Cooperative Activation of TLR2 and Bradykinin B2 Receptor Is Required for Induction of Type 1 Immunity in a Mouse Model of Subcutaneous Infection by Trypanosoma cruzi


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We have previously reported that exogenous bradykinin activates immature dendritic cells (DCs) via the bradykinin B2 receptor (B2R), thereby stimulating adaptive immunity. In this study, we show that these premises are met in a model of s.c. infection by *Trypanosoma cruzi*, a protozoan that liberates kinins from kininogens through its major protease, cruzipain. Intensity of B2R-dependent paw edema evoked by trypomastigotes correlated with levels of IL-12 produced by CD11c+ dendritic cells isolated from draining lymph nodes. The IL-12 response induced by endogenously released kinins was vigorously increased in infected mice pretreated with inhibitors of angiotensin converting enzyme (ACE), a kinin-degrading metallopeptidase. Furthermore, these innate stimulatory effects were linked to B2R-dependent up-regulation of IFN-γ production by Ag-specific T cells. Strikingly, the trypomastigotes failed to up-regulate type 1 immunity in TLR2−/− mice, irrespective of ACE inhibitor treatment. Analysis of the dynamics of inflammation revealed that TLR2 triggering by glycosylphosphatidylinositol-anchored mucins induces plasma extravasation, thereby favoring peripheral accumulation of kininogens in sites of infection. Further downstream, the parasites generate high levels of innate kinin signals in peripheral tissues through the activity of cruzipain. The demonstration that the deficient type 1 immune responses of TLR2−/− mice are rescued upon s.c. injection of exogenous kininogens, along with trypan- mastigotes, supports the notion that generation of kinin “danger” signals is intensified through cooperative activation of TLR2 and B2R. In summary, we have described a s.c. infection model where type 1 immunity is vigorously up-regulated by bradykinin, an innate signal whose levels in peripheral tissues are controlled by an intricate interplay of TLR2, B2R, and ACE. *The Journal of Immunology*, 2006, 177: 6325–6335.
as angiotensin converting enzyme (ACE; kininase II; CD143) and neutral endopeptidase P (1, 13) and/or by desensitization and sequestration of B2R (8, 14). Inhibitors of ACE (ACEi) such as captopril and lisinopril prevent degradation of kinins thereby prolonging their half-life and enhancing their physiological effects (13). In contrast to B2R, B1R is the prototype of an inducible receptor present in low levels in normal tissues and highly up-regulated in injured or inflamed tissues (9). Generation of B1R agonists depends on enzymatic removal of the C-terminal Arg residue from intact kinins by carboxypeptidase M/N (kininase II) (9).

Immunological studies performed in models of Th2-dependent lung inflammation induced in BALB/c mice immunized with OVA revealed that exogenous kinins (BK/LBK) drive Th1 polarization via the IL-12 pathway (12). Of further interest, we found that in vivo administration of ACEi enhanced IL-12 production by DCs (12), suggesting that endogenous levels of kinin generated by this pathogen, we found evidence that the extent of kinins generated TLR2, B2R, and ACE. Perturbations in kinin homeostasis, here revealed that exogenous kinins (BK/LBK) drive Th1 polarization by a brief step of precipitation with 60% saturated ammonium sulfate, the precipitates were quickly dissolved in PBS, and then applied to the thiol-Sepharose 4B (Pharmacia). Before applying the cruzipain-rich sample to the resin, we added 5 mM DTT for 15 min. After removing the excess of the precipitating agent through a brief step of precipitation with 60% saturated ammonium sulfate, the precipitates were quickly dissolved in PBS, and then applied to the thiol-Sepharose 4B (Pharmacia) equilibrated in 100 mM Tris-HCl and 2 mM EDTA (pH 7.4). The bond protease was eluted from the resin with 20 mM DTT, added to the same buffer. After pooling the enzymatically active cruzipain fractions as described (31), the purified protease was dialyzed against PBS, concentrated by centrifugation in Centri- con 10 filters. After checking for biochemical homogeneity, purified cruzipain was stored at −20°C. GPI-linked mucins (tropomyosin-derivated GPI-anchored mucin (GPI-m)) were purified from TCTs of the Y strain as described (32). Electrospray ionization-mass spectrometry (negative ion mode) analysis did not show indication of LPS/lipid A contamination in the GPI-m sample within the limits of detection of lipid A, estimated as 1–10 femtomoles. In addition, no contaminating *Mycoplasma* lipoproteins were found by positive-ion mode electrospray ionization-mass spectrometry and MALDI-TOF-MS analyses after digestion of GPI-m with proteinase K, as described (26). LPS contamination of these preparations was ruled out using the *Limulus* amebocyte lysate assay (BioWhittaker).

**Isolation and characterization of DC from mice infected with *T. cruzi***

BALB/c mice pretreated, or not, with 10 mg/kg i.p ACEi (captopril) (Sigma-Aldrich) and/or 100 μg/kg s.c. HOE-140 (Aventis), were injected 1 h later in the hind footpads with 5 × 10^7 TCTs. DCs were isolated from popliteal lymph nodes (LN) at 18 h postinfection (p.i.). Briefly, pooled LN fragments were treated with collagenase D (Sigma-Aldrich) and DCs were fully isolating the water-soluble fraction, the sample was dialyzed against PBS (pH 7.2), following extensive dialysis with the same buffer, aggregate-free cleared by centrifugation and the soluble protein fraction (40 ml) was extracted with 80 ml of dehydrated n-butanol at 4°C. After thoroughly isolating the water-soluble fraction, the sample was dialyzed against PBS (pH 7.2) and then subjected to affinity chromatography on a thiol-Sepharose 4B (Pharmacia).

**Materials and Methods**

**Mice and parasites**

Experiments were done with mouse strains BALB/c, J129 WT (B2R−/−), J129 B2R+/−, C57BL/6 WT (TLR2+/−), C57BL/6 TLR2−/− (donated by Dr. S. Akira, Osaka University, Osaka, Japan), C3H/HePAS (TLR4−/−), C3H/HeJ (TLR4+/−), C3H/HeJ (TLR4+/−), and C57BL/6 WT (TLR2+/−) mice. Culture supernatants containing T. cruzi (epimastigote) Ag. Culture supernatants were collected after 48 h and levels of IFN-γ were quantified by ELISA (R&D Systems).

**Kinogen-mediated induction of cytokine production by T cells from infected mice**

BALB/c mice pretreated, or not, with 10 mg/kg i.p ACEi (captopril) and/or 100 μg/kg s.c. HOE-140 were inoculated 1 h later with 1 × 10^7 TCTs (paw). Infection of J129 WT (B2R−/−), J129 B2R+/−, C3H/HePAS (TLR4−/−), C3H/HeJ (TLR4+/−), and C57BL/6 WT (TLR2+/−) mice was done by inoculating 5 × 10^7 TCTs, or alternatively with an equivalent dose of TCTs preincubated (20 min at room temperature) with 10 μM methylpyrazine-Phe-homoPhe-vinylsulfone-benzene (VSPh-TCT) per hind footpad (33). After 10 days, LN T cells were isolated and stimulated with 20 μM/ml soluble *T. cruzi* (epimastigote) Ag. Culture supernatants were collected after 48 h and levels of IFN-γ were quantified by ELISA (R&D Systems).
ACEi-TLR2<sup>−/−</sup> mice were injected with a TCT suspension contained 10 μg/ml BK (Sigma-Aldrich) or 50 μg/ml humane HK (Calbiochem), or alternatively, with VSPH-TCT suspension supplemented with 50 μg/ml HK. After 10 days, LN T cells were isolated and stimulated with 25 μg/ml soluble <i>T. cruzi</i> (epimastigote) Ag. Culture supernatants were collected after 48 h and IFN-γ was quantified by ELISA (R&D Systems).

**Edema assays**

Animals pretreated with 10 mg/kg i.p. ACEi (captopril) and/or 100 μg/kg s.c. HOE-140 were injected 1 h later with 1 × 10<sup>6</sup> TCTs or equivalent number of epimastigotes, as previously reported (31). Where indicated, the mice were inoculated with VSPH-TCT. For polymorphonuclear neutrophils (PMN) depletion, mice were injected i.p. with 0.45 ml of a 1/10 dilution in PBS (34) of rabbit antiserum to PMN (Accurate Chemical) or an equivalent volume of normal rabbit serum (control). TLR2<sup>−/−</sup> and TLR4<sup>−/−</sup> mice, pretreated or not with ACEi and/or HOE-140 as described above, were injected with 10 nM purified GPI-mucin alone or combined with 5 nM purified cruzipain. When indicated, cruzipain was previously inactivated by 5 nM 1-trans-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64). Edema volumes reflecting the difference between injected and contralateral paws were measured by plethysmometer (24).

**Intravital microscopy in the hamster cheek pouch**

To study vascular permeability increases in postcapillary venules of the hamster cheek pouch model, fluorescein-labeled dextran (FITC-dextran, molecular mass ~150,000 Da; 250 mg/kg body weight) was injected i.v. in the pouch tissues superfused with HEPES/bicarbonate-buffered saline solution at a constant rate of 6 ml/min at 35°C, before topical application of parasites, as previously described (7). In some experiments, 1 μM ACEi (captopril) was added to the superfusing medium. The perfusion flow was stopped for 10 min to allow for topical application of TCTs (2.5 × 10<sup>5</sup>) in the cheek pouch. The number of "leaky spots" was counted at various time points after parasite challenge. Where required, the ACEi-treated cheek pouch received 0.5 M HOE-140, before parasite application topically. To study the involvement of kininogens in the permeability-inducing activity of cruzipain, we first established baseline activity levels by applying increasing concentrations of activated cruzipain (range of 0.1–2.5 μM) to the ACEi-treated cheek pouch. No significant leakage was observed at cruzipain concentrations <0.25 μM. The experiments involved six consecutive topical applications of the test samples in the same cheek pouch tissues. This was performed with 30-min intervals between the tests, to permit tissue superfusion with ACEi medium. After testing the effect of activated cruzipain (0.25 μM) alone, the cheek pouch was superfused with ACEi medium for 30 min. This was followed by topically applied HK (500 nM), added immediately before activated cruzipain. After another 30 min of tissue superfusion with ACEi medium, we added HK alone, i.e., without the protease. The fourth test involved HK application following by addition of cruzipain inactivated by E-64. In the fifth test, we checked whether the microvascular bed was still responsive to the combination of HK and activated cruzipain. Finally, ACEi-treated cheek pouch tissues were pretreated with HOE-140, before a final application of cruzipain/HK. Leaks induced by cruzipain were counted at 2, 5, 10, 15, 20, and 30 min. Peak activity responses resulting from the combined addition of HK and cruzipain were consistently observed at 5 min. For each test condition, average number of leaky sites was determined by collecting data from at least five hamsters.

**Results**

**Kinins released in paw tissues infected by <i>T. cruzi</i> induce DC maturation via B<sub>2</sub>R**

In a previous study (31), we reported that mice pretreated with a single-dose of captopril, an ACEi, develop a vigorous paw edema 3 h after inoculation of TCTs. The swelling reaction evoked by TCTs was intense in J129 B<sub>2</sub>R<sup>−/−</sup> mice, but the parasites did not induce a significant vascular reaction in ACEi-treated J129 B<sub>2</sub>R<sup>−/−</sup> mice or in ACEi-J129 B<sub>2</sub>R<sup>+/+</sup> mice pretreated with the selective B<sub>2</sub>R antagonist HOE-140 (31). Here, we used the same s.c. model of <i>T. cruzi</i> infection to test the proposition that endogenously released kinins link innate to adaptive immunity (12). To this end, we inoculated TCTs in the paw of BALB/c mice and isolated CD11c<sup>+</sup> DCs from popliteal LN, 18 h p.i. Analysis of IL-12 production by FACS showed that the percentage of CD11c<sup>+</sup> DCs expressing high levels of IL-12 was drastically increased in mice pretreated with ACEi, but this effect was nullified by HOE-140 (Fig. 1A). By contrast, CD11c<sup>+</sup> DCs isolated from mice infected in the absence of ACEi displayed low IL-12 responses, indicating that the innate effects of released kinins is normally blunted by this major kinin-degrading peptidase. As an additional control, CD11c<sup>+</sup> DCs isolated from noninfected mice pretreated with ACEi did not produce IL-12 (Fig. 1A). Essentially the same profile was obtained when we used ELISA to quantify IL-12p70 produced by CD11c<sup>+</sup> DCs under these conditions (Fig. 1B). Collectively, our results indicate that kinins generated in the infected paw can overtime stimulate IL-12 production by CD11c<sup>+</sup> DCs via the B<sub>2</sub>R-signaling pathway.

**Kinins link innate to adaptive immunity in <i>T. cruzi</i>-infected mice via the B<sub>2</sub>R pathway**

Because the intensity of IL-12 production by DCs in vivo correlates with extent of kinin generation in primary sites of infection, we then checked whether these innate modulatory effects translate into changes of adaptive immunity. Recall responses by LN T cells isolated from BALB/c mice 10 days after infection showed that ACEi-BALB/c produced over 4-fold more IFN-γ than mice infected in the absence of the ACEi (Fig. 2A). In the absence of <i>T. cruzi</i> infection to test the proposition that endog-
Ag, the LN T cells only secreted baseline levels of IFN-γ (data not shown). We then checked whether blockade of B₂R activation at the onset of infection could prevent ACEi-dependent up-regulation of type 1 responses. Indeed, T cells derived from ACEi-BALB/c mice pretreated with HOE-140 produced low levels of IFN-γ upon in vitro stimulation with T. cruzi Ag (Fig. 2A). IL-4 was not detected in mice pretreated with the B₂R antagonist, irrespective of ACEi administration (data not shown). To further assess the immunoregulatory role of kinins, we then compared the impact of ACEi treatment in adaptive responses of wild-type (WT) (B₂R+/−/−) J129 mice vs J129 B₂R−/−/−. Similar to BALB/c mice, T cells isolated from draining LN of ACEi-treated B₂R+/−/− mice vigorously up-regulated IFN-γ production upon stimulation with T. cruzi Ag in vitro, whereas T cells derived from HOE-140-treated B₂R−/−/− mice only produced baseline levels of IFN-γ (Fig. 2B). Cultures run in the absence of T. cruzi Ag only showed baseline levels of IFN-γ (data not shown). In contrast, recall assays performed with T cells isolated from infected ACEi-B₂R−/−/− mice showed that IFN-γ production was not up-regulated (Fig. 2B). Collectively, these results support the proposition that ACEi promotes increased generation of innate kinin signals in the primary sites of infection, thereby stimulating adaptive immunity via the B₂R/IL-12 pathway.

**FIGURE 2.** Kinins link innate to adaptive immune responses. A. ELISA determination of IFN-γ production by popliteal LN T cells isolated from infected BALB/c male mice, following in vitro stimulation with soluble T. cruzi (epimastigote) Ag. The mice were pretreated (i.p.) with ACEi (●) or not (□), 1 h before paw injection of 1 × 10⁶ TCTs. Where indicated, the mice were pretreated (s.c.) with HOE-140. The data are representative of three independent experiments, with similar results. B. Ag-specific IFN-γ production by popliteal LN T cells isolated from infected B₂R+/−/− and B₂R−/−/− mice (n = 4 each) pretreated with ACEi (●) or not (□). Where indicated, the mice were pretreated 1 h before infection with HOE-140. Results are representative of two independent experiments with similar results. Statistical differences between mean values were evaluated by ANOVA, and pair-wise comparisons (represented by a and b) were done by the Tukey test (*, p < 0.01). ●, Mice pretreated with ACEi; □, mice pretreated with PBS.

**Generation of immunoregulatory kinins depends on cooperation of TLR2 and B₂R**

Considering that host resistance in mice infected with T. cruzi by the i.p. route depends on pathogen recognition by TLRs (24), we asked whether generation of immunoregulatory kinins in infected paw tissues might be subordinated to parasite-induced activation of TLR4 and/or TLR2 (25–29). As internal controls, separate groups of mice were injected with TCTs pretreated with VSPh, a potent irreversible inhibitor of the kinin-releasing cysteine protease, cruzipain (33). First, we tested whether interference with kinin homeostasis in TLR4+/+/+ (C3H/HePAS) and TLR4−/− (C3H/HeJ) mice resulted in modulation of IFN-γ production by Ag-specific T cells. ELISA data showed that the recall responses of both mice strains were significant, albeit of low intensity, when the TLR4+/+/+ or TLR4−/− mice were infected by TCTs in the absence of ACEi pretreatment. In contrast, LN-borne T cells from infected ACEi-TLR4+/+/+ or ACEi-TLR4−/− mice inoculated with TCT-VSPh failed to up-regulate
IFN-γ production (Fig. 3A), supporting the notion that the parasites generate kinins through the proteolytic activity of cruzipain. Thus, our results suggest that ACEi up-regulates type 1 immune responses by increasing the half-life of the innate kinin signals which cruzipain generates in the infected paw, without requirement for TLR4 signaling. We then compared the Ag-specific T cell responses of TLR2+/− mice infected with TCTs: in contrast to the strongly up-regulated IFN-γ production observed in ACEi-treated TLR2+/− mice, the production of the type 1 cytokine was reduced in ACEi-TLR2−/− mice (Fig. 3B). Notably, the reduced IFN-γ response of T cells from ACEi-TLR2−/− mice was not due to an intrinsic defect in the B2R signaling cascade because the production of the type 1 cytokine was rescued by inoculating trypomastigotes in suspensions containing exogenous bradykinin, while HOE-140 canceled these effects (Fig. 3B). These internal controls indicate that B2R signaling induced by provision of exogenous bradykinin to infection sites has compensated for the deficient adaptive response of TLR2−/− mice.
Similar to data obtained with TLR4+/+ mice, the injection of TCT-VSPH failed to up-regulate the type 1 response in ACEi-TLR2+/+ mice, thus further suggesting that generation of the innate kinin signal in peripheral tissues is dependent on cruzipain activity (Fig. 3B). Collectively, these data suggest that type 1 immune responses depend on cooperative activation of TLR2 and B2R, by mechanisms involving an intricate interplay of cruzipain and ACE at early stages of infection.

**TLR2 is required for overt activation of the kinin system by TCTs**

After showing that induction of type 1 immune responses depends on cooperative activation of TLR2 and B2R, we asked whether the parasite’s ability to activate the kinin system was dependent on TLR recognition of the pathogen. We first addressed this question by measuring the intensity of parasite-evoked paw swelling (kinin/B2R dependent) in mice displaying functional deficiency of TLR2 or TLR4. First, we compared the intensity of trypomastigote-evoked edema in ACEi-TLR4 WT mice (TLR4+/+, C3H/HePAS) vs TLR4-deficient mutant mice (TLR4−/−, C3H/HeJ). In both strains, trypomastigotes elicited weak but significant edema in the absence of ACEi (Fig. 4A) and the vascular reaction was drastically enhanced upon pretreatment with ACEi. Intriguingly, HOE-140 almost completely abolished the inflammation in ACEi-treated TLR4−/−, while partially reducing the anti-inflammatory effect in ACEi-TLR4+/+ mice (Fig. 4A). These results observed in TLR4−/− mice suggest that this strain may compensate TLR4 deficiency by dominantly engaging B2R-dependent pathways of inflammation. We then compared the magnitude of swelling in ACEi-treated WT TLR2+/+ vs TLR2−/− mice inoculated with TCTs (Fig. 4B). Paw swelling was markedly reduced in ACEi-TLR2−/− mice as compared with ACEi-TLR2+/+. This differential effect is not due to an impaired B2R function in the TLR2−/− mice because injection of synthetic bradykinin induced a prominent edema in such animals (Fig. 4B). Thus, our results suggest that parasite engagement of TLR2, but not TLR4, is mandatory for overt activation of the kinin pathway in the s.c. model of *T. cruzi* infection.

**Leakage of plasma supplies kininogens to infected peripheral tissues, thus allowing for cruzipain-mediated generation of kinins**

To investigate the mechanisms underlying plasma leakage evoked by trypomastigotes, we studied the dynamics of inflammation induced by topical application of the pathogen in the microvascular beds of the hamster cheek pouch. Addition of TCTs provoked significant leakage of FITC-dextran in ACEi-treated cheek pouch within 2–3 min of application (Fig. 5A), coinciding with early signs of PMN adherence to the endothelium (our unpublished observation). The microvascular permeability response induced by TCTs peaked at 15 min p.i., fading thereafter and reaching basal levels within 30–40 min. Similar to the B2R-driven edema responses which TCTs induced in ACEi-mice (Fig. 4), the vascular permeability responses in the ACEi-treated cheek pouch were canceled by HOE-140 (Fig. 5A). In addition, topically applied VSPh-TCTs only elicited weak microvascular leakage responses, suggesting that the parasite’s proedematogenic activity depends on generation of kinins by cruzipain (data not shown). To further investigate this possibility, we verified whether topical application of activated cruzipain could directly promote microvascular leakage in the ACEi-treated cheek pouch. Our results showed that activated cruzipain evoked minor vascular permeability increases (Fig. 5B). We thus reasoned that, in the “steady state,” the pouch extravascular tissues might not contain sufficient levels of endogenous kininogens, thus precluding release of significant levels of kinins by cruzipain. Indeed, by exogenously applying purified HK along with activated cruzipain into the ACEi-treated cheek pouch, we could record a massive increase in vascular permeability (Fig. 5B). Our results showed that activated cruzipain evoked minor vascular permeability responses in the same ACEi-treated pouch (Fig. 5B). Controls performed by consecutive applications of HK, either alone or combined with E-64-inactivated cruzipain, caused negligible vascular permeability responses in the same ACEi-treated pouch (Fig. 5B). To make sure that tissues were not exhausted at the end of these consecutive applications, the pouch was rechallenged with activated cruzipain and HK. Significant responses were again observed, and furthermore, HOE-140 blocked these effects (Fig. 5B). Together, these in vivo studies suggest that availability of plasma-borne kininogens in peripheral tissues is a prerequisite for release of kinins by cruzipain. To further investigate the proinflammatory role of cruzipain, we turned to the mouse model to compare the edemagenic responses of TCTs vs VSPh-TCT. As previously reported (24), our positive controls (Fig. 6A) showed that TCTs injected s.c. in ACEi-treated C57BL/6 mice induced a powerful paw swelling that was canceled by HOE-140. In contrast, VSPh-TCTs were unable to evoke a significant edema in ACEi-treated mice, supporting the notion that cruzipain enzyme activity is necessary for kinin generation in vivo. Next, we injected A

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**FIGURE 4.** TCTs evoke inflammation by stimulating cooperative activation of TLR2 and B2R. A. Paw edema (3 h) induced by injection of 1 × 10⁶ TCTs in the paw of TLR4+/+ and TLR4−/− mice. The mice were pretreated with ACEi (■), or not (□), 1 h before parasite injection. Where indicated, the mice were also pretreated with HOE-140. B. Edema (3 h) induced by injection of TCTs (10⁶) or 10 nM BK in ACEi-TLR2+/+ or ACEi-TLR2−/− mice. The results (means ± SD) are representative of three independent experiments (*n = 5*). Statistical differences between mean values were evaluated by ANOVA, and pair-wise comparisons (represented by a and b) were done by the Tukey test (*p < 0.01).
epimastigotes in the paw of ACEi mice and noted that these non-infective forms of *T. cruzi* failed to induce significant paw edema, irrespective of ACEi pretreatment (Fig. 6A). At first sight, this result seemed to be contradictory because epimastigotes express high levels of cruzipain (35). To further evaluate this issue, we injected purified cruzipain into ACEi-treated mice and found that, similar to cheek pouch assays, the protease did not induce significant paw swelling (Fig. 6A). Because epimastigotes or cruzipain per se are unable to activate the kinin system in vivo, we reasoned that tGPI-m and cruzipain may promote plasma leakage by activating PMNs (36), each step controlling cruzipain-mediated generation of vasoactive kinins.

**FIGURE 5.** Availability of kininogens in extravascular tissues is a limiting step controlling cruzipain-mediated generation of vasoactive kinins. A, Determination of mean number of FITC-dextran venular leaks evoked by multiple injections (numbers are given in brackets) of 1 × 10^8 TCTs, with 30-min intervals each, in the hamster cheek pouch tissues superfused by plasma-borne kininogens (cruzipain substrate) into peripheral tissues. The cheek pouch tissues are washed, after being exposed to the parasites. BK was added (250 nM) at 60 min as internal control (see arrow). B, Mean number of leaks in postcapillary venules of ACEi-treated cheek pouch tissues superfused with HEPES buffer. ACEi (1 μM captopril) was applied for 5 min, before application of TCTs. DTT, Data represent means ± SD of at least five independent experiments per point (n = 5).

Considering that tGPI-m is a TLR2 microbial signature expressed by tissue-culture trypanostigmates, but not by epimastigotes (26-28), we wondered whether the combined injection of tGPI-m and activated cruzipain may induce swelling, thus recapitulating the proinflammatory effects of TCTs. We used TLR4-def mice to exclude the possibility that traces of endotoxin eventually contaminating cruzipain could possibly interfere with our assay system. Control experiments run in the absence of ACEi showed that combined injection of tGPI-m and cruzipain produced only a minor edema in TLR4-def mice (Fig. 6B). However, a striking swelling was observed when we injected tGPI-m and cruzipain in ACEi-treated mice, while injection of tGPI-m or cruzipain alone had minor effects (Fig. 6B). Notably, the vigorous synergistic responses elicited by tGPI-m/cruzipain were canceled when ACEi-TLR4-def mice were pretreated with HOE-140 or when cruzipain was previously inactivated by the irreversible inhibitor E-64 (Fig. 6B). Notably, the potent edema that tGPI-m/cruzipain induced in ACEi-treated TLR2+/− mice was markedly attenuated in TLR2−/− mice (Fig. 6C).

**TLR2 and neutrophils are required for overt activation of the kinin system**

Awareness that tGPI-m drives neutrophil recruitment via TLR2 (36) led us to examine whether kinin system activation was subordinated to TLR2-mediated activation of PMNs. We investigated this possibility by treating TLR2+/− mice with specific anti-PMN Abs before inoculation of trypanostigmates. After 18 h, a single dose of ACEi was applied to the PMN-depleted mice, and the animals were subsequently challenged with TCTs. Infected animals pretreated with nonimmune serum developed an appreciable paw edema (13 ± 1 μl; n = 5; data representative of three independent assays) while PMN-depleted mice displayed negligible responses under these conditions (< 1 μl; n = 5). Consistent with these data, injection of tGPI-m/cruzipain failed to induce swelling in PMN-depleted BALB/c mice irrespective of presence of ACEi (our unpublished observation). Combined, these results suggest that tGPI-m and cruzipain may promote plasma leakage by activating PMN through mechanisms that may involve a long-distance "cross-talk" between TLR2 and B2R.

**TLR2-driven kininogen influx into peripheral tissues is the limiting step for B2R-dependent up-regulation of adaptive immunity**

Earlier in this work, we showed evidences that ACEi-treated TLR2−/− mice failed to up-regulate type 1 immune responses in response to TCTs (Fig. 3B). Internal controls showed that TLR2−/− mice do not have any intrinsic defect in B2R signaling because injection of exogenous BK along with TCTs restored the deficient type 1 immune responses of infected TLR2−/− mice (Fig. 3B). Because TCTs evoked a mild edema in the paw of ACEi-TLR2−/− mice (Fig. 4B), we reasoned that reduced influx of plasma-borne kininogens into the peripheral tissues probably accounted for the deficient phenotypes. To test this hypothesis, we sought to "bypass" the requirement for TLR2-dependent plasma leakage by injecting TCTs into ACEi-TLR2−/− mice along with exogenous HK. Our results showed that administration of exogenous HK to TCT suspension led to a drastic increase in IFN-γ levels produced by T cells from ACEi-TLR2−/− mice at day 10 p.i. (Fig. 7). Of note, HOE-140 abrogated the HK-dependent rescuing effect of TCTs. We used TLR4−/− mice to exclude the possibility that traces of endotoxin eventually contaminating cruzipain could possibly interfere with our assay system. Control experiments run in the absence of ACEi showed that combined injection of tGPI-m and cruzipain produced only a minor edema in TLR4−/− mice (Fig. 6B). However, a striking swelling was observed when we injected tGPI-m and cruzipain in ACEi-treated mice, while injection of tGPI-m or cruzipain alone had minor effects (Fig. 6B). Notably, the vigorous synergistic responses elicited by tGPI-m/cruzipain were canceled when ACEi-TLR4−/− mice were pretreated with HOE-140 or when cruzipain was previously inactivated by the irreversible inhibitor E-64 (Fig. 6B). Notably, the potent edema that tGPI-m/cruzipain induced in ACEi-treated TLR2+/− mice was markedly attenuated in TLR2−/− mice (Fig. 6C).
immunity observed in ACEi-TLR2−/− mice is restored by kinins liberated from the exogenously supplied kininogen through the activity of cruzipain. Thus, it appears that TCTs must activate TLR2/PMNs to drive the influx of blood-borne kininogens into the site of infection, thereby allowing for peripheral generation of kinins through the proteolytic activity of cruzipain (Fig. 8). Further downstream, the short-lived kinins act as maturation signals for DCs (12), thus linking innate to adaptive immunity.

Discussion

Our studies add substance to the understanding of molecular mechanisms underlying innate recognition of protozoan parasites. The s.c. infection model presented herein proposes the cooperation of TLR2 and B2R as signaling effectors of immunity. Our mechanistic studies revealed that TLR2 activation by microbial signatures present in tissue culture trypomastigotes promote diffusion of plasma-borne kininogens to the sites of infection, allowing for cruzipain-mediated liberation of kinin “danger” signals in peripheral tissues. Acting further downstream, endogenously released kinins stimulate IL-12 production by LN CD11c+ DCs via the B2R-signaling pathway, and mount a full-fledged type 1 adaptive response.

The evidence for an intertwined role of TLR2 and B2R as positive modulators of type 1 immunity in the s.c. model of infection contrasts with the relatively marginal role which TLR2 plays as an effector of innate immunity in mice infected by i.p. route (30). Of further interest, we recently found that B2R−/− mice infected by the i.p. route exhibit a highly susceptible phenotype (our unpublished observation). In this context, it has been recently documented that MyD88−/− mice infected with T. cruzi via i.p. also display heightened susceptibility to T. cruzi infection (30). Pertinently, it was shown that IFN-γ−/− mice infected by the i.p. route displayed a more accentuated parasitemia and mortality than MyD88−/− mice, suggesting that IL-12 and IFN-γ...
The finding that trypomastigotes evoked a weak edema in ACEi-TLR2<sup>-/-</sup> mice, along with evidence that these animals failed to up-regulate type 1 immunity, suggested that TLR2 triggering by the pathogen is critically required for full-fledge activation of the kinin system. Clues to understand the role of TLR2 in kinin system activation came from comparative analysis of the edematogenic responses induced by different developmental forms of <i>T. cruzi</i>. Unlike the potent responses induced by tissue-culture trypomastigotes, epimastigotes failed to evoke significant edema via the kinin/B<sub>2</sub>R pathway despite the fact that this avirulent form characterizes as potent TLR2 stimulators, being conspicuously expressed by tissue-culture derived trypomastigotes while absent in epimastigotes (27). Indeed, epimastigotes failed to up-regulate type 1 immune responses in TLR2<sup>-/-</sup>, irrespective of ACEi administration (data not shown), despite the high levels of cruzipain expressed by this parasite form. Considering that tGPI-m activate inflammatory phenotype of the tissue culture trypomastigotes was recapitated by injecting purified tGPI-m along with activated cruzipain in WT but not in TLR2<sup>-/-</sup> mice. Although we do not

![Figure 7](image.png)

**FIGURE 7.** Exogenous kininogen rescues type 1 cytokine production in TLR2<sup>-/-</sup> mice. Recall responses of LN T cells isolated from ACEi-TLR2<sup>-/-</sup> mice (<i>n</i> = 4/group) injected 10 days earlier with suspensions of TCTs (1 × 10<sup>6</sup>), or with the same inoculums of VSPh-TCTs, in the presence or absence of exogenous HK (50 μg/ml). Where indicated, the ACEi mice were pretreated with HOE-140 (s.c.). T cells isolated from draining LN (10 days p.i.) were stimulated in vitro with 25 μg/ml soluble <i>T. cruzi</i> Ag, and the IFN-γ levels in culture supernatants were quantified by ELISA. ACEi-TLR2<sup>-/-</sup> mice were used as positive controls. One control group consisting of noninfected ACEi mice was injected with HK alone. Results represent means ± SD of two independent experiments obtained by pooling cells from four mice per group. Statistics were done by ANOVA and pair-wise comparisons were done by the Tukey test (*, p < 0.01).

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claim here that tGPI-m is the only PAMP driving TLR2 activation by T. cruzi in vivo (25, 28), our data suggest that tGPI-m and cruzipain may act cooperatively, steering the activation of the kinin system via the TLR2-B2-R axis.

The critical involvement of neutrophils in the TLR2-B2-R axis is supported by data showing that paw edema induced either by TCTs was nullified in PMN-depleted mice. These results were consistent with intravascular microscopy observations in the hamster cheek pouch model indicating that leukocytes adhere to the endothelium within 1 min of trypomastigote application to the microvascular bed. Increased microvascular leakage was observed shortly thereafter, suggesting that resident sentinel cells (e.g., macrophages and/or mast cells) perhaps secreting TNF-α and/or chemokines (36) may promote endothelial/leukocyte adherence and drive plasma extravasation via CAP37/azurocidin (38). Thus, by controlling the influx of plasma-borne kininogens into peripheral tissues, TLR2/PMM may link the initial inflammatory responses elicited by the pathogen to subsequent kinin system activation, achieved through proteolytic release of kinins from interstitial kininogens, possibly through cruzipain (Fig. 8). In animals pretreated with ACEi, vasoactive kinins accumulate and thus intensify plasma extravasation through B2-R activation (14). In addition, production of reactive oxygen species via NAD(P)H oxidase (39) may up-regulate endothelial expression of TLR2. These conditions, if met, may sustain inflammation by promoting a transcellular “cross-talk” between B2-R and TLR2, similarly to mechanisms recently described for TLR4 and TLR2 (39).

The findings that ACEi-treated TLR2−/− mice fail to up-regulate type 1 immunity raised the possibility that DCs from this mouse strain “sense” TCTs via TLR2-independent pathways. Alternatively, the deficient phenotype of infected TLR2−/− may solely reflect impaired generation of kinin “danger” signals in peripheral tissues of such mice. Evidence in favor of the latter possibility was obtained upon injection of exogenous HK, along with trypomastigotes, in the paw of ACEi-treated TLR2−/− mice. This bypass maneuver rescued the defective type 1 immune response, suggesting that TLR2/PMM controls a limiting step governing the kinin-generation mechanism, i.e., the efflux of plasma-borne kininogens into peripheral sites of infection.

Previously, we have reported that exogenous kinins induce DC maturation through stimulation of their B2-R, and proposed that kinins may function as endogenous “danger” signals that induce TH1 polarization via the IL-12 pathway (12). Here, we validated these premises by interfering with kinin homeostasis through the administration of a single-dose of ACEi shortly before onset of s.c. T. cruzi infection. Our results show that IL-12 production by CD11c+ DCs is vigorously induced in a kinin/B2-R-dependent manner. This effect may reflect ACEi-dependent inhibition of the kinin-degrading activity of ACE (CD143) displayed by immature DCs (15), rendering these APCs hyperresponsive to kinin maturation through stimulation of their B2-R and vanilloid receptor signaling pathways (40), as this would indirectly provide “danger” signals for the innate immune system (20).

In conclusion, our analysis of the molecular mechanisms responsible for the endogenous generation of kinins in sites of infection appointed an intricate interplay of TLR2, B2-R, and ACE, here characterized as modulators of antiparasite immunity.

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Disclosures

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