Thromboxane A₂ is a key regulator of pathogenesis during Trypanosoma cruzi infection

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Chagas’ disease is caused by infection with the parasite Trypanosoma cruzi. We report that infected, but not uninfected, human endothelial cells (ECs) released thromboxane A₂ (TXA₂). Physical chromatography and liquid chromatography–tandem mass spectrometry revealed that TXA₂ is the predominant eicosanoid present in all life stages of T. cruzi. Parasite-derived TXA₂ accounts for up to 90% of the circulating levels of TXA₂ in infected wild-type mice, and perturbs host physiology. Mice in which the gene for the TXA₂ receptor (TP) has been deleted, exhibited higher mortality and more severe cardiac pathology and parasitism (fourfold) than WT mice after infection. Conversely, deletion of the TXA₂ synthase gene had no effect on survival or disease severity. TP expression on somatic cells, but not cells involved in either acquired or innate immunity, was the primary determinant of disease progression. The higher intracellular parasitism observed in TP-null ECs was ablated upon restoration of TP expression. We conclude that the host response to parasite-derived TXA₂ in T. cruzi infection is possibly an important determinant of mortality and parasitism. A deeper understanding of the role of TXA₂ may result in novel therapeutic targets for a disease with limited treatment options.

Chagas’ disease is caused by infection with the protozoan parasite Trypanosoma cruzi. This parasite has a complex life cycle that involves mammalian hosts and insect vectors (1). The vectors become infected after taking a blood meal from an infected host that has circulating, non-dividing, blood form trypomastigotes (BFT). The BFT transform into epimastigotes, which multiply extracellularly, inside the insect midgut and differentiate into nondividing metacyclic trypomastigotes that enter the host during the insect’s next blood meal. Trypomastigotes penetrate a variety of host cell types and multiply intracellularly as amastigotes (2). When amastigotes fill the host cell, they differentiate into BFT, which are released as the cell ruptures. The BFT invade adjacent tissues and/or spread via the lymphatic and circulatory systems to distant sites where they undergo further cycles of intracellular multiplication. In this way, humans maintain a parasitemia infective for vectors, thus completing the transmission cycle. T. cruzi may also be transmitted by other means such as by blood transfusion, laboratory accidents, organ transplantation, and from mother to fetus (3, 4).
10–30% of *T. cruzi*-infected individuals develop chronic symptomatic cardiomyopathy. Chagas cardiomyopathy is a major cause of mortality and disability in endemic areas of Mexico, Central, and South America (1) and is now recognized as an opportunistic infection in individuals with HIV/AIDS (5). *T. cruzi* infects many cell types in the cardiovascular system, including cardiac myocytes, cardiac fibroblasts, endothelial cells (ECs), and vascular smooth muscle cells. Infection results in acute myocarditis, associated with parasite pseudocysts, myonecrosis, focal ischemia, enhanced platelet activation/aggregation, and thrombus formation (1, 6). Infection also induces vasculitis in multiple vascular beds resulting in focal endothelial denudation and perturbation of the normal quiescent endothelial state associated with EC activation, loss of normal vascular tone, increased vasospasm, and reduced tissue perfusion (7–9). Chronic cardiomyopathy is associated with inflammation and fibrosis; however, few parasites are observed during the chronic phase.

Many of the sequelae associated with *T. cruzi* infection are reminiscent of the effects of the eicosanoid thromboxane A2 (TXA2). TXA2 is a potent proinflammatory agent that activates and facilitates cytokine production by monocytes (10). TXA2 perturbs the normal quiescent phenotype of ECs resulting in increased vascular permeability, increased adhesion molecule expression on ECs, and leukocyte adhesion to the vessel wall, important components of the inflammatory response. TXA2 also promotes platelet activation/aggregation and degranulation as a part of its prothrombotic properties. In smooth muscle cells, TXA2 promotes proliferation and migration contributing to neointima formation (11).

TXA2 is the most potent vasoconstrictor known, producing substantial narrowing of coronary arteries and resistance vessels and mediating cyclic coronary flow variants in experimental models of myocardial infarction and unstable angina (11–13). Indeed, thromboxane A2 receptor (TP) blockers and TXA2 synthase (TXA2S) inhibitors attenuate the effect of and the damage caused by ischemic injury and inflammation in several organs, including heart (11). TP expression and plasma levels of TP ligands are elevated, both locally and systemically, in several vascular and thrombotic diseases, including unstable angina, myocardial infarction, and various vasculopathies (11, 12, 14).

Previous studies have reported increased expression of proinflammatory cytokines (15), chemokines (16), vascular adhesion molecules (15), and endothelin-1 in *T. cruzi* infection both in vivo and in vitro (6, 8, 17). All of these factors promote inflammation and vascular injury (9), and many of these parameters result from or require the activation of TXA2 receptors for full effect (10, 18–20). The role of TXA2 in the pathogenesis of *T. cruzi* infection remains unexplored.

In the present study, we report that TP modulates host response to *T. cruzi* infection and that the parasite enzymatically metabolizes prostaglandin (PG) H2 to produce TXA2, which is biologically active and able to modulate host response. Parasite-derived TXA2 perturbs the normal endothelial state producing an inflammatory phenotype, denoted by increased intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM) expression. Moreover, infection of TP-deficient mice results in increased parasitemia and early death of the host. This was caused by the absence of TP in somatic cells which was replicated in vitro with ECs from TP-null mice displaying higher intracellular parasitemia than WT mice. This indicates that parasite-derived TXA2 is important in modulating disease pathogenesis in the absence of host-derived TXA2. These results indicate that TXA2 is an important factor in Chagas’ disease that controls parasite proliferation and the resulting inflammatory response to *T. cruzi* infection.

### RESULTS

**Infected HUVECs produce more TXA2 than uninfected HUVECs**

Eicosanoids are produced by multiple organisms in addition to mammals such as fungi, cestodes, trematodes, nematodes, and protozoans, including trypanomastids (21–25). The rate of TXA2 release from human umbilical vein endothelial cells (HUVECs) infected with the Brazil strain of *T. cruzi*, measured as the stable metabolite thromboxane B2 (TXB2), was sixfold higher than that from uninfected HUVECs (Fig. 1 A). Western blotting revealed a time dependent increase of TXA2S expression in infected HUVECs, a requirement for the synthesis of TXA2 (Fig. 1 B). ECs do not usually express TXA2S expression (26) (Fig. 1 D); thus, we hypothesized that intracellular amastigotes may be the source of the TXA2. The release of TXA2 from purified cultured trypomastigotes (Brazil strain) was ~5-fold greater than that from infected HUVECs (Fig. 1 C), and trypomastigote extracts contained ~10-fold more TXA2S than equivalent extracts (30 μg) from infected HUVECs (Fig. 1 D). The related, and more virulent, Tulahuen strain of *T. cruzi* also released TXA2, expressed TXA2S, and increased TXA2 release from infected HUVECs. Media from uninfected L929 cells used to grow the trypomastigotes, was devoid of TXA2 indicating that the release was not a contaminant from co-culture with another cell type. Thus, the majority of TXA2 released from infected HUVECs was most likely derived from the parasite.

**TXA2 is the main eicosanoid released during all life stages of *T. cruzi***

Because trypomastigotes could release TXA2 we determined which developmental stages of the parasite expressed TXA2. TXA2 release (Fig. 1 E) was highest in lysates of purified, extracellular amastigotes (90 pg TXA2/mg-protein). Epimastigotes (Epi) and trypomastigotes (Trp) exhibited similar TXA2 release (Trp, 40 pg/mg; Epi, 33 pg/mg). For comparison, TXA2 levels in amastigote lysates were approximately half those in platelet extracts (Fig. 1 E), indicating that the TXA2S content of amastigotes is substantial. Analysis by liquid chromatography-tandem mass spectrometry (LC-MS-MS) established the presence of TXB2, the stable hydrolytic product of TXA2, in lysates of *T. cruzi*. Identification was
based on matching retention times in two chromatographic systems with synthetic TXB₂. The prominent ions of diagnostic value obtained with synthetic TXB₂ included M/Z 169, 195, 307, 325, 333, 351, 369 (M-H), which matched prominent diagnostic ions in the lysates from Epi (Fig. 1 F) and Trp and establish its identity as TXB₂. These diagnostic ions were also present in T. cruzi–conditioned media but neither in naive nor L6E9-conditioned media.

Comparison with synthetic prostanoids revealed two clearly distinguishable prostanoid species in T. cruzi extracts. Based on the retention times, in addition to TXB₂, material also separated with prostaglandin F₂α (PGF₂α) (Fig. 2 A). T. cruzi extracts were able to catalyze exogenous [¹⁴C]-arachidonic acid (AA) to either TXA₂ or PGF₂α with TXA₂ production four-times greater than PGF₂α (Fig. 2 B). The conversion was enzymatic as neither product was found without enzyme or upon heat treatment of the lysates (100 °C, 1 h; n = 3). Thus, TXA₂ is the main eicosanoid produced by T. cruzi.

The only eicosanoid synthase previously reported in T. cruzi was old yellow enzyme (TcOYE), a potential PGF₂α synthase (27). To eliminate TcOYE as the putative TXA₂S we produced recombinant TcOYE and examined its biochemical properties. Recombinant TcOYE did not cross-react with an antibody against human TXA₂S (Fig. 2 C) nor did it result in TXA₂ biosynthesis (Fig. 2 D). These findings rule out TcOYE as the putative TXA₂S; however, recombinant TcOYE catalyzed PGF₂α biosynthesis (Fig. 2 D) confirming its previous identification as a PGF₂α synthase (27).

Parasite-derived TXA₂ is biologically active and produces changes in host cells consistent with the pathology observed in Chagas’ disease. Release of TXA₂ by the parasite indicated a potential to contribute to the pathogenesis of Chagas’ disease. The potential for parasite-derived TXA₂ to impact host physiology was examined using Ca²⁺ mobilization (Fig. 3 A). Exposure of TPα-expressing, indo-1–loaded CHO cells to freshly isolated parasite-conditioned media (orange line) resulted in robust Ca²⁺ release similar in kinetics to that observed with the TP ligand IBOP ([IS-1α,2α(Z),3β(1E,3S*),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) (black line). Moreover, this ligand-induced Ca²⁺ release was blocked by pretreatment of the cells with the TP blocker SQ29548 ([1S[1α,2α(Z),3α,4α]-7-[[2-[(phenylamino)
carbonyl[hydrazine[methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) (28) (blue line). No effect of SQ29548 was observed on Ca\(^{2+}\) handling in CHO cells (unpublished data). Thus, the parasite-derived TXA\(_2\) appears to affect host response through traditional mammalian TPs.

TXA\(_2\) release has a variety of consequences for a mammalian host, one of which is perturbation of the normally quiescent EC surface to produce an inflammatory state (20). A coculture system was used to examine whether parasite-derived TXA\(_2\) has functional consequences on the host vasculature (Fig. 3 B). We observed that a molecule secreted by trypomastigotes induced the expression of ICAM-1 and VCAM on HUVECs. This soluble mediator was as potent as TNF-\(\alpha\) (10 U/ml) and accounted for all the inflammatory properties of cocultures where the endothelium was infected by the parasite. Moreover, the perturbation of the HUVECs by this factor was ablated by the TP blocker, SQ29548, indicating that parasite-derived TXA\(_2\) was responsible (Fig. 3 B). These data indicate that direct infection of ECs by the parasite may be unnecessary to perturb the vascular system and manifest the changes observed during \textit{T. cruzi} infection.

Parasite-derived TXA\(_2\) accounts for the majority of circulating TXA\(_2\) in experimental \textit{T. cruzi} infection

Circulating TXA\(_2\) levels (measured as the stable metabolite TXB\(_2\)) in infected mice were measured to establish the contribution of parasite-derived TXA\(_2\) to disease pathogenesis. TXB\(_2\) was detectable in the plasma of WT mice (Fig. 4 A). Circulating plasma TXB\(_2\) levels in uninfected WT mice were very low (<1 ng/ml) with most of the TXB\(_2\) detected reflecting a minor activation of platelets during sampling. This value increased to 4.2 ng/ml in infected mice 14 d after infection and stabilized at around 2.5–3 ng/ml for the remainder of the time course examined. As expected, TXB\(_2\) levels were absent in plasma obtained from uninfected TXA\(_2\) synthase-null mice. Surprisingly, the rate of increase in circulating...
TXB₂ in TXA₂S-null mice was similar to that found in WT mice during the acute phase of infection (reaching 2.5 ng/ml after 14 d of infection; Fig. 4 A). At longer times (14–28 d) the amount of circulating TXA₂ was almost identical in the TXA₂S-null and WT mice. TXA₂S-null mice are incapable of generating TXA₂ (29), indicating that parasite-derived TXA₂ may account for up to 90% of the circulating levels in WT mice during acute infection. The mortality (Fig. 4 A, insert) and parasitemia (Fig. 4 B) of TXA₂S-null mice was similar to WT mice upon infection, indicating that parasite-derived TXA₂ can compensate for any deficits in host response derived from the inability of the host to produce TXA₂.

Inability of the host to respond to parasite-derived TXA₂ increases mortality and parasitemia during acute infection

To examine whether host response to parasite-derived TXA₂ was an important regulator in murine models of T. cruzi infection we used TP-null mice. Increased mortality was observed in both the C57/BL6 (Fig. 5 A) and BalbC (Fig. 5 B) strain of TP⁻/⁻ mice compared with strain-controlled WT mice. Plasma TXB₂ levels in infected WT and TP-null mice were similar (Fig. 5 C). TP-null mice display more severe cardiac pathology (Fig. 5, F–I) than their age/sex-matched WT counterparts. The myocardium of infected TP-null mice had increased inflammatory cell infiltrates (Fig. 5, F, H, and I) and myonecrosis (Fig. 5 I) compared with the myocardium of WT mice (Fig. 5 G). As the ensuing damage to the heart of infected TP-null mice is greater, we hypothesize that these cardiac anomalies are the cause of acute death in the TP-null mice.

The increased mortality in TP-null mice was accompanied by elevated levels of parasite pseudocysts in the myocardium (Fig. 5, F–I, black arrow). Quantitative PCR confirmed a 2.7-fold increase in parasite number in the myocardium of infected TP-null mice (Fig. 5 J), and the circulating levels of parasites were increased fourfold (Fig. 5 D). Inactivation of the TP gene in the TP-null mice was achieved by insertion of a neomycin resistance cassette (30). As such, TP expression is deleted in all cells. This results in deficits in both platelet (30) and monocyte activation (11, 31), both of which potently regulate parasite number (32–34). To dissect the role of TP on somatic cells versus cells engaged in innate immunity we transplanted BM cells into irradiated mice to repopulate the hematopoietic-derived cells and examined mortality after T. cruzi infection (Fig. 5 E). Transplantation of TP⁻/⁻ BM into WT-C57/BL6 mice (WT→knockout [KO]) resulted in a survival rate identical to both the normal C57/BL6 and C57/BL6 mice transplanted with WT marrow (WT→WT). Similarly, repopulation of the TP-null mice with WT BM (KO→WT) did little to decrease the higher mortality observed in TP-null mice recapitulated with TP⁻/⁻ BM (KO→KO). These data clearly indicate that although hematopoietic cells may be important in controlling T. cruzi infection, the expression of TP on and response of hematopoietic cells to TXA₂ is not a critical determinant of host survival or parasitism; however, TP presence on the somatic cells is a critical determinant of parasite replication.

The absence of the TP in somatic cells appears to be important in determining the mortality and parasitemia resulting from T. cruzi infection. To assess the validity of this hypothesis and the possible role of TP in the pathogenesis of disease we infected ECs derived from the TP-null and WT mice and followed intracellular parasite growth in vitro using Giemsa staining (Fig. 6 A) and quantitative PCR (Fig. 6 B) to determine the extent of intracellular parasitism. Over 72 h, the proliferation of intracellular amastigotes in TP⁻/⁻ ECs was clearly greater than in WT ECs by Giemsa staining, and the rate of proliferation was up to threefold higher as assessed by quantitative PCR. Reconstitution of TP expression into ECs from TP-null mice normalized proliferation rates (Fig. 6 B).
Figure 5. Failure of the host somatic cells to respond to T. cruzi–derived TXA2 results in increased parasitemia and enhanced mortality. (A and B) Survival curves for WT (○) and TP−/− (●) mice in both the C57BL6 (A) and BalbC (B) backgrounds after inoculation of 5 × 10^4 and 10^5 trypomastigotes of the Brazil strain, respectively. (C) Plasma TXB2 levels in infected WT and TP−/− mice (C57BL6 strain, day 21) showing equivalent TXB2 levels. (D) Parasitemia in the blood of WT and TP−/− mice (BalbC) 14 d after infection with 10^5 trypomastigotes (Brazil strain). (E) Adoptive bone marrow transfer of WT and TP−/− marrow into WT and TP−/− mice (C57/B6 strain). Mice were irradiated to kill BM and unfractionated marrow transplanted to change the phenotype of the hematopoietic cells. Repopulation of WT and TP-null mice with marrow from the same genotype (WT→WT; Ko→Ko) did not change the susceptibility to death compared with uninstrumented animals. Transfer of TP-null marrow into WT mice (WT→Ko) did not alter their survival in response to infection, nor did transfer of WT marrow into TP-null mice (Ko→WT). Results with non-irradiated WT mice are also shown. This data indicates that survival is dependent on TP expression in somatic cells and not cells of the hematopoietic lineage (including platelets and monocytes). [F–I] Representative sections of the myocardium of TP-null (F) and WT (G) mice stained with hematoxylin and eosin. High power view of the hearts of TP−/− mice indicating extensive inflammation (H) and myonecrosis (I). Black arrows denote parasite pseudocysts located in the myocardium. (J) Quantitative PCR indicating parasite levels in the myocardium of WT and TP−/− mice. Data (mean ± SD) are derived from at least five mice per group. Data (mean ± SD) are from three individual experiments. * indicates significance (P ≤ 0.005) from control (WT) group. Bars, 125 μm.
**Figure 6.** Gαq signaling by host TP in response to parasite-derived TXA2 suppresses intracellular parasite growth. (A and B) Overnight infection of EC from T. cruzi (black bars) C57/BL6 mice with trypomastigotes (Brazil strain, MOI 2:1) results in a rate of intracellular parasite growth three times greater than that in EC from WT (white bars) mice by Giemsa staining (A) and PCR (B). Bars, 20 μm. Reconstitution of murine TP expression in TP−/− ECs (black bars) normalizes the number of amastigotes per cell (B) indicating host response to parasite-derived TXA2 is an important regulator of parasite proliferation. (C) Examination of pathways involved in the suppression of intracellular parasite proliferation by host TP. Reconstitution of ECs from TP-null mice with either human isoform, TPα, or TPβ, or a truncation mutant containing only the common domain, TP328, suppress parasite growth in vitro. However, two TPα mutants devoid of Gαq coupling, R60L and R130V, did not suppress parasite growth indicating the mechanism is mediated by Gαq activation. Examination of ERK activation by immunoblotting supports the role of these two residues in Gαq activation. Data (mean ± SD) and autoradiographs are from three individual experiments. * and # indicate significance (P ≤ 0.005) from control (WT) and TP−/− groups, respectively.

These data indicate that TP signaling by host cells directly affects the rate of intracellular amastigote growth and suggests a role for TP-induced signaling pathways in the control of parasitism during T. cruzi infection.

To determine the signaling pathway(s) involved we reconstituted ECs from the TP-null mouse with each of the human isoforms, TPα and TPβ, a truncation mutant devoid of the intracellular tail, TP328, and two point mutants, TPα-R60L and TPα-R130V, that cannot activate Gαq-dependent pathways (Fig. 6 C). ELISA assay, using an antibody derived against the NH2 terminus of human TP, indicated that expression of all constructs was not significantly different to endogenous TP expression in WT ECs. Compared with TP-null EC expression of TPα, TPβ and TP328 suppressed parasite growth in vitro (Fig. 6 C). However, the two TPα point mutants were unable to suppress parasite growth, indicating the response was linked to the activation of Gαq. To confirm signaling through Gαq was compromised in the TPα-R60L– and TPα-R130V–reconstituted cells, we examined ERK activation in response to IBOP (100 nM). ERK activation, as measured with a phospho-specific antibody, was enhanced in response to IBOP in the TPβ–, TPα–, and TP328–expressing cells but not in the TP-null, TPα-R60L–, and TPα-R130V–expressing ECs, indicating Gαq coupling was truly compromised in these cells. The suppression of parasite growth is mediated through Gαq signaling downstream of TP and indicates that TP signaling and not TP expression itself is responsible for the control of parasite proliferation in somatic cells during T. cruzi infection.

**DISCUSSION**

Eicosanoids, many of which are vasoactive, are produced by several parasitic organisms (22–25, 35) and represent ideal modulators of vasculopathy during infection. In addition to vasospasm, TXA2 mediates many of the other pathophysiological features of chagasic cardiomyopathy. In this study we documented, using ELISA, TLC, and LC-MS-MS, that the parasitic protozoan T. cruzi releases TXA2. TXA2 is the predominant prostanoid released by the parasite. However, TXA2 is not the only bioactive lipid produced. T. cruzi also produce and release PGF2α, and we have confirmed that TcOYE, the only eicosanoid synthase identified to date in T. cruzi, is a PGF2α synthetase. In addition, we established that TcOYE does not biosynthesize TXA2. Both LC-MS-MS and [14C]-AA conversion/labeling assays have identified other potential bioactive lipids released from T. cruzi (unpublished data), many of which are unidentified, that could also play a role in the pathogenesis of T. cruzi infection.

TXB2 levels are elevated in mice infected with T. cruzi (36). The present results identify TXA2 as a parasite-derived molecule that modulates survival and disease progression. T. cruzi–derived TXA2 elicits a robust response from host cells and influences host cell activation. TP-null mice had an increased mortality and their coronary artery ECs had a higher intracellular parasitism compared with controls. It is clear that TXA2 plays a prominent role in the acute stages of the disease; however, the primary issue has been the belief that the enhanced TXA2 levels manifest through a host response to infection, such as inflammation and platelet activation. Results from the TXA2−/− mice clearly identify the parasite as the source of up to 90% of the circulating TXA2 in experimental Chagas’ disease. In addition, it suggests that parasite-derived
TXA2 is sufficient to stimulate host TP to ensure normal disease progression, parasitemia, and host survival.

The pathology of the T. cruzi–infected TP-null mice indicates that the biosynthesis and release of TXA2 by the parasite may have multilevel actions in the host (Fig. 7). On one hand, the resulting TP activation on host cells initiates a signaling cascade that controls parasite growth and permits parasite replication at a rate that enables continued survival of the host (Figs. 5 and 7). In addition, the response of the host to parasite-derived TXA2 appears to be largely anti-inflammatory, with the WT mice displaying minimal pathology (Fig. 5). The combination of these effects allows for a balance between the needs of the parasite (proliferation and survival through evading the host immune response) and the host (to survive the initial infection and largely limit collateral damage to organs during acute infection). These findings appear counterintuitive at first. The actions of TXA2 are normally considered proinflammatory as it enhances monocyte activation and cytokine production (10), leukocyte adhesion molecule expression on ECs (20), and vascular permeability (37, 38). However, the dramatic antiinflammatory effect of TXA2 may result from the suppression of NFKB activation by other inflammatory mediators in the more complex setting of T. cruzi infection in vivo. We previously demonstrated that TP stimulation inhibits the proinflammatory effects of TNF-α via a Gαq-mediated mechanism (20). The present study is the first in vivo demonstration of the anti-inflammatory properties of TP stimulation and builds on our previous observations on the role of TXA2 as an inflammatory regulator.

Other host responses elicited by parasite-derived TXA2 account for many of the substantial physiological changes that occur during the acute stage of T. cruzi infection (Fig. 7). TXA2 is a potent vasoconstrictor due in no small part to its inhibition of endothelial-derived vasorelaxants, such as nitric oxide (11, 39) (Fig. 7). TXA2 is also a potent stimulus for vascular smooth muscle cell proliferation and migration (11) which explains the proliferative phenotype observed in the arteries from infected mice (40) (Fig. 7). TXA2 increases vascular permeability (37, 38) and promotes platelet activation/aggregation (11) (Fig. 7) which may explain the enhanced adhesiveness of platelets to ECs and the enhanced reactivity of platelets from infected mice (36). Finally, TP activation is a direct contributor to tissue damage in multiple organs, including heart (11), which could increase the degree of cardiac myocyte apoptosis and result in the increased likelihood of a
dilated cardiomyopathy and progression to heart failure, a major cause of death in patients with this disease (Fig. 7).

In addition to the maelstrom of changes during the acute phase, the ongoing secretion of TXA₂ may also impact chronic T. cruzi infection. During chronic disease T. cruzi are morphologically absent in most tissues; however, molecular techniques demonstrate considerable levels of parasites in the blood (41) and multiple organs, including eye (42), adipose (43), connective tissue (44), and heart (45). In general, parasite burden during the acute phase correlates well with persistence during the chronic stage (44). It has been proposed that tissue-resident parasites sustain the infection and produce the ongoing systemic symptoms (46, 47). Indeed, it is possible that chronic exposure to high levels of T. cruzi–derived TXA₂ may produce many of the clinical manifestations that are characteristic of chronic Chagas’ disease. For instance, changes to protein expression profiles in the chronically infected myocardium indicate substantial oxidative stress and ongoing apoptosis (48–50). TXA₂ has been linked to the induction of myocardial apoptosis and increases oxidative stress in tissues (11). In the retina, TP are expressed on multiple cell types including photoreceptors and ECs (51). The production of TXA₂ by parasites in the orbit may therefore directly contribute to the retinal dysfunction and decreased vascularization observed in chronic Chagas’ disease (52). Thus, the persistence of TXA₂–producing parasites in multiple tissues may directly contribute to the development of the clinical symptoms observed in chronic Chagas’ disease.

Parasite-derived TXA₂ may also inhibit the host’s adaptive immune response enhancing progression to the chronic disease (Fig. 7). Chronic infection of mice with this parasite attenuates dendritic and T cell functions (53) and reduces the number of IFN-γ-producing CD8⁺ T cells through selective loss of immature thymocytes (54–56). TP stimulation negatively regulates dendritic cell–T cell interactions preventing maturation and antigen presentation (57) and induces apoptosis in immature thymocytes (58) indicating parasite–derived TXA₂ may mediate the loss of T cell function and number. Immature B cells are also targeted for apoptosis by an unknown agent responsible. Thus, TXA₂ release by the parasite may be responsible for restructuring the number and function of cells involved in acquired immunity during chronic T. cruzi infection.

It is well known that several parasitic species produce PGs (23, 25, 35) but the contribution of these modulators to disease pathogenesis remains largely unexplored. If parasite PGs act as immunoregulatory agents for the host, similar to TXA₂, then our present observation may have larger implications by highlighting a common mechanism used by intracellular parasites to affect host response. This data potentially clarifies the mechanism behind the previously unexplained clinical phenomena that cyclooxygenase inhibition enhances mortality rates in patients (60, 61). In this case, these pharmacological agents that regulate TXA₂ generation or responsiveness would essentially mimic the TP-null mice enhancing parasitemia and mortality rates.

TXA₂ biosynthesis and release were observed in all three life stages of T. cruzi. It is clear that in the infectious forms of the parasite, multiple functions exist for this bioactive lipid. The most important may be to control parasite proliferation and ensure further transmission of the parasite through continued survival of the host (Fig. 6). Collectively, the enhanced mortality observed in TP-null mice, suppression of amastigote proliferation by host TP activation, and elevated expression of TXA₂S in amastigotes (intracellular forms) suggest the intensity of TP signaling may act as a quorum sensor for the parasite. A cell with few intracellular amastigotes releases little parasite-derived TXA₂ providing few signals from host TP that regulate growth. Paracrine TP signaling on the host cell surface would increase proportionally to amastigote number, with the intensity of TP activation indicating the number of intracellular amastigotes. This environmental cue eventually exceeds a threshold which slows amastigote proliferation and initiates differentiation into the trypomastigote stage in preparation for another round of infection. This mechanism is self-contained and operates independently of host physiology, such as the level of activation of the host immune response. The most likely mediator of these effects downstream of TP is Gₛₐ₃ (Fig. 6) and little is known of the role of this important signaling pathway in T. cruzi infection.

The sensing mechanism is not a direct effect of TXA₂ on parasite physiology as TXA₂ mimetics added to epimastigotes has little effect on parasite growth (unpublished data), and only the absence of host TP causes deranged parasite proliferation. Thus, the proposed mechanism is unique as it intertwines host biochemistry and parasite biology as opposed to other agents where receptors on the parasite direct behavior. Another question is what advantage is afforded to the epimastigote by TXA₂ release? In organisms with insect vectors it has been hypothesized that parasite-derived PGs suppress immunity and permit the chronic habitation of the vector. TXA₂ has already been shown to inhibit acquired immunity in mice (57) and may also act to protect the parasite in a similar fashion in the insect. Moreover, TXA₂ may aid in the colonization of the gut by producing mucosal injury (62) and increase the potential spread of the parasite through increasing gut motility (63).

In conclusion, our results demonstrate for the first time that T. cruzi–derived TXA₂ is an important modulator of survival and disease progression in mammalian host and functions, through the activation of TP in somatic cells, as a quorum sensor to control parasite proliferation and parasitemia in vivo. These findings advance our understanding of host–parasite relationships and reveal a potential new avenue for pharmacological treatment for a disease with few therapeutic options.

MATERIALS AND METHODS

Materials. Sterile plasticware was purchased from Costar (Cambridge, MA). Tissue culture materials and reagents, excluding pooled human serum (Gemini Bio-Products Inc.), were from Invitrogen. The TXA₂ mimetic IBOP and the TP blocker SQ29548 were obtained from Cayman Chemical. All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.
Mice and BM transplantation studies. TP-null (both C57/BL6 and Balb/C) and TXAS-null (C57/BL6) mice were obtained from Dr. Thomas Coffman (Duke University) and Dr. Kenneth Wu (University of Texas Health Science Center), respectively. WT, TP-, and TXAS-null mice were bred in house. BM transplants were performed as previously described (64). In brief, recipient mice (6 wk old C57/Bl6) received 9 Gy of γ-radiation, administered as a split dose, after which 5 × 10^8 cells of WT or TP−/− bone marrow was injected into the tail vein. Bone marrow was obtained from the femurs of male WT and TP-null mice by flushing the marrow cavity with HBSS, the resulting suspension dissociated using a 25-gauge needle. Male mice were infected 28 d later with the Brazil strain of T. cruzi by intraperitoneal route at the inoculums indicated. Mortality was monitored daily and blood drawn for the determination of parasitemia at the intervals stated. All animal experiments were performed under protocols approved by the Institutional Animal Care and use Committee of the Albert Einstein College of Medicine.

Parasites. The Brazil and Tulahuen strains of T. cruzi were maintained in C3H/HeJ and A/J mice (Jackson Laboratories), respectively. In vitro experiments used both the Brazil and Tulahuen strains. No significant differences were observed between the two strains regarding infection and growth in ECs. Epimastigotes and trypomastigotes were propagated, and extracellular amastigotes were produced as previously described (65).

Isolation of primary human and murine ECs. The isolation and expansion of HUVECs was performed as previously described (20). HUVECs at passage 1 were used for the present study. Primary murine ECs were isolated from the hearts of 6-8-wk-old WT and TP-null mice. After excision, hearts were minced into pieces and digested until completion with collagenase (100 U/ml in Hank’s balanced salt solution). Magnetic particles (Imag; BD Biosciences) were coated with saturating amounts of G. simplicifolia isolectin B4 (Sigma-Aldrich) and added to the resulting cell suspension. Cells and beads were incubated for 30 min at 4°C, and ECs were isolated using a magnetic field and cultured on fibronectin-coated plates.

The R^KO1/2 mutant was a gift from Dr. Valeria Capra (University of Milan, Milan, Italy). Other TP mutants were cloned using PCR-based site-directed mutagenesis (Quick change II kit; Stratagene). Transfection of various TP constructs into TP-null ECs was performed using the GenePorter reagent (Gene Therapy Systems) and resulted in transfection rates of ≥70%. Stable expression was obtained under antibiotic selection (G418, 200 μg/ml). Transfection of various TP constructs into TP-null ECs was performed using the GENEporter reagent (Gene Therapy Systems) and resulted in transfection rates of ≥70%. Stable expression was obtained under antibiotic selection (G418, 200 μg/ml). To maintain the integrity of the EC phenotype cells were used from passage 1–4. Passage 1 and 4 ECs are comparable in appearance, CD31 expression, and rate of migration and differentiation. TP expression was determined using cell surface ELISA assay using an antibody developed in house against the NH2 terminus of the human TP.

Infection of HUVECs and primary mouse EC cultures. To examine the potential host response to parasite-derived TXA2 and its pathological role in experimental T. cruzi infection we monitored TXA2 release from ECs (HUVECs) and the proliferation of parasites in ECs derived from WT and TP-null mice with or without reconstitution of murine TP expression. All ECs were infected with trypomastigotes of the Brazil and Tulahuen strains, harvested as previously described (65), at a multiplicity of infection (MOI) of 2:1. TXA2 release was monitored by ELISA (see Identification of TXB2– section) 48 h after infection. Parasitism was followed by PCR, and Giemsa staining.

Quantification of intracellular parasitism. Quantitative real-time PCR was used to determine the number of parasites/cell using the T. cruzi forward and reverse primers as previously described (43). A parallel reaction using murine-specific microglobulin primers (β2F2 and β2R2) was performed to normalize the amount of mouse DNA present, e.g., number of cells, in each analysis (43). A standard for murine β2-microglobulin concentration was developed from a serial dilution of DNA (PCR product murine genomic DNA). The mean number of parasites per cell was calculated by dividing the number of parasites (copy number of T. cruzi DNA obtained by real-time PCR) by number of cells (number of copies of B2-microglobulin).

Identification of TXB2 by LC-MS-MS, ELISA, and TLC. Crude cell extracts for analysis were prepared from washed parasites (epimastigotes, trypomastigotes, and amastigotes) or human platelets. Cells were resuspended at 10^9 cells/ml in lysis buffer (20 mM Tris-HCl, pH 7.5; mM DTT plus protease inhibitors), passed through a 26-gauge needle, homogenized by six strokes in a Dounce homogenizer, and sonicated three times for 15 s each to ensure complete lysis. Before further analysis lysates were clarified by centrifugation (10 min, 17,000 g).

For LC-MS-MS, samples were prepared for analysis by solid-phase extraction using C-18 SPE 500-mg columns (Alltech). LC-MS-MS identification was acquired with a LCQ quadrupole ion trap spectrometer system equipped with an electrospray ionization probe (ThermoFinnigan). Samples were suspended in mobile phase and injected into the HPLC component, which consisted of a SpectraSYSTEMS P4000 (ThermoFinnigan) quaternary gradient pump, with a LUNA Prodigy ODS (3) (100 × 2 mm, 5 μm) column (Phenomenex). The column was eluted at a flow rate of 0.2 ml/min with methanol/water/acetic acid (65:34:99.01, vol/vol/vol) from 0 to 8 min, and then a gradient increasing to 100% methanol from 8.01 to 30 min. Synthetic TXB2 was purchased from Cayman Chemical. TXB2 (the stable hydrolytic product of TXA2) was measured by ELISA (GE Healthcare) according to manufacturer’s instructions.

TLC was performed as in reference 66 on parasite lysates extracted with ethyl ether/methanol/0.2 M acetic acid (30:4:1, vol/vol) using the indicated PG standards (5 μg of each) as a marker. Conversion of [3H]-labeled AA was performed using 100 μl of cell extract and 1 Gbq [3H]-AA in 100 mM sodium phosphate (pH 7.0), 2 μM hematin, and 5 mM triphenyl at 37°C. The reaction was quenched by addition of 100 mg/ml stannous chloride in 1 N HCl and analyzed by TLC. The nonenzymatic conversion of AA was examined in the absence of T. cruzi lysates (“no enzyme control”) or by preincubating lysates at 100°C for 10 min to destroy all enzymatic activity (“heat treated control”).

PCR, cloning, and expression of TcOYE. The TrcOYE open reading frame was ampliﬁed using platinum PCR super mix (Invitrogen) and the gene-speciﬁc sense (5′-CGGAATTCTGATGGCGTATCTGAACTCGT-3′) and antisense (5′-CCGGTCGAGTATTGGTTGTCGTCGGTA-3′) primers. The PCR product was cloned into pGEX-4T-1 expression vector (GE Healthcare) using EcoRI and XhoI restriction sites. Recombinant TrcOYE was isolated from Rosetta BL21 E. coli (Novagen) using glutathione (GSH)–Sepharose 4B resin (GE Healthcare) after overnight induction with 1 mM IPTG at 30°C. The recombinant enzyme was separated from the GST component by thrombin digestion while bound to the resin.

Co-culture of HUVECs and trypomastigotes and expression of leucocyte molecules. Transwell inserts in 24-well plates were used to establish the EC-trypomastigote co-culture model. Fibronectin-coated Transwell inserts (0.4 μm pore size; Corning-Costar) were seeded with 3 × 10^5 HUVECs, incubated with trypomastigotes for 24 h with mild agitation to ensure the trypanosomatids remained in suspension. ICAM and VCAM expression were determined by cell-based ELISA as previously described (20). Trypanosomatids were included in the upper chamber (2 × 10^5 per insert) or the lower chamber (4 × 10^5 per insert) in complete media. Media alone and TNF-α (100 U/ml) served as negative and positive controls, respectively. For antagonism of TP on HUVECs, SQ29548 (5 μM) was included in the upper and lower chambers.

Immunoblotting. Whole cell lysates (30 μg protein per lane) were separated by SDS-PAGE under reducing conditions and transferred onto PVDF membrane. Immunoblotting was performed as previously described (26) using antibodies against human TXAS (Cayman Chemical), α-tubulin (Sigma-Aldrich), total and phosphorylated ERK1/2 (Cell Signaling Technology).
Ca\textsuperscript{2+} imaging in CHO cells. CHO cells with stable expression of the α-isofrom of human TP were incubated for 45 min at 37°C with the Ca\textsuperscript{2+} indicator Indo-1 AM (10 μM; Molecular Probes). After harvesting, cells were suspended in Tyrode's solution (mM: 137.0 NaCl; 4.0 KCl; 0.5 MgCl\textsubscript{2}; 2.0 CaCl\textsubscript{2}; 24.0 NaHCO\textsubscript{3}; 1.8 NaH\textsubscript{2}PO\textsubscript{4}; 5.5 glucose; 5.0 Hepes, pH 7.2) at 5 × 10\textsuperscript{5}/ml. Mobilization of intracellular Ca\textsuperscript{2+} was induced by addition of either 100 nM IBOP or 0.2 μm fltered, conditioned media (200 μl) from parasite cultures to the suspended CHO cells. The conditioned media used as the stimulus was fltered directly into the cassette containing labeled CHO cells because of considerations of the aqueous half-life of TXA\textsubscript{2}. This process minimized the degradation of secreted TXA\textsubscript{2}, as the time elapsed for the emission fiux was measured by spectroflourometry using standard excitation and emission flters for Indo-1.

Statistical analysis. Data were pooled and statistical analysis was performed using the Mann–Whitney U-test using Sigma Stat Version 2.0 (SPSS).

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