Thromboxane A_2 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 A_2 (TXA_2). Physical chromatography and liquid chromatography–tandem mass spectrometry revealed that TXA_2 is the predominant eicosanoid present in all life stages of *T. cruzi*. Parasite-derived TXA_2 accounts for up to 90% of the circulating levels of TXA_2 in infected wild-type mice, and perturbs host physiology. Mice in which the gene for the TXA_2 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 TXA_2 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 TXA_2 in *T. cruzi* infection is possibly an important determinant of mortality and parasitism. A deeper understanding of the role of TXA_2 may result in novel therapeutic targets for a disease with limited treatment options.

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Abbreviations used: AA, arachidonic acid; BFT, blood form trypomastigote; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; I-BOP, [1S-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; ICAM, intercellular adhesion molecule; KO, knockout; LC-MS-MS, liquid chromatography-tandem mass spectrometry; PG, prostaglandin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SQ29548, [1S[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl] hydrazine]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TcOYE, *T. cruzi* old yellow enzyme; TP, TXA_2 receptor; Trp, trypomastigotes; TXA_2, thromboxane A_2; TXA_2S, TXA_2 synthase; TXB2, thromboxane B_2; VCAM, vascular cell adhesion molecule.

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, nondividing, 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).

A.W. Ashton and S. Mukherjee contributed equally to this work.

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10–30% of *T. cruzi*–infected individuals develop chronic symptomatic cardiomyopathy. Chagasic 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 A_2 (TXA_2). TXA_2 is a potent proinflammatory agent that activates and facilitates cytokine production by monocytes (10). TXA_2 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. TXA_2 also promotes platelet activation/aggregation and degranulation as a part of its prothrombotic properties. In smooth muscle cells, TXA_2 promotes proliferation and migration contributing to neointima formation (11).

TXA_2 is the most potent vasoconstrictor known, producing substantial narrowing of coronary arteries and resistance vessels and mediating cyclic coronary flow variations in experimental models of myocardial infarction and unstable angina (11–13). Indeed, thromboxane A_2 receptor (TP) blockers and TXA_2 synthase (TXA_2S) 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 TXA_2 receptors for full effect (10, 18–20). The role of TXA_2 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) H_2 to produce TXA_2, which is biologically active and able to modulate host response. Parasite-derived TXA_2 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 TXA_2 is important in modulating disease pathogenesis in the absence of host-derived TXA_2. These results indicate that TXA_2 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 TXA_2 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 TXA_2 release from human umbilical vein endothelial cells (HUVECs) infected with the Brazil strain of *T. cruzi*, measured as the stable metabolite thromboxane B_2 (TXB_2), was sixfold higher than that from uninfected HUVECs (Fig. 1 A). Western blotting revealed a time dependent increase of TXA_2S expression in infected HUVECs, a requirement for the synthesis of TXA_2 (Fig. 1 B). ECs do not usually express TXA_2S (26) (Fig. 1 D); thus, we hypothesized that intracellular amastigotes may be the source of the TXA_2. The release of TXA_2 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 TXA_2S than equivalent extracts (30 μg) from infected HUVECs (Fig. 1 D). The related, and more virulent, Tulahuen strain of *T. cruzi* also released TXA_2, expressed TXA_2S, and increased TXA_2 release from infected HUVECs. Media from uninfected L_6E_9 myoblasts, the feeder cells used to grow the trypomastigotes, was devoid of TXA_2 indicating that the release was not a contaminant from coculture with another cell type. Thus, the majority of TXA_2 released from infected HUVECs was most likely derived from the parasite.

TXA_2 is the main eicosanoid released during all life stages of *T. cruzi*

Because trypomastigotes could release TXA_2 we determined which developmental stages of the parasite expressed TXA_2. TXA_2 release (Fig. 1 E) was highest in lysates of purified, extracellular amastigotes (90 pg TXA_2/mg-protein).

Epimastigotes (Epi) and trypomastigotes (Trp) exhibited similar TXA_2 release (Trp, 40 pg/mg; Epi, 33 pg/mg). For comparison, TXA_2 levels in amastigote lysates were approximately half those in platelet extracts (Fig. 1 E), indicating that the TXA_2S content of amastigotes is substantial. Analysis by liquid chromatography-tandem mass spectrometry (LC-MS-MS) established the presence of TXB_2, the stable hydrolytic product of TXA_2, in lysates of *T. cruzi*. Identification was

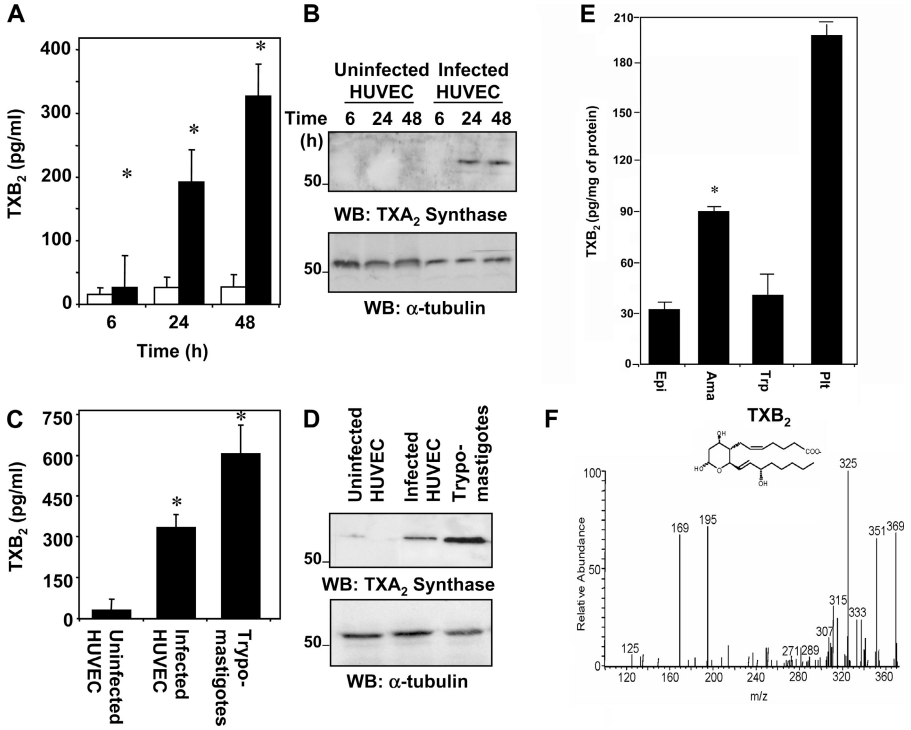

Figure 1. *T. cruzi* infection increases TXA_2 release through the liberation of parasite-derived TXA_2. (A and B) TXB_2 content of conditioned media (A) and TXA_2S expression (B) from uninfected (white bars) and *T. cruzi* infected (black bars) HUVECs (30 μg protein per lane). (C and D) Release of TXA_2 by uninfected and *T. cruzi*–infected HUVECs and extracellular trypomastigotes (10^7 cells) into conditioned media (C), and the expression of TXA_2S (D) was measured by ELISA and immunoblotting, respectively. (E) TXB_2 content of lysates from epimastigotes (Epi), extracellular amastigotes (Ama), and trypomastigotes (Trp) was measured by ELISA. Extracts from the same cells were also probed for TXA_2S by immunoblotting. Platelet extracts (Plt) were used as a source of comparison for TXA_2 release. (F) Identification of TXB_2 content in epimastigotes lysates using LC-MS-MS (as described in Materials and methods) using synthetic TXB_2 as a reference. Data (mean ± SD) and blots are from at least three experiments. Numbers to the side of immunoblots indicate migration of molecular weight standards (kD). * indicates significance ($P \leq 0.005$) from uninfected HUVECs and other *T. cruzi* life stages.

based on matching retention times in two chromatographic systems with synthetic TXB_2. The prominent ions of diagnostic value obtained with synthetic TXB_2 included M/Z169, 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 TXB_2. These diagnostic ions were also present in *T. cruzi*–conditioned media but neither in naive nor L_6E_9-conditioned media.

Comparison with synthetic prostanoids revealed two clearly distinguishable prostanoid species in *T. cruzi* extracts. Based on the retention times, in addition to TXB_2, material also separated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Fig. 2 A). *T. cruzi* extracts were able to catalyze exogenous [^{14}C]-arachidonic acid (AA) to either TXA_2 or $PGF_{2\alpha}$ with TXA_2 production four-times greater than $PGF_{2\alpha}$ (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, TXA_2 is the main eicosanoid produced by *T. cruzi*.

The only eicosanoid synthase previously reported in *T. cruzi* was old yellow enzyme (TcOYE), a potential $PGF_{2\alpha}$ synthase (27). To eliminate TcOYE as the putative TXA_2S we produced recombinant TcOYE and examined its biochemical properties. Recombinant TcOYE did not cross-react with an antibody against human TXA_2S (Fig. 2 C) nor did it result in TXA_2 biosynthesis (Fig. 2 D). These findings rule out TcOYE as the putative TXA_2S; however, recombinant TcOYE catalyzed $PGF_{2\alpha}$ biosynthesis (Fig. 2 D) confirming its previous identification as a $PGF_{2\alpha}$ synthase (27).

Parasite-derived TXA_2 is biologically active and produces changes in host cells consistent with the pathology observed in Chagas' disease. Release of TXA_2 by the parasite indicated a potential to contribute to the pathogenesis of Chagas' disease. The potential for parasite-derived TXA_2 to impact host physiology was examined using Ca^{2+} mobilization (Fig. 3 A). Exposure of TPα-expressing, indo-1–loaded CHO cells to freshly isolated parasite-conditioned media (orange line) resulted in robust Ca^{2+} mobilization similar in kinetics to that observed with the TP ligand IBOP ([1S-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 Ca^{2+} release was blocked by pretreatment of the cells with the TP blocker SQ29548 ([1S[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)

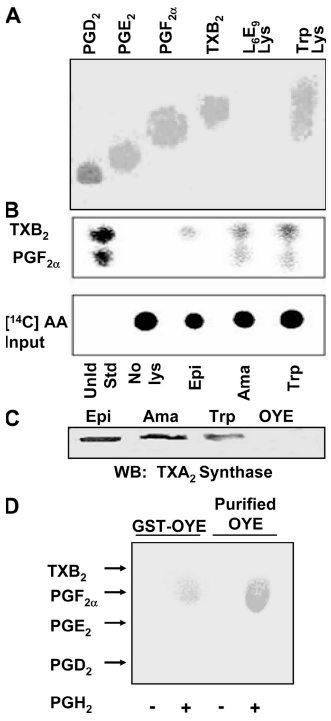

Figure 2. TXA_2 is the primary eicosanoid produced by *T. cruzi*. (A and B) TLC examining the diversity of eicosanoids produced by trypomastigotes (Trp) (A and B), epimastigotes (Epi), and amastigotes (Ama) (B). Eicosanoids generated upon incubation of [^{14}C]-AA with extracts from epimastigotes, extracellular amastigotes, and trypomastigotes are shown (B). Incubation of [^{14}C]-AA without lysate was used as a control. Amount of [^{14}C]-AA is shown to denote equal loading ([^{14}C]-AA input). Standards for PGD_2, PGE_2, TXB_2, and $PGF_{2\alpha}$ were used to identify the species present in *T. cruzi* lysates. PGs were not detected in L_6E_9 myoblasts lysates. (C and D) Recombinant TcOYE does not react with TXA_2S antisera (C) and is incapable of synthesizing TXA_2 but can readily metabolize PGH_2 to $PGF_{2\alpha}$ (D). Immunoblot performed using 30 μg of protein lysate per lane. Numbers to the side of immunoblots indicate migration of molecular weight standards (kD). The autoradiographs shown are representative of four individual experiments. No Lys, no lysate; Unld Std, unlabeled standard.

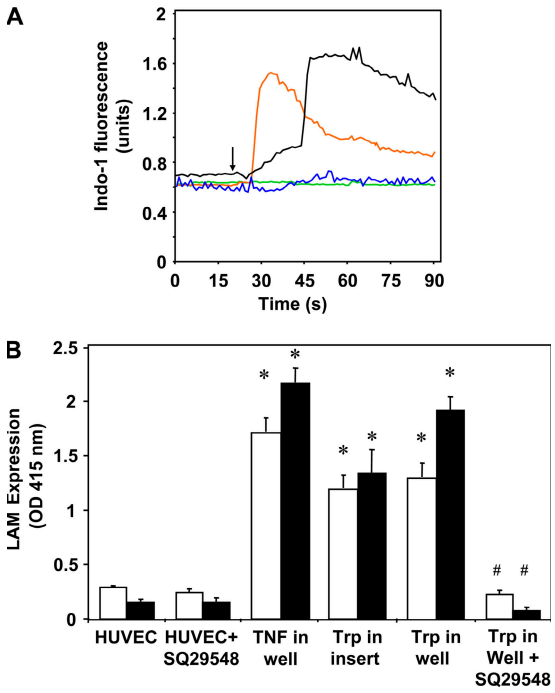

Figure 3. Trypomastigote-derived TXA_2 elicits a pathological response from host cells. (A) Mobilization of intracellular Ca^{2+} in CHO cells expressing the α-isoform of human TP in response to unconditioned (green line) or trypomastigote-conditioned media (orange line). The role of TXA_2 was assessed by preincubating the cells with the TP blocker SQ29548 (5 μM, blue line). Response to the TXA_2 mimetic IBOP (100 nM) is also shown (black line). Arrow denotes the point at which conditioned media or IBOP was added. (B) HUVECs were cocultured with trypomastigotes for 24 h and ICAM-1 (white bars) and VCAM (black bars) expression assessed by ELISA. The role of TP was determined by including SQ29548 (5 μM) in the media. TNF-α (10 U/ml) was used as a positive control to induce ICAM-1 and VCAM expression. All data are representative of three independent experiments. * and # indicate significance ($P \leq 0.005$) from uninfected and infected HUVECs, respectively.

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-α (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 *T. cruzi* infection.

Parasite-derived TXA_2 accounts for the majority of circulating TXA_2 in experimental *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

TXB_2 in TXA_2S-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 TXA_2 was almost identical in the TXA_2S-null and WT mice. TXA_2S-null mice are incapable of generating TXA_2 (29), indicating that parasite-derived TXA_2 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 TXA_2S-null mice was similar to WT mice upon infection, indicating that parasite-derived TXA_2 can compensate for any deficits in host response derived from the inability of the host to produce TXA_2.

Inability of the host to respond to parasite-derived TXA_2 increases mortality and parasitemia during acute infection

To examine whether host response to parasite-derived TXA_2 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 TXB_2 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 TXA_2 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).

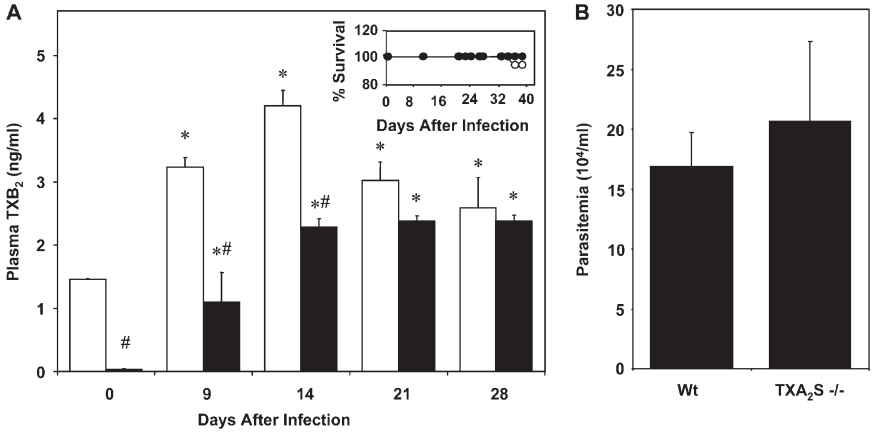

Figure 4. *T. cruzi*–derived TXA_2 accounts for the majority of plasma TXB_2 levels in *T. cruzi*–infected mice. (A) Time course of plasma TXB_2 levels in infected WT (white bars) and TXA_2S-null (black bars) C57/BL6 mice. Levels of circulating TXB_2 between the strains are not significantly different after 21 d of infection. Insert shows the survival of WT (○) and TXA_2S-null (●) mice over 40 d of infection. (B) Parasitemia (day 17) for WT and TXA_2S-null mice infected with 10^5 trypomastigotes of the Brazil strain. Data (mean ± SD) are derived from at least five mice per group. * and # indicate significance ($P \leq 0.005$) from basal values and from WT mice, respectively.

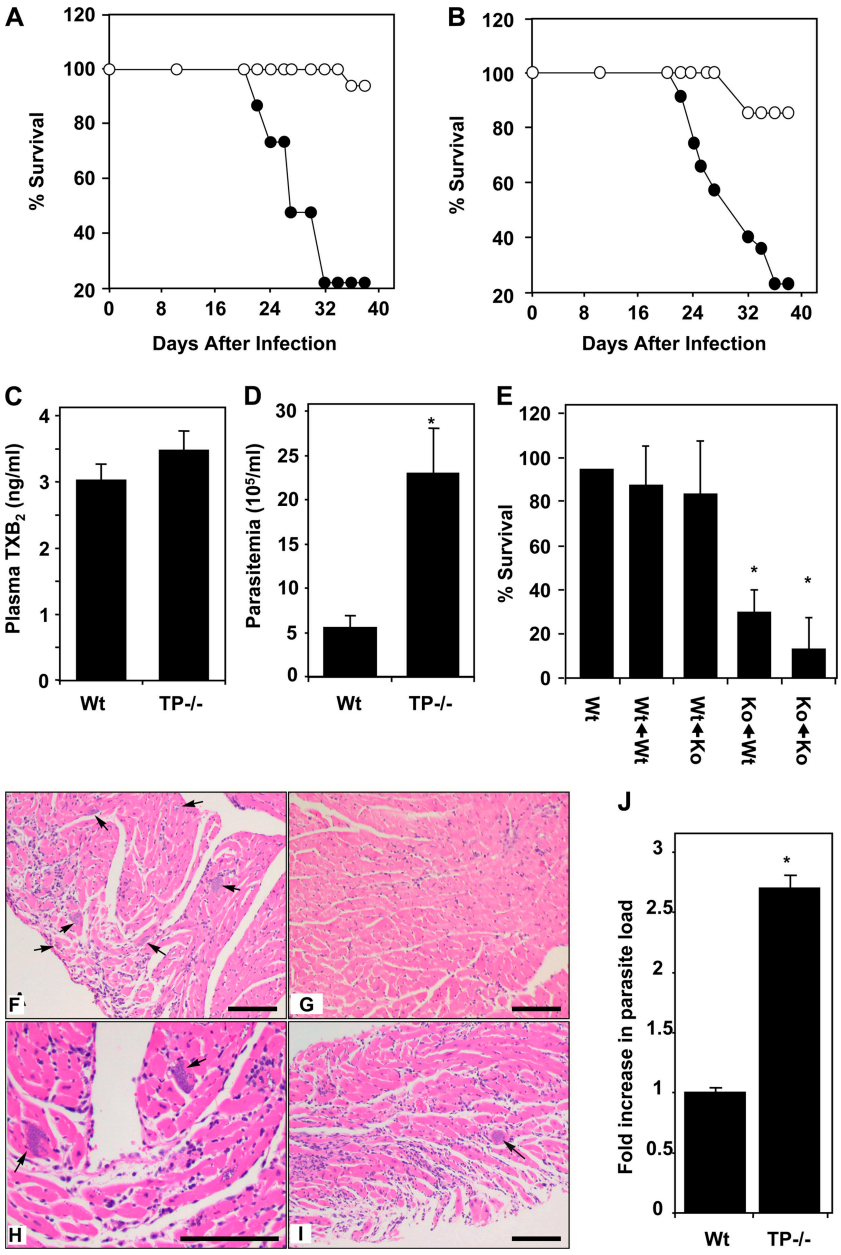

Figure 5. Failure of the host somatic cells to respond to *T. cruzi*–derived TXA_2 results in increased parasitemia and enhanced mortality. (A and B) Survival curves for WT (○) and $TP^{-/-}$ (●) mice in both the C57/BL6 (A) and BalbC (B) backgrounds after inoculation of 5×10^4 and 10^5 trypomastigotes of the Brazil strain, respectively. (C) Plasma TXB_2 levels in infected WT and $TP^{-/-}$ mice (C57/BL6 strain, day 21) showing equivalent TXB_2 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/BL6 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 ($P \leq 0.005$) from control (WT) group. Bars, 125 μm.

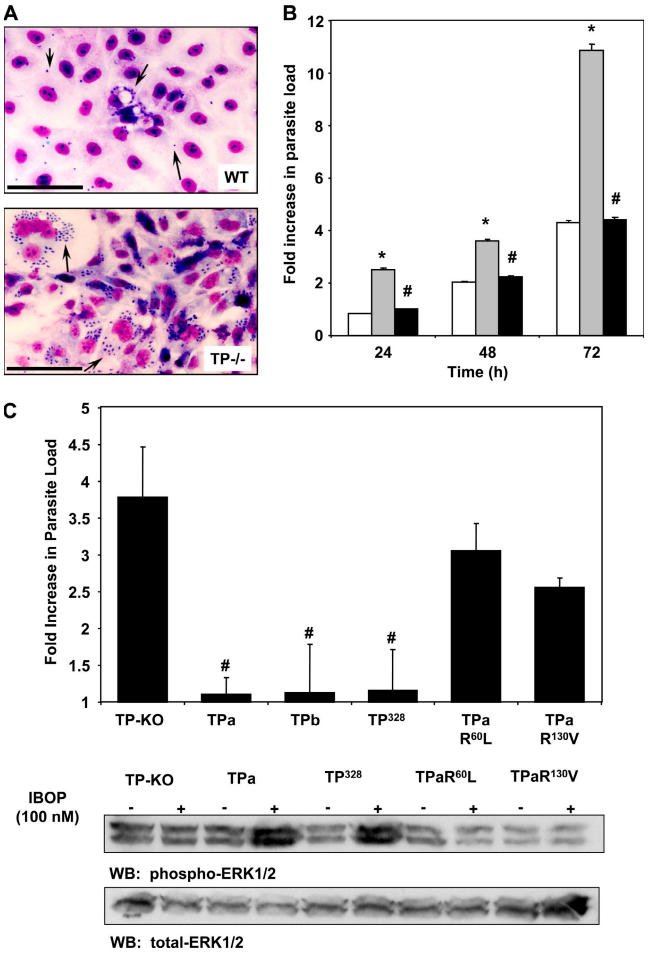

Figure 6. $G_{\alpha q}$ signaling by host TP in response to parasite-derived TXA_2 suppresses intracellular parasite growth. (A and B) Overnight infection of EC from $TP^{-/-}$ (black bars) C57/BL6 mice with trypomastigotes (Brazil strain, MOI 2:1) results in a rate of intracellular parasite growth 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 TXA_2 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, TP^{328}, suppress parasite growth in vitro. However, two TPα mutants devoid of $G_{\alpha q}$ coupling, $R^{60}L$ and $R^{130}V$, did not suppress parasite growth indicating the mechanism is mediated by $G_{\alpha q}$ activation. Examination of ERK activation by immunoblotting supports the role of these two residues in $G_{\alpha q}$ activation. Data (mean ± SD) and autoradiographs are from three individual experiments. * and # indicate significance ($P \leq 0.005$) 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 parasitemia 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, TP^{328}, and two point mutants, TPα-$R^{60}L$ and TPα-$R^{130}V$, that cannot activate $G_{\alpha q}$-dependent pathways (Fig. 6 C). ELISA assay, using an antibody derived against the NH_2 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 TP^{328} 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 $G_{\alpha q}$. To confirm signaling through $G_{\alpha q}$ was compromised in the TPα-$R^{60}L$– and TPα-$R^{130}V$–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 TP^{328}-expressing cells but not in the TP-null, TPα-$R^{60}L$–, and TPα-$R^{130}V$–expressing ECs, indicating $G_{\alpha q}$ coupling was truly compromised in these cells. The suppression of parasite growth is mediated through $G_{\alpha q}$ 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, TXA_2 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 TXA_2. TXA_2 is the predominant prostanoid released by the parasite. However, TXA_2 is not the only bioactive lipid produced. *T. cruzi* also produce and release $PGF_{2\alpha}$, and we have confirmed that TcOYE, the only eicosanoid synthase identified to date in *T. cruzi*, is a $PGF_{2\alpha}$ synthase. In addition, we established that TcOYE does not biosynthesize TXA_2. Both LC-MS-MS and [^{14}C]-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.

TXB_2 levels are elevated in mice infected with *T. cruzi* (36). The present results identify TXA_2 as a parasite-derived molecule that modulates survival and disease progression. *T. cruzi*–derived TXA_2 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 TXA_2 plays a prominent role in the acute stages of the disease; however, the primary issue has been the belief that the enhanced TXA_2 levels manifest through a host response to infection, such as inflammation and platelet activation. Results from the TXA_2S-null mice clearly identify the parasite as the source of up to 90% of the circulating TXA_2 in experimental Chagas' disease. In addition, it suggests that parasite-derived

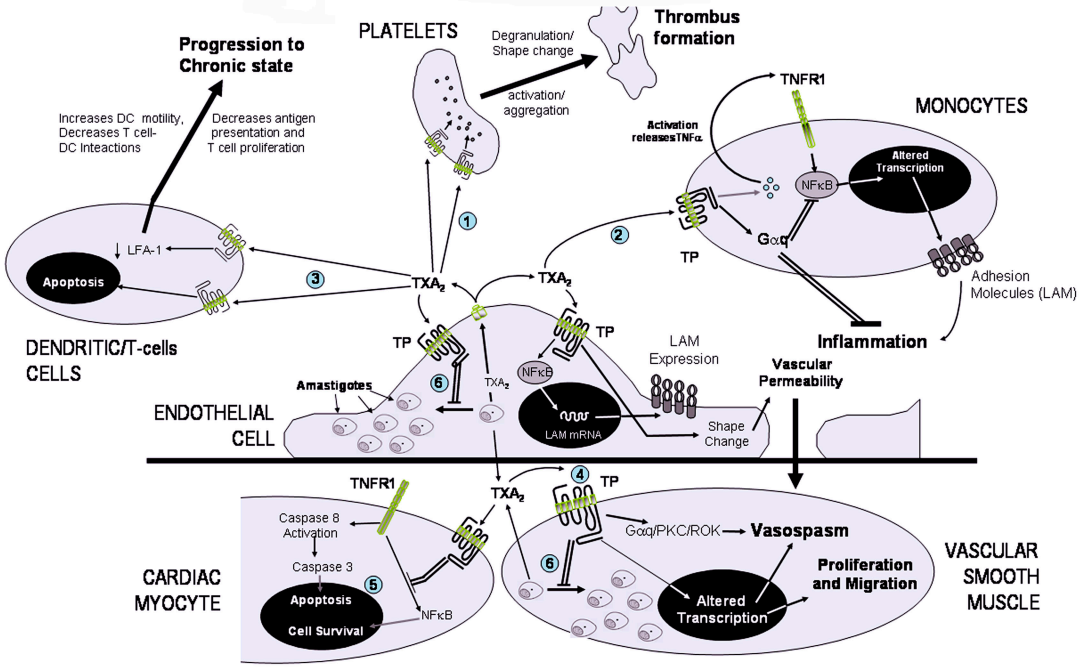

Figure 7. Schematic showing the basis for the role of TP in the pathogenesis of Chagas' disease. TXA_2 liberated from the intracellular amastigote is transported to the extracellular environment by the host cell where it binds to and initiates signaling from the host TP. TP signaling produces a variety of effects: (no. 1) activation of platelets and endothelial cells resulting in a diffuse thrombus formation, perturbation of the quiescent EC state, increased vascular permeability, and accompanying apoptosis; (no. 2) suppresses monocyte/macrophage activation through inhibiting the effects of inflammatory cytokines such as TNF-α; (no. 3) suppresses long-term acquired immunity by preventing antigen presentation to DCs enabling transition to the chronic state; (LFA-1, lymphocyte function–associated antigen 1); (no. 4) produces vascular smooth muscle cell activation with the associated vasoconstriction and limitation to blood flow; (no. 5) produces cardiac myocyte apoptosis resulting in dilated cardiomyopathy; and no. 6) serves to indicate the intracellular parasite load to regulate amastigote proliferation. Collectively, these events produce the plethora of pathophysiological changes that result in the complicated phenotype of acute and chronic Chagas' disease.

TXA_2 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 TXA_2 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 TXA_2 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 TXA_2 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 TXA_2 may result from the suppression of NKκB 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 $G_{\alpha q}$- 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 TXA_2 as an inflammatory regulator.

Other host responses elicited by parasite-derived TXA_2 account for many of the substantial physiological changes that occur during the acute stage of *T. cruzi* infection (Fig. 7). TXA_2 is a potent vasoconstrictor due in no small part to its inhibition of endothelial-derived vasorelaxants, such as nitric oxide (11, 39) (Fig. 7). TXA_2 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). TXA_2 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 TXA_2 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 TXA_2 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). TXA_2 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 TXA_2 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 TXA_2-producing parasites in multiple tissues may directly contribute to the development of the clinical symptoms observed in chronic Chagas' disease.

Parasite-derived TXA_2 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 TXA_2 may mediate the loss of T cell function and number. Immature B cells are also targeted for apoptosis by an unknown cyclooxygenase-derived metabolite during chronic *T. cruzi* infection (59). Our current data suggests that TXA_2 may be the agent responsible. Thus, TXA_2 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 TXA_2, 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 TXA_2 generation or responsiveness would essentially mimic the TP-null mice enhancing parasitemia and mortality rates.

TXA_2 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 TXA_2S 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 TXA_2 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 $G_{\alpha q}$ (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 TXA_2 on parasite physiology as TXA_2 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 TXA_2 release? In organisms with insect vectors it has been hypothesized that parasite-derived PGs suppress immunity and permit the chronic habitation of the vector. TXA_2 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, TXA_2 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 TXA_2 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 TXA_2 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 BalbC) and TXA_2S-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 TXA_2S-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 5×10^5 cells of WT or $TP^{-/-}$ bone marrow was injected through 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 an 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 $R^{130}V$ mutant was a gift from Dr. Valerie 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). 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 NH_2 terminus of the human TP.

Infection of HUVECs and primary mouse EC cultures. To examine the potential host response to parasite-derived TXA_2 and its pathological role in experimental *T. cruzi* infection we monitored TXA_2 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 of ∼2:1. TXA_2 release was monitored by ELISA (see Identification of TXB_2... 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 is 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 β2-microglobulin).

Identification of TXB_2 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-phased 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 (65:34.99:0.01, vol/vol/vol) from 0 to 8 min, and then a gradient increasing to 100% methanol from 8.01 to 30 min. Synthetic TXB_2 was purchased from Cayman Chemical. TXB_2 (the stable hydrolytic product of TXA_2) 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 [^{14}C]-labeled AA was performed using 100 μl of cell extract and 1 Gbq [^{14}C]-AA in 100 mM sodium phosphate (pH 7.0), 2 μM hematin, and 5 mM tryptophan 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 TcOYE open reading frame was amplified using platinum PCR super mix (Invitrogen) and the gene-specific sense (5′-CGGAATTCATGGCG ACGTTCCCTGAACT-TC-3′) and antisense (5′-CCGCTCGAGTTATTTGTTGTACGTCGGG-TA-3′) primers. The PCR product was cloned into pGEX-4T-1 expression vector (GE Healthcare) using EcoRI and XhoI restriction sites. Recombinant TcOYE 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 leukocyte adhesion 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 trypomastigotes remained in suspension. ICAM and VCAM expression were determined by cell-based ELISA as previously described (20). Trypomastigotes were included in the upper chamber (2×10^6 per insert) or the lower chamber (4×10^6 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 (20) using antibodies against human TXA_2S (Cayman Chemical), α-tubulin (Sigma-Aldrich), total and phosphorylated ERK1/2 (Cell Signaling Technology).

Ca^{2+} imaging in CHO cells. CHO cells with stable expression of the α-isoform of human TP were incubated for 45 min at 37°C with the Ca^{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 $MgCl_2$; 2.0 $CaCl_2$; 24.0 $NaHCO_3$; 1.8 NaH_2PO_4; 5.5 glucose; 5.0 Hepes, pH 7.2) at 5×10^5/ml. Mobilization of intracellular Ca^{2+} was induced by addition of either 100 nM IBOP or 0.2 μm filtered, conditioned media (200 μl) from parasite cultures to the suspended CHO cells. The conditioned media used as the stimulus was filtered directly into the cassette containing labeled CHO cells because of considerations of the aqueous half-life of TXA_2. This process minimized the degradation of secreted TXA_2, as the time elapsed for the entire procedure was ∼5 s, and removed contaminating trypomastigotes. SQ25948 (5 μM) was used to assess the role of the TP in this response. Ca^{2+} flux was measured by spectrofluorimetry using standard excitation and emission filters 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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