The kallikrein-kinin system in experimental Chagas disease: a paradigm to investigate the impact of inflammatory edema on GPCR-mediated pathways of host cell invasion by Trypanosoma cruzi

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INTRODUCTION

Aflicting nearly 10 million people in Latin America (Coura and Dias, 2009), Chagas disease is a pleomorphic clinical entity caused by Trypanosoma cruzi, a parasitic protozoan that undergoes obligate intracellular development in the mammalian host. Extremely polymorphic (Macedo and Pena, 1998), the natural populations of T. cruzi have been recently subdivided into six discrete taxonomic units (DTUs) named T. cruzi I to T. cruzi VI (Zingales et al., 2009), of which at least four are known to be involved with human pathology (Miles et al., 2009). Whether transmitted to humans via mucosal wounds inflicted by hematophagous vectors of the reduviid family or, indirectly, by oral ingestion of contaminated juices (Coura and Dias, 2009; Cortes et al., 2012), the insect-derived infective forms (metacyclic trypomastigotes) induce an acute phase that may be asymptomatic, or life-threatening. Characterized by high blood parasitemia, the sequelae of severe acute disease may include heptosplenic pathology, myocarditis, and more rarely, encephalitis. Lasting a few months, the acute symptoms subside with the onset of immunity, but the effector response is not capable of eradicating the intracellular parasites, leading to a chronic infection, characterized by low-grade parasitemia, parasiticism and positive serology. Several years later, about 30% of the patients develop a full-blown chronic chagasic myocardopathy (CCM), characterized by the presence of inflammatory T cell infiltrates, myocardial fibrosis, complex arrhythmias, thromboembolism, and ventricular aneurysms (Marin-Neto et al., 2007). Patients with severe forms of CCM may have heart failure and sudden death, while the remaining chagasic patients (indeterminate stage) remain asymptomatic for decades. In the south cone of America, chagasic patients may also develop digestive system abnormalities (megacolon and/or megaesophagus), albeit in lower frequency than CCM.

CCM: CONVERGING PATHOGENIC MECHANISMS

Nearly a century after the discovery of Chagas disease, we have come to realize that the mechanisms responsible for the variable
clinical manifestations during the chronic phase are still elusive. Cardiac parasympathetic depopulation, microvascular derangement, and low-grade myocardial inflammation directly induced by parasites and T cell-dependent immunopathology seem to converge in the genesis of CCM. After decades of debate, there are persuasive arguments supporting the notion that the primary cause of CCM is a low-grade, persistent parasitism of the myocardium (Tarleton, 2001). A large body of studies in mice and humans indicated that chronic myocarditis is critically dependent on the recruitment of parasite-specific (type 1) effector CD8 T cells to the infected cardiac tissues (Padilla et al., 2009; Silverio et al., 2003; Monteiro et al., 2006; Schmitz et al., 2009; Scharfstein et al., 2000; Toddore et al., 2005; Andrade et al., 2012). Before outlining the arguments supporting this working hypothesis, we will present readers with an overview of the essential structural and functional features of the KKS.

MOLECULAR BASIS OF KKS ACTIVATION AND REGULATION

Recently implicated in thrombo-inflammatory processes (Müller et al., 2009; von Brühl et al., 2012), the KKS is a hub-like network of proteolytic enzymes which, among other biological functions, release the proinflammatory “kinin” peptides from an internal segment of their plasma-borne precursors, the kininogens. Generation of kinins may involve multiple processing enzymes: in the bloodstream, plasma kallikrein (PK) releases the nonapeptide BK from high molecular weight kininogen (HK) upon activation of the contact system by negatively charged surfaces, such as platelet-derived polyporphosphates (Figure 1A). In the extravascular spaces, hydrolase (LBK) is excised from low molecular weight kininogen (LK) or HK by tissue kallikrein, a serine protease that is constitutively expressed in multiple tissues. It is also known that kinins can be generated by alternative proteases. For example, in chronic inflammation kininogens may be processed by the concerted action of neutrophil elastase and mast cell tryptase, leading to the release of a slightly larger kinin, Met-LBK (Kozik et al., 1998). In the context of infections, kinins can be directly liberated from the kininogens by the action of microbial cysteine proteases, such as gingipain from Porphyromonas gingivalis (Imamura et al., 1994), staphopain A from Staphylococcus aureus (Imamura et al., 2003), streptopain from Streptococcus pyogenes (Herwald et al., 1996), and cystein protease (De Nery et al., 1997; Scharfstein et al., 2000; Monteiro et al., 2006).

The biological responses mediated by BK and L BK are mediated by bradykinin B2 receptor (BK2R), a subtype of heterotromeric G-protein-coupled receptors (GPCRs). The rationale of this hypothesis lies on two fundamental premises: (i) due to the low-grade parasitism observed in chronic infection, there are intermittent “flares” of plasma leakage in the inflamed myocardium (ii) the microvascular edema in the inflamed heart to potentiate their infectivity via cooperative signaling of multiple G protein-coupled receptors (GPCRs). Since excessive activation of BK2R can be critical to the function of ETs, although the paper does not provide a specific reference for this statement, it suggests that the activation of BK2R may be involved in the regulation of ET expression in infected tissues.

Studies in various experimental models indicated that tissue culture-derived trypomastigotes (Dm28c) swiftly activate microvascular beds through the activation of the KKS (Toddore et al., 2005; Monteiro et al., 2006; Schmitz et al., 2009; Scharfstein and Andrade, 2011; Andrade et al., 2012). Based on these initial observations, we predicted that the sudden diffusion of plasma-borne kinins (antibodies, complement components, kininogens, ETs) through parasite-laden tissues may affect the delicate host/parasite balance established in the chronically infected myocardium. Although the flagellated trypomastigotes released from pseudocysts may rapidly move away from the primary foci of infection, infection, hence seeking for safer targets elsewhere in the myocardium, we proposed that the transient rise of plasma protein levels in the edematous interstitial spaces may favor generation of infection-promoting peptides, such as bradykinin (BK), in the peripheral tissues (Scharfstein et al., 2000; Toddore et al., 2005; Andrade et al., 2012).

While not dismissing the relevance of intracardiac infiltrates in the progression of CCM, vascular pathologists argued that low-grade infection could lead to the accumulation of microvascular lesions in the chagasic heart, ultimately resulting in myocardial hyponxia, which in turn may aggravate collateral injury inflicted by pathogenic T cells infiltrating the heart (Morris et al., 1990; Rossi, 1990; Higuchi et al., 1999, 2003). Subsequent studies in experimentally infected animals shed light on the mechanisms by which T. cruzi induces microvascular pathology (Andrade et al., 1994; Tanowitz et al., 1999). Initial observations ascribed the formation of vascular lesions to the pathogenic activity of endothelins (ET), a potent class of vasoconstrictor polypeptides (Tanowitz et al., 1999). Of further interest, these workers reported that endothelin-1 (ET-1) expression is up-regulated in parasitized cardiovascular cells (Petkova et al., 2000). Follow-up studies in chronically infected mice demonstrated that cardiac remodeling significantly ameliorated in transgenic lines in which the ET gene was specifically removed from cardiomyocytes, while ablation of this gene in endothelial cells has not significantly reduced heart fibrosis (Tanowitz et al., 2005). Of further interest, the plasma levels of ETs are elevated both in chagasic patients and infected mice (Petkova et al., 2000; Salomon et al., 2003).
(ii) degradation of intact kinins by various metallopeptidases, including angiotensin converting enzyme (ACE, kininase II) – a transmembrane di-peptidyl carboxypeptidase (Skidgel and Erdos, 2004) highly expressed in the endothelium lining and, to a less extent, in other cell types, including innate sentinel cells such as monocytes and DCs (Dandlov et al., 2003). Besides degrading intact kinins, thus reducing hypotension, ACE increases blood pressure through the formation of angiotensin II, a potent vaso-pressor octapeptide. Noteworthy, the transmembrane (somatic) enzyme undergoes cleavage by diisnegrin and metalloproteinase (ADAM)-type “sheddase” (Parkin et al., 2004), leading to the accumulation of soluble forms of ACE in the blood and other body fluids.

In contrast to the constitutive BK2R, the expression of BK1R is strongly – but transiently – up-regulated during inflammation (Marceau and Bachvarov, 1998; Figure 1A). For example, ET-1 and angiotensin II induce BK1R expression through the signaling of ET1R and AT1 (angiotensin 1 receptor) respectively, leading to activation of phosphoinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) cascade (Medeiros et al., 2004). BK1R is also induced by IL-1β, TNF-α, and IFN-γ via the NF-κB transcription factor (Marceau and Bachvarov, 1998; Medeiros et al., 2004; Moreau et al., 2007). Differently from BK2R, the inducible BK1R is not triggered by “intact” kinins (BK/LBK). Instead, BK1R is activated by the kinin metabolites [des-Arg]-BK or [des-Arg]-LBK, both of which are generated by carboxypeptidase N (CPN)/carboxypeptidase M (CFM) (kinase I)–mediated cleavage of the C-terminal arginine residue of BK/LBK (Figure 1A).

**BIOLOGICAL FUNCTIONS OF BKRs**

In the cardiovascular system, kinins/BK1R control the blood flow through nitric oxide (NO)-dependent vasodilation. In contrast to the beneficial role of kinins in cardiovascular homeostasis, there is evidence that dysregulated BK1R signaling drives myocardial fibrosis and impairs heart function in different experimental models (Opplmann and Tschop, 2012).

Beyond inducing detrimental cardiac responses, it is well-established that BK1R plays a major role in hyperalgesia (Calixto et al., 2004; Galera et al., 2005; Cunha et al., 2007). Studies in mice subjected to traumatic brain injury revealed that blood-brain leakage and recruitment of inflammatory leukocytes to the CNS is blocked by a specific BK1R antagonist (Raslan et al., 2010). Although inflammation is usually initiated through the signaling of the constitutive BK2R, the sustenance of the inflammatory response depends on the signaling of endothelial BK1R. McLean et al. (2000) were the first to report that the trans-endothelial leukocyte migration is enhanced as result of up-regulated expression/signaling of endothelial BK1R. Recent progress in studies of experimental autoimmune encephalitis (EAE) demonstrated that BK1R increases the recruitment of pathogenic effector T cells into the CNS (Dutra et al., 2011; Gobel et al., 2011; Table 1).

More recently, a growing number of studies indicated that immune resistance against infection by parasitic protozoan (Monteiro et al., 2006, 2007; Svensjö et al., 2006; Nico et al., 2012; Scharfstein and Svensjö, 2012) and bacterial pathogens (Kaman et al., 2003; Svensjö et al., 2006).
Table 1 | Role of BKRs in immunity: literature update.

| Experimental System                  | Bradykinin receptor subtype | Functional Roles of BKRs                                                                                   | Reference                          |
|--------------------------------------|-----------------------------|-----------------------------------------------------------------------------------------------------------|-----------------------------------|
| T. cruzi infection (mice)            | BK2R                        | Induction of CD11c+ DC (splenic) maturation and upregulation of effector (type 1) CD4 and CD8 T cells       | Monteiro et al. (2006); Monteiro et al. (2007) |
| L. donovani and L. chagasi (hamsters and mice) | BK2R                        | Inflammatory edema induced by promastigotes, stimulation of promastigote uptake and modulation of the intracellular growth of leishmania in macrophages | Svensjö et al. (2006)             |
| Visceral Leishmaniasis (mice)        | BK2R                        | Immune resistance to acute infection                                                                       | Nico et al. (2012)                |
| L. monocytogenes infection (mice)    | BK2R                        | Modulation of innate immunity and infection control                                                        | Kaman et al. (2008)               |
| P. gingivalis infection-bucal (mice) | BK2R                        | Upregulation of Th1 and Th17 responses (submandibular lymph nodes)                                        | Monteiro et al. (2009)            |
| In vivo leukocyte trafficking across mesenteric postcapillary venules (mice) | BK2R                        | Trans-endothelial leukocyte migration                                                                     | McLean et al. (2000)              |
| EAE (mice)                           | BK2R                        | TH1 and Th17 responses development and clinical progression of EAE                                         | Dutra et al. (2011)               |
|                                      | BK1R                        | Induction of blood-brain-barrier disruption and T cell transmigration into the CNS                          | Göbel et al. (2011)               |
|                                      |                             | Limiting infiltration of pathogenic subsets of effector CD4 T cells into the CNS                           | Schulze-Toppoff et al. (2009)     |
| In vitro DC migration (human)        | BK2R                        | Stimulation of DC migration                                                                                 | Bartum et al. (2007)              |
|                                      | BK1R                        | Inhibition of DC migration                                                                                  | Gulliver et al. (2011)            |
| Allergic inflammation (mice)         | BK1R/BK2R                   | Induction/inhibition of migration and activation of eosinophils                                             | Vasquez-Pinto et al. (2010)       |
|                                      | BK2R                        | Induction of DC maturation and IL12-dependent Th1 polarization                                              | Aliberti et al. (2003)            |

et al., 2009) is critically dependent on activation of innate sentinel cells via the BK2R pathway (Table 1).

**BKRs: DOMAIN COMPARTMENTALIZATION, REGULATION, AND SIGNAL TRANSDUCTION PATHWAYS**

Stably expressed at the cell surface, the constitutively expressed BK2R is rapidly desensitized/internalized via GRK4-mediated receptor phosphorylation upon ligand binding, thus yielding a transient signaling response (Blaukat et al., 2001). Using BK2R-fusion constructs, Enquist et al. (2007) revealed that upon BK binding, the internalized receptors colocalize with transferring in endosomes, prior to entry in the arrestin-dependent, clathrin-mediated recycling pathway. A second trafficking pathway, described in smooth muscle cells and fibroblasts, indicated that BK also promotes the redistribution of BK2R and their coupling G proteins to caveolar rafts (de Weerd and Leeb-Lundberg, 1997).

Although the complex systems vary from one report to another, it is well-established that BK binding to BK2R promotes the formation of BK2R homodimers and oligomer complexes of higher order (Leeb-Lundberg et al., 2005). Kang et al. (2004) reported that BK2R and BK1R form heterodimers in transfected HEK293 cells. Notably, BK2R also forms heterodimeric complexes with type-1 angiotensin receptors (AT1) in vascular smooth muscle cells and transfected HEK293 (AbdAlla et al., 2001).

As to the signal transduction pathways, it is well established that BK frequently induces BK2R-dependent [Ca2+]i transients and PLC-β-dependent hydrolysis of phosphoinositides, both of which commonly coupled to the activation of PKC isoenzymes α, ε, and ξ. Although BK2R often signals through Gaq and Gai, in some cellular systems BK-driven activation of the constitutively expressed BKR is coupled to Gas and Ga12/13 (Leeb-Lundberg et al., 2015). Similar to the effects induced by other GPCR agonists, BK sequesters BK2R along with Gaq and Gai in caveolar compartments of smooth muscle cells (de Weerd and Leeb-Lundberg, 1997). In fibroblasts, a cell-type often used in models studies of T. cruzi interaction with non-professional phagocytic cells, BK2R activation is associated with transient tyrosine phosphorylation.
ACE inhibitors.

All the components of the KKS are present in the heart. In addition, BK2R signaling improves cardiac function by (i) reducing apoptosis and chamber dilatation (McKerrow et al., 2009), (ii) optimiziong the efficiency of the kinin-release reaction in peripheral sites of infection.

Previous characterized as a therapeutic target of Chagas disease (Mackernow et al., 2009), cruzipain is a remarkably versatile virulence factor of T. cruzi. Beyond activation of the KKS (see below), the proteolytic activity of cruzipain was implicated in mechanisms of parasite virulence/pathogenicity, such as hirucinogenase activity of soluble HK in persipilin whereas reciproci increesing the catalytic efficiency of the parasite protease, measured with synthetic peptides flanking the BK sites. Consistent with these results, heparan sulfate potentiated HK processing by cruzipain, generating multiple HK breakdown products and promoting accelerated kinin release (Figure 1B). Combined, these biochemical studies suggested that the substrate specificity of the parasite protease was re-directed as result of reciprocal interactions between sulfated proteoglycans with the substrate (HK) and protease (cruzipain) molecules (Lima et al., 2002), hence increesing the efficiency of the kinin-release reaction in peripheral sites of infection.

**T. cruzi TRYPOMASTIGOTES LIBERATE KININS FROM SURFACE-BOUND KININOGENS VIA CRUZIPAIN**

The first clues suggesting that T. cruzi is equipped with a kininogenase came from enzymatic analysis of the substrate specificity of cruzipain (Del Nery et al., 1997), a lysosomal-like cysteine protease classified as member of clan A of the C1 peptidase family (Cazzulo et al., 2001; Alvarez et al., 2012). At first sight, the discovery that cruzipain acted as a “kininogenase” seemed paradoxical because kininogens are members of the cystein family of cysteine protease inhibitors, hence rely on cystatin-like domains to potenitly inactivate papain-like enzymes, including cruzipain itself (Stoka et al., 1995). Consistent with this, in vitro data showed that cruzipain hydrolyzes soluble forms of HK at slower rates as compared to tissue kaliriken. This comundrum was settled by awareness that HK binds to negatively charged sulfated proteoglycans, such as heparan or chondroitin sulfates via the histidine-rich positively charged motif (DψH) localized at the C-terminal end of the BK (D4) sequence (Renné et al., 2000; Renné and Muller-Esterl, 2001). Based on this information, Lima et al. (2002) hypothesized that the spatial orientation of cell-bound HK docked to heparan sulfate proteoglycans was not suitable for cruzipain binding and inactivation by the cystatin-like inhibitory domain (Figure 1B). Indeed, model studies performed with cruzipain and HK in the test tube offered circumstantial support to this hypothesis (Lima et al., 2002): the addition of heparan sulfate (at optimal concentrations) drastically reduced the cysteine inhibitory activity of soluble HK on cruzipain while reciprocally increasing the catalytic efficiency of the parasite protease, measured with synthetic peptides flanking the BK sites. Consistent with these results, heparan sulfate potentiated HK processing by cruzipain, generating multiple HK breakdown products and promoting accelerated kinin release (Figure 1B). Combined, these biochemical studies suggested that the substrate specificity of the parasite protease was re-directed as result of reciprocal interactions between sulfated proteoglycans with the substrate (HK) and protease (cruzipain) molecules (Lima et al., 2002), hence increasing the efficiency of the kinin-release reaction in peripheral sites of infection.
Infectors at the cost of limited self-tissue destruction. Perturbations of steady-state tissue homeostasis are sensed by sentinel cells of the innate immune system through specialized pattern-recognition receptors (PRRs; Medzhitov and Janeway, 1997). In many infections, the activation of PRRs lead to rapid secretion of pre-formed vasoactive mediators by innate sentinel cells (e.g., eosinophils, leukotrienes, chemokines, TNF-α), which then activate the endothelium lining, rendering them sticky for circulating neutrophils. Further downstream, vasoactive mediators generated/released at the neutrophil/endothelial interface impair the integrity of the endothelial barrier, hence opening the “blood gates” (DiStasi and Ley, 2009).

Studies in a mouse model of subcutaneous (footpad) infection provided the first evidence that Dm28c TCTs (tissue culture trypanosomastigotes) induce inflammatory edema through the activation of the kinin system (Todorov et al., 2003; Figure 2A). A footpad edema potentiated by the ACE inhibitor captopril was observed 2 h p.i. in wild-type B6 mice, but not in BK2R-deficient mutants. Using pharmacological tools, we showed that the constitutively expressed BK2R orchestrated the early phase (2 h) edema, whereas BK2R (acting in “cross-talk” with BK1R) sustained the inflammatory response (24 h; Todorov et al., 2003). Intriguingly, Dm28c epimastigotes failed to induce a conspicuous inflammatory edema via the kinin pathway despite the fact that these vector-borne non-infective forms express abundant levels of cruzipain. In a subsequent study, it became clear, for reasons that will be explained further below, that cruzipain was necessary but not sufficient to generate kinins in hamster cheek pouch (HCP) topicaly exposed to Dm28c epimastigotes.

Insight of the functional interplay between Toll-like receptor (TLR)-driven innate responses and the KKS emerged from analysis of the mechanisms underlying TLR-driven microvascular leakage responses induced bytopically applying the pathogen to the HCP (Monteiro et al., 2006). This unconventional system proved advantageous because it dispensed the use of needle to inject parasites, hence avoiding activation of the KKS by bleeding and traumatic injury. Using intravital microscopy, we found that Dm28c TCTs induced discrete plasma leakage responses in the HCP within a few minutes of parasite application. Noteworthly, the microvascular reactions were attenuated by HOE-140 (BK2R antagonist) or by pre-treating the TCTs with an irreversible cruzipain inhibitor N-methyl-piperazine-Phe-homoPhe-vinyl sulfone (K11777), an anti-parasite drug that will be soon tested in clinical trials (Engel et al., 1998; McKerrow et al., 2009).

Monteiro et al. (2006) highlighted the fact that purified cruzipain (activated) topically applied to the HCP failed to induce significant plasma leakage even in the presence of ACE inhibitors. However, purified (activated) cruzipain induced a strong BK2R-driven leakage response when it was applied in combination to purified HK. The dependence on a supply of exogenous HK suggested that, in resting state conditions (i.e., in the absence of inflammation), the levels of endogenous kinin precursor proteins in the interstitial spaces are insufficient to allow for significant activation of the kinin system. Based on these observations, Monteiro et al. (2006) predicted that Dm28c TCTs might bear developmentally regulated proinflammatory molecules (i.e., absent in Dm28c epimastigotes) which control the rate-limiting step of KKS activation in parasite-laden tissues: the influx of the plasma-borne kininogen (cruzipain substrate) into peripheral sites of T. cruzi infection (Figure 2A). Seeking to identify these factors, Monteiro et al. (2006) then turned their attention to tGPI, a mucin-linked lipid anchor previously characterized by Almolda and Gazzinielli (2001) as a TLR2 ligand that was expressed at high-levels exclusively in TCTs. Consistent with the working hypothesis, Dm28 TCTs failed to evoke inflammatory edema in TLR2−/− mice or in BK2R−/− mice (Monteiro et al., 2006). In contrast, these infective parasites evoked a prominent edema both in wild-type mice and TLR4 mutant (C3H/HeJ). These results argued against a role for GPI, a lipid anchor of epimastigotes previously characterized as TLR4 ligand (Oliveira et al., 2004), in the activation pathway that generates vasoactive kinins in peripheral sites of infection.

Next, Monteiro et al. (2006) studied the functional interplay between TLR2 and the KKS by injecting TLR2-deficient mice with a parasite suspension supplemented (or not) with purified HK. Strikingly, the injection of HK in infected TLR2−/− mice rescued the edema response. Moreover, the edema responses which HK induced in TLR2−/− infected mice were abolished by HOE-140, or alternatively, by pre-incubating the TCTs with the irreversible cruzipain inhibitor K11777. Combined, these results supported the hypothesis that TCTs induce the initial leakage/accumulation of the HK (cruzipain substrate) in peripheral tissues via TLR2, whereas cruzipain amplifies this inflammatory response through the release of BK2R agonist, at the downstream end of the inflammatory cascade (Figure 2A).

In order to further confirm this hypothesis, Monteiro et al. (2006) injected (i.e.) purified tGPI, combined or not to activated cruzipain, in wild-type naïve mice or in TLR2−/− or BK2R−/− mutants. Edema measurements showed that tGPI/cruzipain potentely induced microvascular responses via the TLR2/BK2R-pathway and these proinflammatory effects were further potentiated by ACE inhibitors. Beyond their effects on the microcirculation, the released kinins link TLR2-driven inflammation to innate immunity by triggering BK2R up-regulated on resident/migrating DCs, converting them into Th1-directing antigen-presenting cells (APCs; Monteiro et al., 2006). Conversely, ACE counter-modulates Th1-polarization via the trans-cellular the TLR2/BK2R pathway by degrading BK2R, an endogenous signal that drives DC maturation (Alberti et al., 2003; Monteiro et al., 2006, 2007; Scharfstein et al., 2007).

**NEUTROPHIL LINKS TLR2/CXCR2 TO THE PROTEOLYTIC (KKS) PHASE OF INFLAMMATION**

A key event at early stage of infection, the interaction of circulating neutrophils with the endothelium has profound effects on the outcome of the inflammatory process. One of the most common consequences of the interactions that take place on the luminal side of post-capillary vessels is the increased vascular permeability, a biological response that leads to the accumulation of protein-rich edema fluid in interstitial spaces. Although the list of endogenous soluble factors that increase vascular permeability is extensive, they usually impair the integrity of the endothelial barrier through the triggering of [Ca2+], the via signaling of heterotrimic
FIGURE 2 | Influence of interstitial edema on host/parasite balance in the myocardium: lessons from studies with the Dm28c strain of T. cruzi.

(A) Despite the scanty parasitism in the myocardium of chronic chagasic patients, parasite-containing pseudocysts occasionally burst, releasing high numbers of pro-inflammatory trypomastigotes into the surrounding interstitial spaces. (2) Innate sentinel cells, such as macrophages and/or mast cells,
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G-proteins, i.e., a pathway required to induce myosin-dependent contraction and functional disruption of the endothelial cells.

In the HCP studies described in the previous section, Monteiro et al. (2006) observed that circulating leukocytes rapidly adhered to the luminal face of post-capillary venules. Complementary studies performed in neutrophil-depleted wild-type mice revealed that Dm28c TCTs failed to evoke a conspicuous edema in these animals. Similar to the pharmacological maneuvers employed with TLR2−/− mice, already described, the deficient phenotype of neutrophil-depleted mice was rescued upon injection of TCT supplemented with HK (Monteiro et al., 2006). Collectively, these results supported the concept that Dm28c TCTs may directly activate ETAR antagonist-blanched plasma leakage in the hamster cheek pouch and blocked the inflammatory edema in T. cruzi-infected mice. Collectively, these results indicated that ETAR antagonists blunted plasma leakage in the hamster cheek pouch and blocked the inflammatory edema in T. cruzi-infected mice. 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leukotriene C4 (Yamamura et al., 1994), and TNF-α (Coulombe et al., 2002). Of further interest, there is evidence that ET-1 induces metalloproteinase-driven ventricular remodeling in models of chronic heart pressure/volume overload (Murray et al., 2004; Janicki et al., 2006) and modulate cardiac contractility through the induction of mast cell degranulation via ETaR (Esladi et al., 2008). Although the role of the ETaR/mast cell axis was not directly investigated in CCM, there is evidence that plasma levels of ET-1 are elevated both in chagasic patients and experimentally infected mice (Petricca et al., 2000; Sakamoto et al., 2003), presumably reflecting increased shear stress or other infection-associated hemodynamic alterations in these individuals. Furthermore, as highlighted earlier in this text, the expression of ETs is up-regulated in parasitized cardiomyocytes (Tanowitz et al., 2003). Thus, irrespective of the source of ET-1, it is conceivable that the sudden rise in the extravascular levels of this potent prooxidative mediator may lead to the activation of perivascular ETaR-positive mast cells in the chagasic heart. As such, this hypothetical scenario, mast cell degranulation via ETaR may release histamine, chemokines, along with a myriad of vasoactive mediators, in the parasite-laden cardiac tissues (Figure 2B).

More recently, Nascimento et al. (2012) have started to investigate this hypothesis by interfering with mast cell function in HCP topically exposed to Dm28C TCTs. Using mast cell stabilizers, we found that plasma leakage (BK2R-driven) was indeed inhibited, thus suggesting that mast cell degranulation is required for overt activation of the KKS in peripheral sites of chagasic infection (Figure 2B).

Thus far, the mechanisms by which mast cells may propagate the KKS cascade in peripheral sites of T. cruzi infection remain unknown. In an interesting precedent coming from mouse models of allergic inflammation, Oshatz et al. (2011) have recently proposed that heparin (a mast cell storage product) acts as a typical endogenous “contact” activator, i.e., it activates PKa in FXIIIa/HK-dependent manner, thereby releasing BK. Alternatively, parasite-induced activation of cardiac mast cells may activate the KKS through the formation of DNA-containing extracellular traps (von Kockritz-Blickwede et al., 2008). Further studies may determine if the ET/mast cell/KKS pathway plays an important role in the pathogenesis of CCM.

BK2R IS ESSENTIAL FOR DEVELOPMENT OF ACQUIRED IMMUNE RESISTANCE TO CHAGASIC INFECTION

Comprising a heterogeneous population of professional APCs, DCs are widely but sparsely distributed in peripheral tissues and lymphoid organs (Shortman and Naik, 2007). Strategically positioned in T cell-rich areas of secondary lymphoid tissues, the resident DCs are specialized in antigen-presentation to CD4+ and CD8+ T cells. In steady-state conditions, immature DCs present MHC-restricted antigen peptides to virgin T cells and lymphoid organs (Sela et al., 2011). However, during infection, the immature DCs sense "danger" motifs expressed by pathogens through distinct families of PRRs, such as TLRs or NOD2-like receptors (NLR; Akira, 2009). In addition, conventional DCs may sense the threat to tissue integrity via receptors for endogenous proinflammatory mediators, such as ATP, uric acid (Sansonetti, 2006), and BK (Aliberti et al., 2003; Monteiro et al., 2007). Stabilized by cognate interactions with co-stimulatory molecules (CD80/86, CD40, and MHC), the prolonged encounters between antigen-bearing DCs and naive T cells are essential for TCR activation. During the course of DC/T cell interaction, the "mature" APCs deliver T17 polarizing cytokines, such as IL-12p-70, which is critically required for T17 development.

Efforts to characterize the activation pathways controlling DC maturation in the context of T. cruzi infection have initially converged to nucleic acid-sensing TLRs (3, 7, and 9). In a key study, Caetano et al. (2011) showed that transgenic mice strains which lack functional UNC93B1 as well as functional endosomal TLRs (TLR3, 7, and 9), were susceptible to T. cruzi infection as mice deficient in TLR3/7/9 (Caetano et al., 2011). In the same study, it was documented that T. cruzi-infected macrophages and DCs from 3 day mice displayed low IL-12p40 and INF-γ responses. Based on these results, it was inferred that recognition of intracellular parasites require UNC93B1-driven translocation of the nucleic acid-sensing TLRs from the endoplasmic reticulum to the endolysosomes (Caetano et al., 2011). In spite of this conceptual advance, other studies suggest that DCs might sense T. cruzi through TLR-independent pathways. For example, Koyama et al. (2009) showed that T. cruzi induces maturation (up-regulation of MHC class II, CD40, and CD86) of MyD88−/− TRIF−/− mice bone-marrow-derived DCs as efficiently as wild-type bone-marrow-derived DCs. Using fetal liver DCs as target cells, these authors linked the T. cruzi-induced responses (IFN-γ production and DC maturation) to the activation of the NFATc1 pathway. These results implied that innate immunity is not exclusively controlled by nucleic acid-sensing TLRs.

Several years ago, we reported that BK, acting as a typical endogenous danger signal (i.e., T17-directed endogenous adjuvant) induced DC maturation (IL-12 and up-regulated expression of co-stimulatory molecules) via BK2R (Alberti et al., 2003). In a key finding, we subsequently reported that BK2R-deficient mice succumbed to acute challenge by Dm28C TCTs (i.p. route). Analysis of the immune dysfunctions underlying the susceptible phenotype of BK2R−/− mice at early stages of infection showed a modest, but significant drop in the frequency of intracardiac type-1 effector T cells. Intriguingly, however, as the acute infection progressed in BK2R−/− mice, the immune deficiency was intensified and generalized, involving both the extra-lymphoid and lymphoid compartment. Of note, the delayed T17 response of BK2R−/− mice was accompanied by a corresponding rise in IL-17-producing T cells (T17). The premise that the defective adaptive response of BK2R−/− mice was a secondary manifestation resulting from impaired BK2R−/− DC maturation was confirmed by systemically injecting wild-type BK2R+/+ DCs into the susceptible BK2R−/− mice, prior to pathogen injection. Remarkably, this DC transfer maneuver rendered the recipient BK2R−/− mice resistant to acute T. cruzi challenge, and restored their capability to generate protective IFN-γ-producing CD4+ CD44+ and CD8+ CD44+ effector T cells, while conversely suppressing the potentially detrimental T17 CD4+ subset anti-parasite responses.

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In the same study, Monteiro et al. (2007) further demonstrated that Dm28c TCTs potently activated BK,R\(^{-/}\) CD11c\(^+\) DCs, using IL-12 secretion and expression of co-stimulatory molecules (CD86, CD80, CD40) as read-out for DC maturation in vitro. Of further interest, K11777-treated trypomastigote failed to robustly activate wild-type DCs, thus linking generation of the BK,R agonist (DC maturation signal) to the proteolytic activity of cruzipain. Noteworthy, Dm28c TCTs also induced the maturation of (splenic) TLR2\(^+\) CD11c\(^+\) and TLR4 mutant (C3H/HeJ) via BK,R, thus precluding cooperative signaling between this GPCR and either one of these surface PRRs. Admittedly, these results do not exclude the possibility that enhanced parasite uptake via the BK,R pathway might have indirectly facilitated nucleic acid-sensing by TLRs residing in endolysosomes of immature splenic DCs. In any case, whether acting as a classical sensor receptor, and/or as a upstream pathway that potentiates TLR-signaling by parasite DNA or ssRNA, these results are in line with the concept that kinin “danger” signals proteolytically released by TCTs convert splenic BK,R\(^+\) DCs into inducers of type 1 immunity (Monteiro et al., 2007). Considering that the splenic parenchyma is continuously exposed to plasma proteins, it is conceivable that flagellated trypomastigotes navigating through the splenic stroma might be faced with abundant levels of blood-borne kininogens, most likely associated to ECM or cell-surface sulfated proteoglycans. Accordingly, we may predict that antigen-bearing CD11c\(^+\) DCs (bearing T. cruzi antigens) residing in the spleen (and/or liver) stroma are converted into TLR1 inducers following exposure to high-levels of kinin “danger” signals. To this date, studies of BK,R function in human DCs were limited to lineages derived from monocytes exposed to granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-4. Using this experimental model, Bertram et al. (2007) reported that BK,R signaling promotes DC migration although BK, per se, did not induce the maturation of human DCs. However, in subsequent studies Kaman et al. (2009) suggested that BK,R signaling potentiates the maturation of DCs previously “primed” via TLR4. In view of the marked phenotypic heterogeneity of DCs (Shortman and Naik, 2007), studies with a more representative range of human DCs are required to determine whether BK,R is a key sensor of T. cruzi.

In conclusion, the analysis of BK,R function in different models of acute T. cruzi infection strongly suggests that activation of the kinin system fuels anti-parasite immunity. In the next section we will review evidences indicating that generation of kinins in sites of infection may also benefit T. cruzi, hence translating into mutual benefits to the host–parasite equilibrium.

**TCTs RELY ON CRUZIPAIN ACTIVITY TO INVADE CARDIOVASCULAR CELLS VIA THE BK,R PATHWAY**

As mentioned earlier, studies of T. cruzi interaction with mouse cardiomyocytes showed that membrane-permeable irreversible inhibitors of cruzipain impaired trypomastigote invasion and halted intracellular growth of amastigotes (Morielles et al., 1992). While the studies on cruzipain-mediated pathways of invasion were in progress, Tardieux et al. (1992) reported that T. cruzi invades non-phagocytic cells through the induction of calcium-regulated pathway of lysosomal exocytosis. Subsequent studies revealed that the oligopeptidase B-mediated processing of a polypeptide precursor accumulating in the T. cruzi cytoplasm generated the calcium-inducing signal (Cüler et al., 1996; Morry et al., 1999) that propelled parasite internalization. In a parallel development, Leite et al. (1998) reported evidences that GPCRs were the signal transducers of the (Ca\(^{2+}\))-inducing signals generated by the oligopeptidase B-dependent peptide. Consistent with their working hypothesis, parasites genetically deficient in oligopeptidase B showed impaired infectivity, suggesting that proteolytic generation of the cytoplasmic Ca\(^{2+}\)-inducing signal was indeed required for the development of infective phenotype. In another interesting study, Santana et al. (1997) described the biochemical properties of Tc80, a serine protease displaying collagenase activity. Subsequently characterized as a proP1 oligopeptidase, Tc80 localizes in a vesicular compartment close to the flagellar pocket of trypomastigotes. Synthetic inhibitors of Tc80 potently inhibited T. cruzi (Y strain) invasion of non-phagocytic cells without interfering with the (Ca\(^{2+}\))-inducing activity of parasite extracts (Grellier et al., 2001).

Further exploring the role of cruzipain as a virulent factor, Scharfstein et al. (2000) reported that activation of BK,R potentiates parasite uptake by non-phagocytic cells. Whether using CHO transfected cells, human umbilical vein endothelial cells (HUVECs), primary cultures of mouse (neonatal) cardiomyocytes (Todorov et al., 2003), or primary culture of human smooth muscle cells (HSMCs, Andrade et al., 2012), these studies indicated that the parasites relied on cruzipain activity to generate a (Ca\(^{2+}\))-inducing signal (BK,R agonist) from HK displayed on host cell surfaces. Although the genetic ablation of the monocopy cruzipain genes remained a technical obstacle, experiments performed with membrane-permeable irreversible inhibitors of cruzipain and parasites overexpressing the cruzipain gene strongly suggested that the trypomastigotes critically depended on the enzymatic activity of cruzipain to stimulate parasite uptake by BK,R-positive target cells (Scharfstein et al., 2000). The authors hypothesized that upon trypomastigote attachment (posterior end) to host cell surfaces, the enzymatically active forms of cruzipain – localized in the flagellar pocket (Murtz et al., 1999; Santos-Padrão et al., 1999) – rapidly diffused into the sealed spaces formed by the juxtaposed host/parasite membranes (Scharfstein et al., 2000). Once confined to “synapses” (Tyler et al., 2005; Butler and Tyler, 2012), the active protease should be protected from targeting by natural inhibitors, such as cystatins, soluble forms of kininogens (Stoka et al., 1998), and m2-macroglubulin (Araújo-Jorge et al., 1994; Morrot et al., 1997) present in interstitial fluids. Considering that BK,R is sequestered to membrane rafts/caveolae (de Weerd and Lerb-Lundberg, 1997; Haesemann et al., 1998), we hypothesized that surface-bound HK (cruzipain substrate) may translocate in the plasma membrane before being delivered to the site of synapse (Figure 3), thus ensuring efficient kinin release/BK,R-mediated signal transduction (Scharfstein et al., 2008; Santos et al., 2006; Scharfstein and Andrade, 2011; Andrade et al., 2012).

While analyzing the outcome of infection in cultures of HUVECs (resting state), we found that BK,R did not promote parasite uptake in the absence of ACE inhibitors. This was not unexpected, because expression of ACE, a transmembrane...
metalloproteinase that potently degrades intact kinins, is strongly upregulated by endothelial cells. As predicted, BK$_2$R-dependent parasite uptake by HUVECs is potentiated by ACE inhibitors (Scharfstein et al., 2000; Todorov et al., 2003; Andrade et al., 2012).

Noteworthy, ACE inhibitors were not essential for BK$_2$R-driven parasite invasion of cardiomyocytes or smooth muscle cells (Todorov et al., 2003; Andrade et al., 2012). Although the levels of ACE were not measured, it is likely that these muscle cells express lower levels of ACE as compared to HUVECs. Along similar lines, TCTs induced IL-12 responses on splenic CD11c$^+$ DCs via BK$_2$R irrespective of presence/absence of ACE inhibitors (Monteiro et al., 2007). In summary, the outcome of Dm28c TCT interaction with cells that constitutively express BK$_2$R is controlled by ACE in cell-specific manner.
THE INDUCIBLE BK1R: A UBQUITOUS GATEWAY FOR T. CRUZI INVASION OF INFLAMED TISSUES?

A major challenge in Chagas disease research is to predict the clinical outcome of the chronic cardiomyopathy. About 30% of these patients develop a progressive form of cardiomyopathy characterized by the presence of diffused inflammation/fibrosis. As mentioned in the introduction, pathologists noticed the presence of microvascular abnormalities and altered ECM patterns in the heart of CCM patients (Higuchi et al., 1999). To our knowledge, BK1R expression was not systematically investigated in human autopsy studies of chagasic patients despite the indications that dysregulated BK1R function might be detrimental to the heart in other disease models. For example, in animal models of STZ-induced diabetic cardiomyopathy (Westermann et al., 2009), BK1R-deficient mice showed attenuated cardiomyopathy as compared to wild-type mice, as evidenced by the decrease of cardiac inflammation, fibrosis, oxidative stress, and significant improvement of left ventricular function. Pertinent to studies linking heart remodeling to the ET up-regulation by parasympathetic cardiomyocytes (Tanowitz et al., 2005), there is indication that oxidative stress induced by ET-1 (ETAR-driven) and angiotensin I (AT1-driven) up-regulates BK1R, leading to activation of PI3K and MAPK in smooth muscle (Morand-Contant et al., 2010). Although not directly examined, it is conceivable that NF-kB induction by proinflammatory cytokines secreted by antigen-specific intracardiac CDB8+ T cells (Padilla et al., 2009; Silverio et al., 2012) might also up-regulate BK1R in the chagasic heart.

Todorov et al. (2003) were the first to demonstrate that Dm28c TCTs evoke interstitial edema via the sequential activation of BK1R/BK2R. In the same study, they highlighted the dichotomic nature of kinin signaling pathways: the parasites were able to invade activated target cells via the BK1R/CPM pathway (Figure 1B). Differently from results observed in resting HUVECs (which critically depend on ACE blockade to efficiently internalize TCTs via BK1R), the trypomastigotes invade lipopolysaccharide (LPS)-treated HUVECs through interdependent signaling ("cross-talk") between BK1R and BK2R (Todorov et al., 2003). Notably, the "cross-talk" between BK1R and BK2R was also observed in invasion assays performed with primary mouse cardiomyocytes (Todorov et al., 2003), which are cell types that spontaneously express BK1R in culture systems.

Although studies with non-specific kininase I inhibitors have tentatively linked BK1R-dependent parasite uptake to the processing activity of these carboxypeptidases, the role of the transmembrane carboxypeptidase CPM was not directly addressed in the above mentioned studies. As mentioned in the introduction, BK1R agonists (BK/LBK) are converted into the BK1R agonist that propels parasite invasion. First, they showed that BK1R signaling is responsible for the sustained NO response induced by intact BK (BK1R agonist), implying that CPM converted the intact BK to ([des-Arg]2)-kinins. Using cells transfected with genes encoding for CPM and BK1R, the authors disrupted lipid rafts with methyl-beta-cyclodextrin (MβCD). As predicted, this maneuver reduced the BK1R-dependent increase in [Ca2+]i, in response to stimulation with intact BK1R agonists, whereas addition of cholesterol rescued this BK1R-driven response. After showing that CPM and BK1R co-localized in lipid raft/caveolin-enriched membrane fractions (Zhang et al., 2008), they found that CPM/BK1R physically interact on the cell membrane, based on co-immunoprecipitation, cross-linking, and fluorescence resonance energy transfer analysis.

In an elegant experiment using a novel fusion protein containing CPM at the N-terminus of the BK1R, Zhang et al. (2008) showed that these transfected cells were (Ca2+)i-responsive upon stimulation with intact kinins, but (as predicted) this response was no longer impaired by MβCD or by CPM antibody. As already mentioned, in invasion assays performed with LPS-HUVECs or cardiomyocytes, we found that ACE inhibitors were not required to promote parasite uptake via BK1R, as opposed to the ACE inhibitor-dependent phenotype displayed by resting HUVECs (Todorov et al., 2005; Andrade et al., 2012). This implies that CPM-dependent generation of the BK1R is prioritized over the ACE-dependent pathway of BK/LBK degradation, hence consistent with the findings reported by Zhang et al. (2008). Since both CPM/BK1R and BK1R/BK2R are sequestered into lipid rafts and/or caveolae, it is conceivable that these GPCRs segregate together with HK (bound to heparan sulfate proteoglycans) into specialized plasma membrane microdomains (Figure 3). Of further interest, although BK1R and BK2R signal cells through fairly similar intracellular pathways, the regulation of inducible BK1R differs from BK2R in that the former is desensitized upon agonist binding only to a limited degree (Enquist et al., 2007).

BK1R AND ETRs AS GATEWAYS FOR INVASION OF CARDIOVASCULAR CELLS: FUNCTIONAL LINK WITH THE SPHINGOMYELINASE-DEPENDENT PLASMA MEMBRANE PATHWAY?

In a previous section, we reviewed results of studies showing that Dm28c TCTs induce inflammatory edema through mechanisms involving cooperation between BKRs and ETRs (Andrade et al., 2012). To evaluate whether the parasites could exploit the ET pathway for invasion purposes, Andrade et al. (2012) studied the interaction of Dm28c TCTs with three types of host cells: HUVECs (which only express ETA), primary cultures of HSMCs (which express ETA and ETB), and mouse neonatal cardiomyocytes (expressing both subtypes of ETRs). Using multiple pharmacological tools (subtype-specific ETR antagonists; neutralizing antibodies for each GPCR) and, in addition, interference RNA, Andrade et al. (2012) demonstrated that TCTs invade "resting" HUVECs via ETαR, while invasion of the muscle cells involved activation of both subtypes of ETRs. Notably, the combined treatment of the muscle cell cultures with ETαR and ETβR subtype-specific antagonists failed to decrease parasite infectivity of HSMCs or cardiomyocytes over values induced by the individual drugs, thus recapitulating the "cross-talk" observed between BK1R/BK2R in cardiomyocytes.
Whether using subtype-specific GPCR antagonists, neutralizing antibodies, or iRNA interference (ET AR), our results showed that parasite invasion was efficiently blocked in subtype receptor expression sites, our pharmacological studies support this possibility. Assuming that ligand generation is not a limiting factor, this “space-filling model” predicts that GPCRs that might be present at higher density in such microdomains should have a better chance to coordinate signaling responses upon ligand binding, as seems to be the case for BK,R, ETAR, and ETR (Andrade et al., 2012; Figure 3).

Several years ago, it was demonstrated that cholesterol-depleting drugs reduce host cell susceptibility to T. cruzi infection (Fernandes et al., 2007). In a subsequent study, we explored the possibility that BKRs and ETRs compartmentalize in lipid rafts/caveolae, by treating HSMCs with MβCD (Andrade et al., 2012). As predicted, the cholesterol-depleting drug drastically reduced parasite (Dm28c) entry in HSMCs whereas addition of exogenous cholesterol to MβCD-HSMCs restored ET,R/ETBr/BK,R-dependent pathways of T. cruzi invasion. Although the physical association of these GPCRs was not demonstrated at the molecular level, these results suggest that the cross-talk between ETRs and BK,R may critically depend on the integrity of lipid rafts/caveolae (Figure 3). Interestingly, confocal microscopy studies performed with antibodies to anti-ET,R or anti-ETBr showed that parasite-cell interaction sites contained increased clusters of these GPCRs. These results are consistent with the concept that ET-1 and “kinins” activate their cognate GPCRs in lipid rafts/caveolae, perhaps translocated to synaptic sites (Scharfstein et al., 2000). As explained below, it is also possible that ET-1, ET precursors and HK (bound to heparan sulfate) exert their functional roles as agonists and/or precursors of GPCR ligands after their internalization in ceramide-enriched vesicles (Figure 3). So far, BK,R, ETAR, and ETBR are translocated from lipid and/or caveolar microdomains into lipid rafts of muscle cells (HSMC or cardiomyocytes) or HUVECs, this possibility deserves to be explored in light of evidence that ceramide-enriched microdomains spontaneously fuse to generate large macromdomains containing receptor clusters in multimolecular platforms (Jin et al., 2008). Using endothelial cells derived from coronary arteries, these authors reported that Fas-ligand driven activation of death receptor mediates the formation of redox signaling platforms in lipid rafts via ceramide production by ASM-driven hydrolysis of sphingomyelin. They also showed that formation of ceramide-enriched signaling platforms was canceled in endothelial cells treated with inhibitors of lysosomal function. In other words, the requirements for the formation of multimolecular signaling platforms in ceramide rafts of endothelial cells seem to recapitulate the requirements for parasite invasion via the house-keeping mechanisms of repair from cell wounds described by Fernandes and Andrews (2012). Accordingly, our “space-filling model” (Figure 3), the signal transduction responses coordinated by these various GPCRs (e.g., BK,R/ETAR/ETBR) may either occur (i) within the synthetic sites formed soon after adhesion and/or (ii) after translocation of these GPCRs into ceramide rafts, hence integrating the above mentioned multimolecular signaling platforms. Sufficiently flexible, this GPCR-dependent model of parasite internalization is compatible with linear or converging activation pathways. For example, the linear activation mode predicts that specific GPCR subtypes are differentially targeted to lipid rafts/caveolae or to ceramide-enriched vesicles and/or to nascent parasitophorous vacuoles (Figure 3). Once translocated to nascent parasitophorous vacuoles, some of these GPCR subtypes may generate parasite retention signals through PI3K-dependent remodelling of the actin cytoskeleton (Woolsey and Burleigh, 2004), and/or drive calcium-dependent lysosomal fusion to parasitophorous vacuole (Andrade and Andrews, 2004). Multiple contacts with the pathogen and signals transmitted by soluble agonists available...
at high levels in the edematous extracellular environment, such as ETs, BK, DABK, may then fuel parasite uptake in vivo through converging signaling pathways.

The possibility that these GPCRs might serve as gateways for T. cruzi infection of heart cells is worth exploring. According to Fernandes and Andrews (2012), T. cruzi propensity to invade muscle cells is dictated by the need of muscle cells to constantly repair sarcolemma wounds. If confirmed, the convergence of house-keeping and GPCR-mediated signaling pathways may translate into mutual benefits to the host-parasite relationship, at least so during the indeterminate stage of chronic infection. Interestingly, Petersen et al. (2005) implicated TL2R as the main upstream regulator of hypertrophy which T. cruzi trigger in isolated cardiomyocytes. On the other hand, T. cruzi strains that might express/shed higher levels of tGPI might somewhat blunt this potentially adverse phenotype by favoring low-grade release of cardioprotective kinins via cruzipain as described by Monteiro et al., 2006. In the chronic settings, the benefits of anti-apoptotic effects attributed to purified cruzipain (Aoki Mdel et al., 2006) and BK2R signaling (Chao et al., 2008) may be offset by the upregulation of BK/R, a pathway that may synergize with TL2R and ETs/R, hence fueling inflammatory edema (Figure 2) and cardiac hypertrophy in chronically infected patients.

Another mechanism that may underlie the variable phenotype of T. cruzi strains is the variable expression profiles of cruzipain isoforms (Lima et al., 2001). For example, it is well established that cruzipain 2 (Dm28c strain) has narrow substrate specificity as compared to the major cruzipain isoform, i.e., the parasite kininase (Scharfstein, 2010). Predictably, strain-dependent variability in the ratio of expression between these two cruzipain isoforms may have impact on T. cruzi ability to invade host cells expressing BKRs (influence on tissue tropism) as well as its capacity to induce interstitial edema and TGFβ responses via the kinin pathway. For similar reasons, we may predict that variations in the expression levels of chagasin, a tight-binding endogenous inhibitor of papain-like cysteine proteases – originally described in T. cruzi (Monteiro et al., 2001), may also influence the phenotype of T. cruzi strains. This possibility is supported by evidences (Aparicio et al., 2004) indicating that TCTs of the G strain, which are poorly infective, display increased chagasin/cruzipain ratios as compared to Dm28c. Importantly, the infectivity of the G strain was enhanced upon addition of cruzipain-rich culture supernatants from Dm28c TCTs. In the same study, the authors pointed out that that vesicles shed by TCTs might serve as cruzipain substrates, presumably generating hitherto uncharacterized infection-promoting signals (Scharfstein and Lima, 2008). Hence, strain-dependent differences in the expression levels of tGPI and cruzipain isoforms may have impact on host-parasite relationship, either because kinins influence parasite infectivity, DC function and their ability to steer TGFβ type effectors development.

**CONCLUDING REMARKS**

Host defense to infections depend on the mobilization of two distinct strategies in order to minimize infection-associated pathology: the first, innate immunity, is mobilized at the onset of infection with the purpose to eliminate or at least limit the spread of pathogens to the tissues (Medichthe, 2009). The second strategy, involving products generated by proteolytic cascades and multiple endogenous "danger" signals emanating from injured tissues, has evolved to limit and repair the tissue damage inflicted by the microbial pathogen. The distinction between...
We have proposed that trypomastigotes sharing the Dm28c phenotype DC sensing of the kinin “danger” signal via BK2R was essential for optimal generation of type 1 (host protective) effector T cells (Monteiro et al., 2007). The dual role of the KKS in experimental Chagas disease is so far based on evidences that Dm28c trypomastigotes exploit BKRs/ETRs as (non-exclusive) gateways for cellular invasion of a broad range of non-phagocytic cells, including cardiomyocytes. We have proposed that trypomastigotes sharing the Dm28c phenotype might be able to transiently generate infection-promoting peptidergic ligands for GPCRs, such as BK and ET-1, in inflamed tissues through the induction of interstitial edema.

Interestingly, studies in other models of heart disease have associated KKS/BK2R signaling with protective cardiac functions, such as reducing apoptosis and chamber dilatation in the myocardiun (Chao et al., 2008) and promoting restoration of Sta-mediated sarcoplasmatic Ca2+ uptake by cardiac cells (Tchôpe et al., 2005). Thus, it is tempting to speculate that BK2R signaling may bring about mutual benefits to the host/parasite interaction in this microenvironment. For example, the trypomastigotes navigating through intercellular cardiac spaces might be targeted by high-titered complement fixing/lytic antibodies (Krettli et al., 1982; Almeida et al., 1994) and/or prevent anti-apoptoticote ability to efficiently invade cardiovascular cells (Meirelles et al., 2012). Following a similar line of reasoning, we may predict that ET-1 and [des-Arg] kinins, acting cooperatively, may propel parasite entry in cardiovascular cells through the signaling of BK2R, a subtype ofGPCR that is ubiquitously up-regulated in inflamed tissues.

The sporadic formation of an intramyocardial edema in the proximity of an inflammatory lesion generated by pseudocyst rupture may promote the diffusion of blood-borne ET-1 through perivascular cardiac tissues. If true, these events may in turn trigger cardiac mast cell degranulation via the ET-1/ET, R axis, thus linking the ET pathway to the KKS (Figure 2B). Furthermore, considering that the expression of BK2R is up-regulated by the pro-oxidative EtEs (Morand-Contant et al., 2010), and that ET-1 expression is robustly increased in parasitized cardiomyocytes (Yanovitz et al., 2005), it is conceivable that T. cruzi has evolved strategies to improve its infectivity at expense of increased inflammation, as recently shown in mice infected with the Y strain of T. cruzi (Paiia et al., 2012). Following a similar line of reasoning, we may predict that ET-1 and [des-Arg] kinins, acting cooperatively, may propel parasite entry in cardiovascular cells through the signaling of BK2R, a subtype of GPCR that is ubiquitously up-regulated in inflamed tissues.
receptors (Medeiros et al., 2007) and/or β1-adrenergic receptors (Labovisky et al., 2007). Depending on their titers and fine-specificity, these auto-antibodies may invoke arrhythmia as a secondary consequence of plasma leakage elicited by trypanomastigotes. If confirmed, this hypothesis may legitimate attempts to treat chagasic patients with drugs that target the ET/mast cell/KKS axis: by limiting leakage of auto-antibodies into the myocardium, these drugs may also mitigate cardiac arrhythmia.

Considering the long span of chronic infection, the myocardium of patients exhibiting deficient IL-10 production by macrophages or regulatory T cells (Costa et al., 2009) might be particularly prone to up-regulate the BK-R pathway in response to excessive collateral damage inflicted by pathogenic subsets of T cells. In this hypothetical scenario, the inhibitory type 1 effector CD8+ T cells may relentless fuel the pro-fibrotic pathways coordinated by BK/R/ETR. Of further interest, recent studies of sterile inflammation in tissue necrosis characterized sensor of self-DNA (Ahn et al., 2012). Ongoing studies in chagasic mice treated with BK/R blockers may clarify whether this therapeutic strategy may reduce parasite tissue load as well as myocardial fibrosis. Moreover, it is conceivable that BK/R antagonists may reduce chronic inflammation downregulation of DNAse II, a lysosomal enzyme, fuels sterile inflammation in TLR9-dependent manner, provided that the mice are subjected to pressure overload (Oka et al., 2012). Although not explored in the context of CCM, it may not be surprising if chagasic heart debilitated by microvascular lesions, hypoxia, and immunopathology may be hyper-responsive to TRPV1 signaling induced by T. cruzi microvascular DNA and/or self-DNA (from non-infective cardiomyocytes). If confirmed, it will be intriguing to know if BK/R and ETRs, acting cooperatively in the pro-oxidative environment that prevails in the chagasic heart (Dhiman and Garg, 2011) may up-regulate TRB and/or STING, another recently characterized sensor of self-DNA (Ahn et al., 2012). Ongoing studies in chagasic mice treated with BK/R blockers may clarify whether this therapeutic strategy may reduce parasite tissue load as well as myocardial fibrosis. Moreover, it is conceivable that BK/R antagonists may reduce chronic inflammation downregulation of DNAse II, a lysosomal enzyme, fuels sterile inflammation in TLR9-dependent manner, provided that the mice are subjected to pressure overload (Oka et al., 2012). Although not explored in the context of CCM, it may not be surprising if chagasic heart debilitated by microvascular lesions, hypoxia, and immunopathology may be hyper-responsive to TRPV1 signaling induced by T. cruzi microvascular DNA and/or self-DNA (from non-infective cardiomyocytes). If confirmed, it will be intriguing to know if BK/R and ETRs, acting cooperatively in the pro-oxidative

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