was replaced by a phosphorylation-resistant alanine (**A**; in bold <u>VKKKKIKREIKI</u>AAQRYGRELRRM**A**DEFHV). The penetrating peptide to facilitate cell entry is underlined. As a negative control, S155 (S119 in bovine BAD) was replaced by a phosphorylation-mimic aspartate (**D**; in bold

VKKKKIKREIKIAAQRYGRELRRM**D**DEFHV). All synthetic peptides were labelled on the C-terminus with FITC and HPLC purified at 95%, as described

STAD2 synthesis: Peptides were synthesized and purified as previously described (Wang et al., 2014).

Peptide with conserved JNK-binding domain synthesis: the amino acid of JNK-binding domain is based on the sequences find into the *T. annulata* macroschizont surface protein p104 (in bold) (VKKKKIKREIKITGKIVKL**KRSKSFDDLT**TK). The penetrating peptide to facilitate cell entry is underlined.

4.2 Theileria annulata-infected cell lines and cell culture

Cells used in this study are the Ode vaccine cell line (Singh et al., 2001). The virulent (early passage) Ode corresponds to passage 62 and attenuated (late passage) Ode to passage 309. All cells were incubated at 37°C with 5% CO_2 in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 100 U penicillin, 0.1 mg/ml streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

4.3 Peptide treatments

The SH3 interaction with Grb-2 and its partners was chemically ablated using a specific Grb2-SH3 inhibitor (peptidimer-C) synthesized by Michel Vidal. A concentration of 0.5 µM or 1 µM was used and incubated for 1 h at 37°C in 5% CO_2. The phosphorylation of BAD on S155 was ablated using the non-phosphorylatable S155A peptide described above. A concentration of 10 µM was used for both peptides incubated for 2 h at 37°C in 5% CO_2. A concentration of 1µM or 5µM of JNK binding domain peptide was used and incubated for 24 h at 37°C in 5% CO_2.

4.4 Total RNA extraction and reversed transcription

RNA of *Theileria*-infected macrophages were isolated using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. The quality and quantity of RNA was measured by Nanodrop spectrophotometer. For the reverse transcription, 1µg isolated RNA was diluted by water to a final volume of 12µL, warmed at 65°C for 10 min, then incubated on ice for 2 min. Afterwards, 8 µl of reaction solution (0.5 µL random hexamer, 4 µL 5x RT buffer, 1.5 µL 10 mM dNTP, 1 µL 200 U/µLRT-MMLV (Promega) and 1 µL 40 U/µLRNase inhibitor (Promega) was added to get a final reaction volume of 20 µL and incubated at 37°C for 2 h. The resultant RNA was stored at -20°C.

4.5 Quantitative polymerase chain reaction (qPCR)

mRNA expression levels were measured by qPCR on Light Cycler 480 (Roche) using SYBR Green detection (Thermo). GAPDH was used as internal control to normalize for mRNA levels. The specificity of PCR amplification was confirmed by melting curve analysis.

4.6 Measurement of oxidative stress parameters

Intracellular levels of hydrogen peroxide (H_2O_2)

T. annulata-infected macrophages (1×10^5 cells per well) were seeded in 96-well plates and incubated 18 h in complete medium. Cells were washed in PBS and incubated with 100 µL per wells of 5 µM H2-DCFDA diluted in PBS (Molecular Probes). H_2O_2 levels were assayed by spectrofluorimetry on a fusion spectrofluorimeter (PackardBell). Fluorescence intensity was recorded every hour over a period of 5 h. Excitation and emission wavelengths used for H_2O_2 were 485 and 530 nm. The number of cells was evaluated by the crystal violet assay. Cells were stained in 0.05% crystal violet and 2% ethanol in PBS for 30 min at RT. After four washes in PBS, the stain was dissolved in methanol and measured at 550 nm on Fusion. The

level of H_2O_2 was calculated in each sample as follows: reactive oxygen species rate (arbitrary units$min^{-1}10^5$ $cells^{-1}$) = [fluorescence intensity (arbitrary units) at T300 minutes − fluorescence intensity (arbitrary units) at T0] per 60 min per number of cells as measured by the crystal violet assay.

Catalase activity assay: A dry pellet of *T. annulata*-infected macrophages 1×10^5 cells was lysate in 50 µL PBS, 1% NP40. 50 µL of lysate, 50 µL of antibody-antiperoxydase (1/2000, Sigma) and 50 µL H_2O_2 (1/4000, Sigma) were added to a 96-wells plate and incubated for 10 min at 37°C in 5% CO_2. 50 µL of OPD (SIGMAFAST™, #P9187) was then added and the absorbance was read at 405 nm immediately. Catalase activity assay was assayed on Fusion. Catalase measurement was reported to the amount of proteins in each sample (bovine serum albumin microbiuret assay, Pierce, Bezons, France).

4.7 CRE-driven luciferase assay

Ode cells were transfected by electroporation using the " Amaxa Nucleofector, kit V and program T-017 (Amaxa Lonza protocol). $5x10^5$ cells /ml were washed once with PBS at RT, then cells were suspended in 100 µl of Nucleofector solution V (Amaxa). Cells were co-transfected with 2 µg of luciferase reporter plasmid (6X-CRE), or reporter plasmid (β-galactosidase: β-gal). After transfection, cells were suspended in fresh complete medium and incubated at 37°C, 5 % CO_2 for 24 h.

The measurements of luciferase and β-galactosidase activities were performed using the Dual Light Assay system (life technologies) and luminometer Centro LB 960 (Berthold) according to the manufacturer's instructions.

4.8 Adhesion assay

A 96-well plate was coated with bovine fibronectin (Sigma #F1141), 2 µg/cm^2 diluted in DDW overnight at 4°C. Then washed twice with 100 µl 0.1% BSA in RPMI-1640 and blocked for 1 h at 37°C by 0.5% BSA in RPMI-1640. After two washes, 1X10^4 cells were added to each well and incubated at 37°C, 5% CO_2 for 30min. Non-adherent cells were removed by washing the wells three times before fixing with 100 µl 4% paraformaldehyde for 10 min at RT. Following one further wash, wells were stained with 100 µl crystal violet (1 mg/ml) for 10 min at RT. Wells were extensively washed with distilled water and air dried. Samples were re-suspended by 30 min incubation at RT in 100µl 2% SDS, 2% ethanol before reading the OD at 595 nm.

4.9 Statistical Analysis

Data were analyzed with the Student's t-test. All values are expressed as mean+/-SEM. Values were considered to be significantly different when p values were < 0.05. All experiments were done independently ($n = 3$) from 3 biological replicates.

5. Acknowledgements

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6. Supplementary Data

Supplementary figures and methods can be found in the online version of this article.

7. Figure legends

Figure 1. Disruption of Grb-2 signaling modulates oxidative stress status of *Theileria*-infected macrophages. **A.** The activity of catalase decreased in virulent macrophages treated with 1 µM of Grb-2 non-constrained peptide for 1 h (Vs1), as compared to non-treated macrophages (V). No effect was observed in virulent macrophages treated with scrambled control peptide (Vs0). **B.** H_2O_2 output is increased following the treatment with Grb-2 non-constrained peptide (Vs1) in virulent infected macrophages. $p<0.05$ compared to V; $p<0.005$ compared to V.

Figure 2. Non-constrained CCP triggers apoptosis in *Theileria*-infected macrophages. Infected macrophages were treated for 24 h with 1 µM or 5 µM of non-constrained CCP harboring a JNK binding motif. (A) Estimation of cell survival by trypan blue staining. (B) Percentage of cells undergoing apoptosis estimated by FACS using annexin V/ propidium iodide staining. Infected macrophages were treated with 5 µM of non-constrained CCP. 10^6 of Ode cells are prepared in 1 ml of PBS with 10% FBS in each test tube. After a centrifugation for 5 min at 200 X g and 4°C, cells are resuspended in 100 µl annexin V Binding buffer. 5 µl of annexin V and 5 µl of PI (Propidium Iodide) are added to each tube except single stained control. Ode are incubated 15 min in the dark at room temperature with 400 µl ice cold annexin V binding buffer and then analyzed on the flow cytometry (Accuri C6 - Cflow Plus software). V: virulent Ode, Vp: virulent Ode + peptide. $P<0.05$ compare to V.

Figure 3. Disruption of AKAP-PKA-RII association decrease virulence of *Theileria*-infected macrophages. **A.** Left panel. The expression of ITGA4 (integrin 4 alpha beta 1) decreased in virulent macrophages treated with 8 µM of STAD2 for 1 h (Vs1), as compared to non-treated macrophages (V). No effect was observed in virulent macrophages treated with scrambled STAD2 control peptide (Vs0). Right panel. Adhesion of *Theileria*-infected macrophages to fibronectin decreased following the treatment with STAD2 (8 µM for 1 h). **B.** Transcription of CRE-driven luciferase is lower in virulent macrophages treated with STAD2 (Vs1), as compared to non-treated macrophages (V) and macrophages treated with scrambled STAD2 control peptide (Vs0). $p<0.05$ compared to V; $p<0.005$ compared to V.

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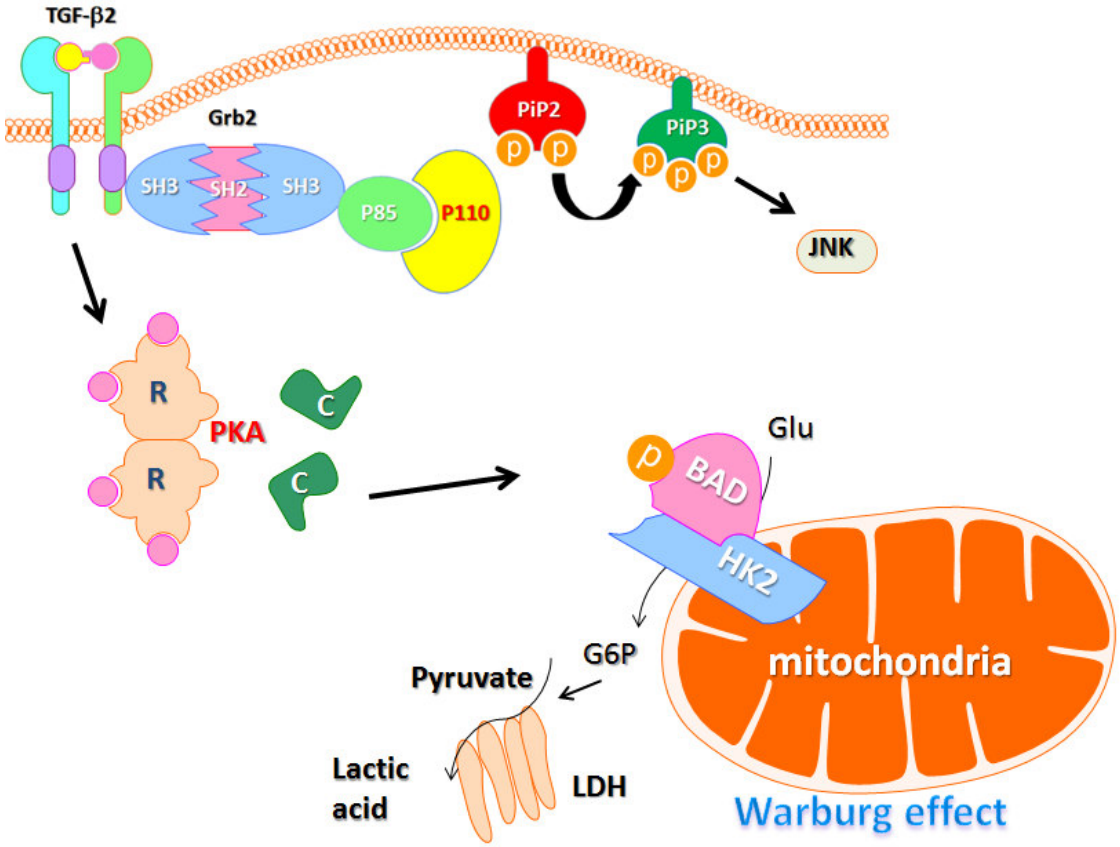
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BAD
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mitochondria
G6P
Pyruvate
Lactic acid
LDH
Warburg effect