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Trypanosoma cruzi Immune Evasion Mediated by Host Cell-Derived Microvesicles

Igor Cestari,*,[1] Ephraim Ansa-Addo,[†] Poliana Deolindo,* Jameel M. Inal,[†] and Marcel I. Ramirez*

The innate immune system is the first mechanism of vertebrate defense against pathogen infection. In this study, we present evidence for a novel immune evasion mechanism of *Trypanosoma cruzi*, mediated by host cell plasma membrane-derived vesicles. We found that *T. cruzi* metacyclic trypomastigotes induced microvesicle release from blood cells early in infection. Upon their release, microvesicles formed a complex on the *T. cruzi* surface with the complement C3 convertase, leading to its stabilization and inhibition, and ultimately resulting in increased parasite survival. Furthermore, we found that TGF-β–bearing microvesicles released from monocytes and lymphocytes promoted rapid cell invasion by *T. cruzi*, which also contributed to parasites escaping the complement attack. In addition, in vivo infection with *T. cruzi* showed a rapid increase of microvesicle levels in mouse plasma, and infection with exogenous microvesicles resulted in increased *T. cruzi* parasitemia. Altogether, these data support a role for microvesicles contributing to *T. cruzi* evasion of innate immunity. *The Journal of Immunology*, 2012, 188: 1942–1952.

*T*rypanosoma cruzi, the causative agent of Chagas disease, has evolved several mechanisms to survive the hostile environments encountered during its life cycle (1). The *T. cruzi* life cycle alternates between an insect vector and a vertebrate host (1). In the insect, *T. cruzi* multiplies as epimastigotes that differentiate to metacyclic trypomastigotes (vertebrate infective stage), which are released on the host skin during transmission by the insect bite. This parasite has to evade the innate immune system and infect host cells to progress in the life cycle. Inside the cells, *T. cruzi* differentiates to amastigotes (multiplicative intracellular stage), which after several rounds of division differentiate to bloodstream trypomastigotes. The latter disrupt the cells and circulate in the blood, infecting other cells or being taken by the insect vector, thereby restarting the life cycle.

One of the main barriers encountered during infection of vertebrates is the complement system (2). The complement system is a protein cascade activated upon pathogen recognition and culminating in pathogen lysis (2). It can be activated by classical, lectin, or alternative pathways, as follows. The classical pathway is activated when IgG/IgM bind to the pathogen and associate with the C1 complex (C1q-r$_2$s$_2$), which cleaves components C2 and C4

and generates C3 convertase (C4b2a). The lectin pathway is activated when mannan-binding lectin (MBL), L-ficolin, and/or H-ficolin recognize pathogen carbohydrates. They associate with MBL-associated serine protease-2 (MASP-2), which cleaves C2 and C4 to form C3 convertase (C4b2a). The alternative pathway is initiated when C3b binds to the pathogen and associates with factor B, forming C3 convertase (C3bBb) (2). C3 convertases are key complexes in the complement system. They cleave C3, thereby amplifying the cascade and leading to C5 convertase formation (C4b2a3b or C3bBb3b). C5 convertase cleaves C5, generating the membrane attack complex (C5b-9) on the pathogen surface, resulting in lysis (2). Although several molecules have been identified that mediate *T. cruzi* complement evasion, other reports have shown that infective stages are lysed by complement (3–7), suggesting that extrinsic factors may contribute to immune evasion. In addition to escaping the complement attack, *T. cruzi* metacyclic trypomastigotes have to invade host cells. Several surface molecules have been described that participate in this process (8). They trigger an increase of intracellular Ca^{2+} in host cells, which induces lysosome recruitment and phagolysosome formation (9). *T. cruzi* metacyclic trypomastigotes are also exposed to several cells in the bloodstream, such as monocytes, lymphocytes, and macrophages. The drastic physiological changes encountered during the infection raise the question of whether host factors contribute to their immune evasion.

Recently, plasma membrane-derived vesicles [PMVs; also known as microvesicles (10), microparticles (11), or ectosomes (12)] have been demonstrated to play a role in several diseases, such as cancer, thrombosis, and pathogen infections (10, 13–17). Several cells, such as monocytes, lymphocytes, erythrocytes, platelets, and endothelial cells, release PMVs (12, 17, 18). PMVs are released from the cell plasma membrane either at basal levels, or upon extracellular stimulus and a concomitant raise in intracellular Ca^{2+} (19). Their main features are as follows: size of 0.1–1 μm, surface-exposed phosphatidylserine, and the presence of actin and other molecules from the cell of origin (18). A role for PMVs in pathogen infection was first shown for HIV (11). This work demonstrated that CCR5 was transferred to HIV-resistant CCR5$^-$ cells by PMVs, rendering them susceptible to HIV infection. PMVs also circulate at high levels in the plasma of patients with

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cerebral malaria and lupus anticoagulant (15, 20, 21), and have also been shown to be involved in the cytoadherence of *plasmodium sp.*-infected erythrocytes to blood vessels (14, 16, 22), indicating their involvement in pathogen infection. In this study, to our knowledge, we show for the first time that *T. cruzi* uses host-derived PMVs to evade the innate immune system. *T. cruzi* induce an increase in PMV release from blood cells in vitro and in vivo. PMVs bind to the complement C3 convertase and inhibit its catalytic activity, thereby conferring protection against complement-mediated lysis. Furthermore, TGF-β–bearing PMVs enhanced *T. cruzi* invasion of eukaryotic cells, and promoted a high infection in mice. Therefore, we propose that PMVs are a host factor used by *T. cruzi* to evade the innate immune system.

Materials and Methods

Cell culture

T. cruzi epimastigotes were cultivated in liver infusion tryptose medium (23). Metacyclic trypomastigotes and tissue culture trypomastigotes were obtained, as previously described (4, 24). Strain Silvio X10/6 was used in all experiments, unless otherwise specified. Vero, HepG2, MCF-7, Jurkat, and THP-1 cells were grown in RPMI 1640 supplemented with 10% FBS at 37°C and 5% CO_2. For PBMCs and erythrocytes, peripheral blood (from adult health volunteer donor) was diluted in RPMI 1640 medium (1:1 v/v) and carefully added to Ficoll-Hypaque (Sigma-Aldrich) at room temperature (RT; 2:1 v/v). Cells were centrifuged at 2000 rpm for 30 min at RT, and the mononuclear fraction (leukocytes) or erythrocytes were collected, washed, and kept in RPMI 1640 medium/5% FBS.

PMV induction and analysis

THP-1–derived PMVs were obtained either by collecting the supernatant from late logarithmic cells or by stimulating cells (1.0×10^6/ml) with *T. cruzi* parasites (5.0×10^6/ml, 5:1 parasites:cell). Cells were preincubated for 1 h in RPMI 1640, washed, and resuspended in RPMI 1640/$CaCl_2$ (1 mM). During stimulus, cells were incubated at 37°C for 1 h and then 5 min on ice. PMVs were obtained by differential centrifugation, as follows: 5 min, $160 \times g$; 2×30 min, $4000 \times g$; and 90 min, $100,000 \times g$. Pellets were resuspended in PBS or HEPES buffer (10 mM HEPES, 140 mM NaCl).

Drug treatments. Cells were preincubated with drugs (100 nM wortmannin, 1.5 μM thapsigargin, or 25 μM genistein, purchased from Sigma-Aldrich) for 30 min. Drugs were washed out prior to parasite addition.

Caspase 3/apoptosis. THP-1 cells (1.0×10^6) were incubated with or without metacyclic trypomastigotes (5.0×10^6) in RMPI at 37°C from 5 to 60 min. Apoptosis was measured using the BD Biosciences apoptosis kit. For caspase 3 activation, the NucView 488 Caspase-3 Assay Kit for Live Cells (Biotium) was used.

Isolation and labeling of PMVs from mice. Mouse blood was collected in 0.1 M sodium citrate and centrifuged to remove cells at $500 \times g$ for 10 min. After that, the supernatant was centrifuged for another 5 min at $1500 \times g$. Afterward, two rounds of centrifugation were performed at $4000 \times g$ for 30 min, and the supernatant was ultracentrifuged for 1 h and 30 min at $100,000 \times g$. The pellet containing the PMVs was analyzed for annexin V-FITC and CD184-allophycocyanin.

PMV labeling with annexin V and CD184 or TGF-β. PMVs from THP-1 cells or obtained from mice were incubated with 5 μl annexin V-FITC (BD Biosciences) in 100 μl HEPES buffer (10 mM HEPES, 10 mM NaCl, 2.5 mM $CaCl_2$) for 1 h at RT. After that, they were diluted to 4 ml in HEPES buffer and ultracentrifuged at $100,000 \times g$ for 1 h. For mice PMVs, further labeling was performed with mAbs anti–CD184-allophycocyanin (1:5, BD Pharmingen). PMVs were washed and analyzed by flow cytometry. For TGF-β, the PMVs were incubated with mAbs anti–TGF-β (1:100; Millipore), washed, and incubated in goat anti-rabbit IgG-PE (1:400). After that, they were washed and analyzed by flow cytometry. PMVs incubated with goat anti-rabbit IgG-PE were used as control.

Measurements of intracellular Ca^{2+}

Calcium measurements were performed, as described (25). Cells were labeled with Fura-2AM dye, and fluorescence was measured with a Hitachi 4500 spectrophotofluorometer.

Transmission electron microscopy

THP-1 cells were stimulated for PMV release and then were fixed in 3% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.2). Cells were se-

quentially incubated in 1% osmium tetroxide solution (Sigma-Aldrich), then 1% aqueous uranyl acetate overnight. Samples were dehydrated in an ascending ethanol series from 70 to 100% v/v absolute ethanol (Sigma-Aldrich), washed in propylene oxide (Agar Scientific), and infiltrated with propylene oxide/agar resin (Agar Scientific). Samples were changed to 100% resin and embedded in capsules for polymerization at 60°C prior to obtaining ultrathin sections. Sections were placed onto copper grids and photographed at the electron microscopy facilities of the London School of Hygiene and Tropical Medicine.

Complement system assays

Normal human serum (NHS) was obtained from healthy voluntary donors, pooled, and stored at −80°C. Complement-mediated lysis assays were performed, as described (23). For assays in the presence of THP-1 cells, metacyclic trypomastigotes (5.0×10^5) were incubated with 50% NHS at 37°C for 60 min with or without THP-1 cells (1.0×10^5 and 5.0×10^4), and survival parasites were quantified using Neubauer chamber. Trypan blue staining and parasite motility were used to distinguish between live and dead parasites. C3b and C4b deposition assays were performed, as described (26). For complement cleavage assays, metacyclic trypomastigotes (1.0×10^6) were incubated in 100 μl 10% NHS with or without PMVs ($0.1–2.5 \times 10^5$/ml) for 10 min at 37°C. Parasites were harvested (5 min, $4000 \times g$), and supernatants (serum) were analyzed for C2 and C4 cleavage. Pellets (parasites) were solubilized in PBS/1% Triton X-100 and analyzed by Western blotting. C2 cleavage by recombinant MASP-2 (rMASP-2) was performed, as decribed (23). For kinetic studies of C2a dissociation, metacyclic trypomastigotes (1.0×10^6 in 100 μl) were incubated in 100 μl 10% NHS with or without PMVs (1.5×10^5/ml) for 1, 2.5, 5, and 15 min at 37°C. Ice-cold PBS was added, parasites were harvested, and protein extracts were obtained with PBS/1% Triton X-100. Proteins were analyzed by Western blotting.

Western blotting

Western blotting was performed as previously described (27).

ELISA experiments

Adsorption of parasites or PMVs to ELISA plates was performed, as described (7).

Binding of C1q, C3, C4, L-, and H-ficolins to T. cruzi. A total of 100 μl 1% NHS (in 10 mM HEPES/140 mM NaCl) was preincubated on ice for 1 h with or without PMVs (1.5×10^5/ml). Sera were added to the wells and incubated for 60 min at 37°C. Polyclonal goat anti-MBL (1:50) (Santa Cruz Biotechnology), polyclonal rabbit anti–L-ficolin (1:500), anti–H-ficolin (1:500), anti-C1q (1:500), and anti-C3 Abs (1:500, in PBS/3% BSA) (Medical Research Council Immunochemistry Unit) were used for detection. Reactions were developed with 100 μl ABTS peroxidase solution (Kirkegaard and Perry Laboratories), and absorbances were obtained through spectrophotometric measurement at 405 nm.

Complement factor binding to PMVs. PMV-coated wells were incubated with NHS (0.1–20%) for 1 h at 37°C. Detection was performed with Abs against MBL, L-ficolins, H-ficolins, C1q, C2, C4, and C3, as above.

Convertase catalytic assay. T. cruzi-coated wells were incubated with 0.5% NHS for 1 h at 37°C (for C4b binding). They were then washed and incubated for 1 h in PBS (for convertase dissociation). Reactions (50 μl in HB) were performed by adding purified C2 (2 μg/ml) (Calbiochem), C3 (5 μg/ml) (28), and rMASP-2 (0.2, 1, and 3 μg/ml). PMVs (1.5×10^5/ml) were added, and reactions were incubated for 90 min at 37°C. Controls were performed by omitting rMASP-2 or C3. Reactions were stopped by washing wells with PBS. C3b detection was performed with anti-C3 mAbs (WM1; Medical Research Council Immunochemistry Unit). Background values of C3b on opsonized parasites were subtracted from values of those incubated with C2, rMASP-2, and C3.

TGF-β measurements. An ELISA cytokine kit (BD Biosciences) was used. A total of 1.0×10^5 PMVs in 100 μl PBS or from mouse plasma was used.

Invasion assays

Vero cells were seeded on 13-mm coverslips in 24-well plates (1.0×10^5 cells/well) and incubated overnight at 37°C with 5% CO_2. Afterward, cells were washed with serum-free RPMI 1640 and preincubated with 1.0×10^5/ml PMVs for 30 min at 37°C with 5% CO_2. *T. cruzi* metacyclic trypomastigotes (5.0×10^5/ml, ratio 5:1, parasites to cells) were added and incubated for 3 h at 37°C with 5% CO_2. Cells were then washed with PBS, fixed with absolute methanol (Merck) for 5 min, washed with H_2O, and stained with Giemsa for 1 h at RT. Afterward, they were washed with H_2O, and slides were mounted with aqueous mounting medium (Biomeda).

Intracellular parasites were quantified by light microscopy, counting at least 500 cells per slide.

Inhibitors. A total of 10 μM SB431542, 100 nM wortmannin, and 1.5 μM thapsigargin was used. Cells were treated for 30 min at 37°C with 5% CO_2 prior to cell invasion.

Assays in cells expressing lysosomal-associated membrane protein-1-GFP. Vero cells were transfected with pLAMP-1-GFP (provided by S. Méresse, Centre d'Immunologie de Marseille-Luminy, Marseille, France) using Lipofectamine 2000 (Invitrogen) 1 d prior to invasion assays with metacyclic trypomastigotes (2.0×10^6, 20:1 parasites:cell) for 30 min at 37°C with 5% CO_2. Cells were then fixed in paraformaldehyde/methanol. Parasites were labeled with anti-3F6 mAbs (1:400), followed by goat anti-mouse AlexaFluor546 Abs (1:400; Molecular Probes). Slides were mounted in Vectashield medium containing DAPI (Vector Laboratories). Intracellular parasites in transfected cells were quantified using a Nikon Eclipse E400 fluorescence microscope.

Simultaneous PMV release and cell invasion. A Transwell plate containing membrane pore size of 0.45 μm (BD Bioscience) was used. Vero cells (1.0×10^5 cells/well) were seeded on 13-mm coverslips on the lower chamber (overnight at 37°C with 5% CO_2 for adherence), and THP-1 cells (1.0×10^6 cells/well) were added to the upper chamber (at the time of the assay). Afterward, *T. cruzi* metacyclic trypomastigotes were added on both chambers (with Vero cells for invasion assay, and with THP-1 cells for PMV induction). PMVs (1.0×10^5/ml) were added to a set of upper chamber wells (which did not receive parasites), whereas to another set of wells (lower chamber) 10 μM SB431542 was added and preincubated with Vero cells for 30 min before parasite addition. For invasion assays, a parasites/cells ratio of 10:1 was used, and for PMV induction, a parasites/cells ratio of 5:1 was used. The assays were incubated for 3 h at 37°C with 5% CO_2. Vero cells were fixed and stained, and intracellular parasites were quantified, as described above.

Animal experimentation

Experiments were performed with BALB/c mice (8–12 wk) in the animal facilities of the Instituto Oswaldo Cruz (CEUA license 11602–SIAPE 0462649). For PMV analysis, mice were infected with metacyclic trypomastigotes (5.0×10^5) by i.p. injections, and blood was collected by cardiac puncture. For parasitemia, mice were infected with metacyclic trypomastigotes (5.0×10^5) and PMVs (1.5×10^5), and blood was collected every 2 d from the tail (by tail pick). All procedures were performed in compliance with the relevant laws and institutional guidelines.

Data presentation and statistical analysis

All data shown are the average of at least three experiments performed in duplicate or triplicate. All data are shown as mean ± SD. Comparisons among groups were made by the unpaired *t* test for repeated measures using GraphPad Prism version 5.00 for Windows (GraphPad Software). The *p* values <0.05 with confidence interval of 95% were considered statistically significant, unless otherwise specified.

Results

T. cruzi *metacyclic trypomastigotes induce blood cells to release PMVs in a Ca^{2+}-dependent fashion*

Because *T. cruzi* is present in the host bloodstream during infection, we reasoned that *T. cruzi* could induce blood cells to release PMVs. To investigate this, THP-1 cells [monocytic cell line broadly used in PMV studies (29, 30)] were incubated with epimastigotes or metacyclic trypomastigotes, and supernatants were analyzed by flow cytometry using annexin V-PE labeling for PMV detection. Metacyclic trypomastigotes, but not epimastigotes, induced a 3-fold increase in PMV release over control (Fig. 1*A*, Supplemental Fig. 1), and this increase was inhibited by Ca^{2+} chelation. Metacyclic trypomastigotes, but not epimastigotes, induced an 8-fold transient increase in intracellular Ca^{2+} in THP-1 cells (Fig. 1*B*). PMVs were labeled with annexin V-PE, indicating surface-exposed phosphatidylserine, and also contained actin (Supplemental Fig. 1), two hallmark characteristics of PMVs. Flow cytometry and microscopy analysis of supernatants from THP-1 cells and *T. cruzi* differentially labeled confirmed that PMVs originated from THP-1 cells, whereas no vesicles were detected being released by *T. cruzi* (Supplemental Fig. 2). Furthermore, transmis-

sion electron microscopy studies revealed that PMVs ranged in size from 200 to 500 nm (Fig. 1*C*). Ultracentrifugation of THP-1 cell supernatant in a 10–40% continuous sucrose gradient showed that PMVs and exosomes migrate at different fractions as detected by specific surface markers and electron microscopy (Supplemental Fig. 2), confirming their different characteristics. *T. cruzi* induction of PMV release from THP-1 cells was dose dependent (Fig. 1*D*), beginning after 5 min of incubation (Fig. 1*E*). This release occurred before caspase-3 activation and without cells undergoing apoptosis (Fig. 1*E*). Furthermore, PMV release was not strain dependent because several *T. cruzi* strains induced PMV release from THP-1 cells (Fig. 1*F*). It is noteworthy that not only metacyclic trypomastigotes, but tissue culture trypomastigotes also induced a 3- to 4-fold increase in PMV release (Fig. 1*G*). *T. cruzi* also induced PMV release from human PBMCs (Fig. 1*H*), but not from erythrocytes (Fig. 1*I*). PMV release was inhibited by wortmannin, genistein, and thapsigargin (Fig. 1*J*), indicating that cell signaling resulting in intracellular Ca^{2+} mobilization is most likely involved in PMV release. Together, these results show that *T. cruzi* trypomastigotes induce PMV release from blood cells in a Ca^{2+}-dependent fashion, most likely via cell signaling cascades.

PMVs inhibit complement-mediated lysis of T. cruzi

During infection in vertebrates, *T. cruzi* has to evade the lytic effect of the complement system and invade host cells to successfully progress in their life cycle. To address whether PMVs play a role in *T. cruzi* metacyclic trypomastigote infection, we analyzed the effect of PMVs on complement activity and cell invasion. Initial lysis experiments were performed with epimastigotes, because this parasite stage is highly sensitive to lysis. Addition of PMVs to human serum inhibited *T. cruzi* lysis in a dose-dependent fashion (Fig. 2*A*). We have recently reported that the metacyclic trypomastigote stage of some *T. cruzi* strains is sensitive to complement-mediated lysis in human serum (7). Therefore, we used *T. cruzi* Silvio X10/6 strain, a complement-sensitive strain (7), to analyze whether PMVs would confer complement protection to metacyclic trypomastigotes in near-physiological conditions (50% serum at 37°C). PMVs (from THP-1 cells, Jurkat cells, or PBMCs) conferred a 3-fold increase of parasite survival (Fig. 2*B*, Supplemental Fig. 3). To determine whether *T. cruzi* induction of PMV release would result in simultaneous complement lysis inhibition, we incubated metacyclic trypomastigotes with THP-1 cells in presence of human serum. Parasite survival was higher in the presence of cells (Fig. 2*C*), and increased from 3- to 5-fold according to the increase of parasite to cell ratio (Fig. 2*C*). Altogether, these results indicate that PMVs can inhibit the complement system in a biologically relevant context.

To determine the complement pathway inhibited by PMVs, ELISA-based C3b and C4b deposition assays (26) were performed. PMVs strongly inhibited C3b deposition by classical and lectin pathways (62 and 56%, respectively) and slightly by the alternative pathway (37%) (Fig. 2*D*). Interestingly, they did not significantly inhibit C4b deposition, indicating that PMVs affect the complement system at C3. To further investigate how PMVs inhibit the complement system, we analyzed whether complement-activating molecules would recognize PMVs. We detected binding of the complement molecules C1q, C3b, C4b, L-, and H-ficolins to PMVs (Fig. 3*A*). PMV addition to serum did not inhibit significantly C1q, C4b, L-, or H-ficolin binding to metacyclic trypomastigotes, but did inhibit C3b deposition (Fig. 3*B*), suggesting that PMVs do not inhibit parasite recognition by complement-activating molecules. The strong inhibition of the classical and lectin pathways at the C3 level suggests that PMVs act prior to C3 cleavage and deposition, but after C4 cleavage. To investigate this,
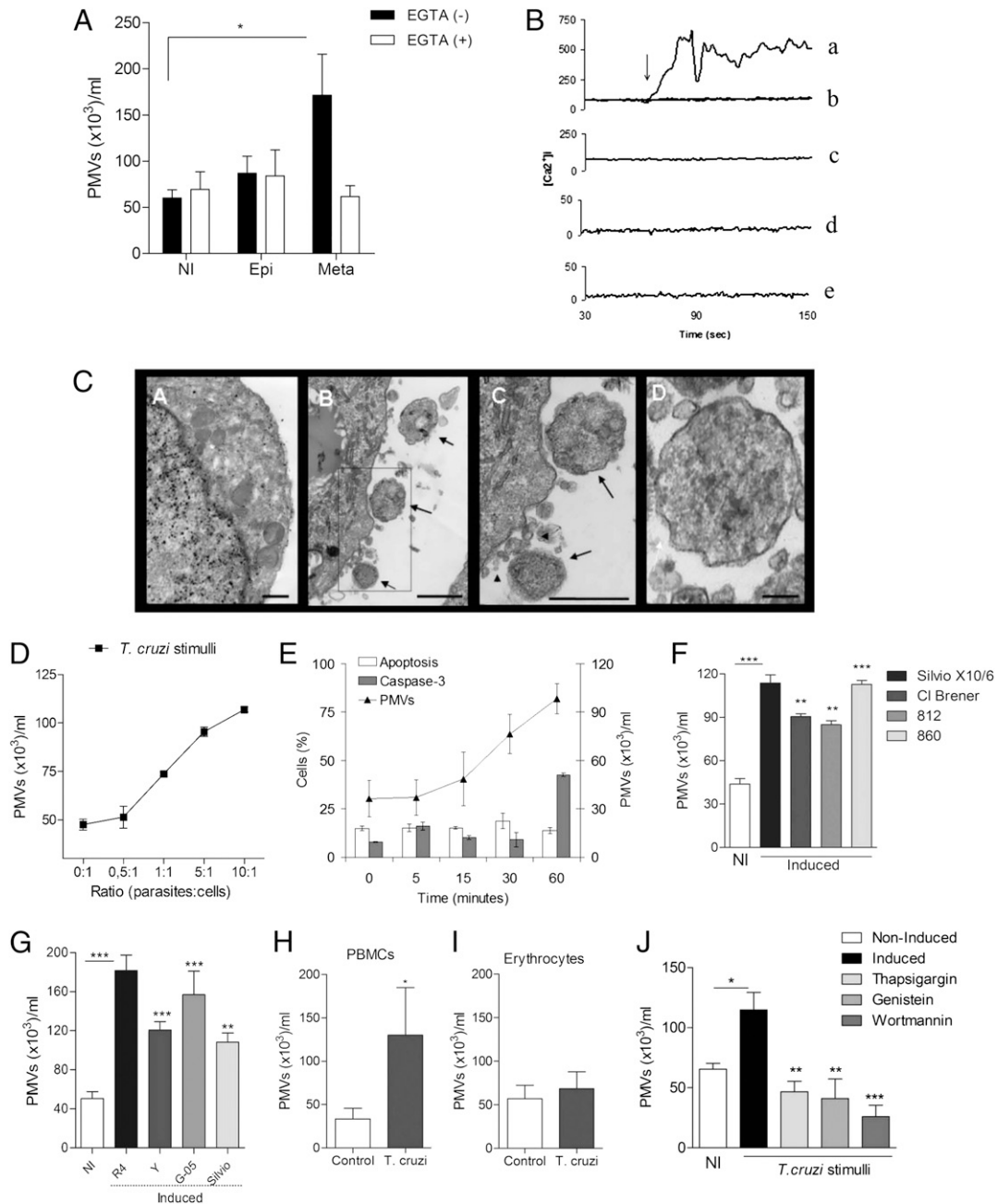
FIGURE 1. *T. cruzi* metacyclic trypomastigotes induce PMV release from blood cells in a Ca²⁺-dependent fashion. *A*, *T. cruzi* (5.0 × 10⁶, epimastigotes or metacyclic trypomastigotes) were incubated with THP-1 cells (1.0 × 10⁶) for 60 min at 37˚C with or without EGTA (5 mM) for PMV induction. Afterward, cell supernatants were analyzed by flow cytometry for PMV quantification. PMV quantification was performed using the ExpressPlus software (from Guava EasyCyte flow cytometer) and stained with annexin V-FITC to determine surface phosphatidylserine (as described in 17) (see also Supplemental Fig. 1 for additional explanation on PMV analysis and quantification). *B*, THP-1 cells (1.0 × 10⁶) loaded with FURA-2AM dye were incubated at 37˚C in HEPES buffer with or without EGTA (5 mM). Parasites (5.0 × 10⁶) were added at 60 s (arrow) to the cells, and the intracellular Ca²⁺ levels were measured over 150 s with a spectrophotofluorometer. *A*, Metacyclic trypomastigotes; *B*, Epimastigotes; *C*, Control (no parasites); *D*, Metacyclic trypomastigotes with EGTA; and *E*, Epimastigotes with EGTA. Ca²⁺ values in nM. *C*, Transmission electron microscopy showing THP-1 cells releasing PMVs. *A*, Noninduced cell; *B*, cell induced with *T. cruzi* metacyclic trypomastigotes for 30 min at 37˚C. Arrows in *C* show PMVs released from the cell surface. *C*, A magnified subfield (black rectangle) of *B*. Arrowheads denote exosomes (smaller vesicles <100 nm) released by THP-1 cells. *D*, A PMV originated from THP-1 cell. Scale bar, panel *C* subfields *A–C*, 500 nm; subfield *D*, 100 nm. *D*, Increasing concentrations of *T. cruzi* metacyclic trypomastigotes were incubated with THP-1 cells (1.0 × 10⁶) for 60 min at 37˚C, and PMVs released in the supernatant were analyzed by flow cytometry. *E*, Metacyclic trypomastigotes (5.0 × 10⁶) were incubated with THP-1 cells (1.0 × 10⁶) at 37˚C for 5–60 min. After collection of PMVs, cells were analyzed by flow cytometry for apoptosis (with annexin V-FITC and propidium iodide) and caspase-3 activation (with NucView 488 caspase-3 assay). *F*, THP-1 cells (1.0 × 10⁶) were incubated at 37˚C for 60 min with metacyclic trypomastigotes (5.0 × 10⁶) of *T. cruzi* strains (Silvio X10/6, CL Brener, 812 and 860), and PMVs released in the supernatant were analyzed by flow cytometry. NI, noninduced. *G*, Experiments were performed as in *F*, but with *T. cruzi* tissue culture trypomastigotes. The strains R4, Y, Gamba 05 (G-05), and Silvio X10/6 (Silvio) were used as stimuli. *H*, *T. cruzi* induce PMV release from human PBMCs. Experiments were performed as in *F*, but using *T. cruzi* Silvio X10/6 strain and human PBMC. Ratio = 5:1, parasites to cells. *I*, Human erythrocytes do not release PMVs under *T. cruzi* stimuli. Assays were performed as in *F*, but using human erythrocytes and *T. cruzi* Silvio X10/6 strain. Ratio = 5:1, parasites to cells. *J*, THP-1 cells (1.0 × 10⁶) were pretreated for 30 min with 100 nM wortmannin, 1.5 μM thapsigargin, and 25 μM (*Figure legend continues*)
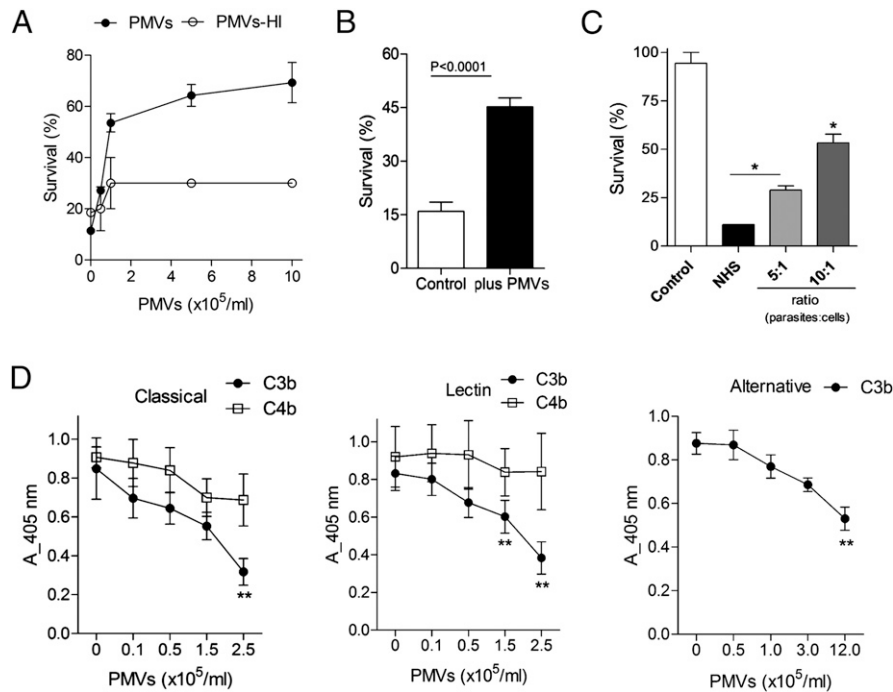
FIGURE 2. PMVs inhibit *T. cruzi* lysis by all complement pathways at C3 level. *A*, Parasites were incubated with 12.5% NHS for 10 min at 37°C with increasing concentrations of PMVs or PMVs heat inactivated (PMVs-HI), and survival parasites were quantified. *B*, PMVs inhibit metacyclic trypomastigote lysis in conditions nearly physiological: 50% NHS at 37°C. Incubations were performed for 60 min and with 1.5×10^5 PMVs/ml. Survival parasites were quantified. Data are shown in percentages, in which 100% survival was calculated using parasites incubated with heat-inactivated serum. *C*, Metacyclic trypomastigotes (5.0×10^5) were incubated with 50% NHS at 37°C for 60 min for complement-mediated lysis in the presence of THP-1 cells (for PMV release, ratios were 5:1 and 10:1, parasites/cells). Survival parasites were quantified. As control, parasites were incubated with heat-inactivated serum and THP-1 cells; NHS indicates parasites incubated with NHS, but without THP-1 cells. *D*, ELISA-based complement C3b and C4b deposition assays were performed to analyze the complement pathways inhibited by PMVs. PMVs were added to serum prior to activation assays, and C3b and C4b deposition were analyzed using their respective polyclonal Abs. For classical and lectin pathways 1% NHS was used, and for alternative pathway 6% NHS was used. The data shown from *A*–*D* represent the mean ± SD from at least three independent experiments. *$p < 0.05$, **$p < 0.01$.

metacyclic trypomastigotes were incubated with human serum for complement activation in the presence or absence of PMVs, and serum C2/C4 cleavage was analyzed. PMVs did not inhibit C2 cleavage (Fig. 3*C*), evidenced by detection of C2 cleavage products C2a (70 kDa) and C2b (30 kDa). Because *T. cruzi* metacyclic trypomastigotes mainly activate the lectin pathway at early stages in human serum (23), we analyzed the effect of PMVs on MASP-2 activity. Interestingly, PMVs did not inhibit active rMASP-2 from cleaving C2 (Fig. 3*D*). PMVs also did not inhibit C4 cleavage, demonstrated by the detection of C4bα′ (Fig. 3*E*) and C4b deposition on the parasite surface (Fig. 3*B*). These data indicate that PMVs could be inhibiting the complement system by interfering with the formation or activity of the C3 convertase, or directly binding to C3.

PMVs bind to the C3 convertase (C4b2a) on the T. cruzi *surface, thereby inhibiting C3 cleavage*

The binding of C3b to the pathogen surface is dependent on C3 cleavage into C3a and C3b by the C3 convertases (2). As we found that PMVs decreased C3b deposition by the classical and lectin pathways without affecting C2 or C4 cleavage, we hypothesized that PMVs affect the formation or activity of C3 convertase (C4b2a). To test this hypothesis, metacyclic trypomastigotes were incubated with human serum and PMVs, and complement com-

ponents bound to parasites were analyzed by Western blotting. Strikingly, addition of PMVs caused increased binding of C4b and C2a to the parasite surface in a dose-dependent fashion (Fig. 4*A*). Interestingly, PMVs bound poorly to purified C3 or C2, but did bind to *T. cruzi* treated with NHS (Fig. 4*B*, Supplemental Fig. 3). It is noteworthy that PMV binding was higher in parasites treated with NHS than on those not treated, and that binding was inhibited with polyclonal anti-C4, but not anti-C2, Abs (Supplemental Fig. 3). These data indicate that PMVs most likely interact with C4b on the parasite surface and that the inhibition of C3b deposition on *T. cruzi* (Figs. 2*C*, 3*B*) does not depend on direct binding of the PMVs to C3. It is possible that PMVs bind to C3 convertase (C4b2a), thereby preventing its dissociation from the *T. cruzi* surface. The pathogen-bound C3 convertase (C4b2a) has a short $t_{1/2}$ (~60 s), resulting in rapid C2a turnover through its dissociation and the association of new C2 molecules with C4b on the pathogen surface (31). Pathogen surface-associated MBL–MASP-2 or C1 complex cleaves C4bC2 and generates active C3 convertase, C4b2a. To determine whether PMVs affect C4b2a dissociation from the *T. cruzi* surface, the kinetics of C2a dissociation from metacyclic trypomastigotes were analyzed during complement activation. C2a was detected at 1-, 2.5-, and 5-min incubation of parasites and serum in the presence of PMVs (Fig. 4*C*). In contrast, C2a was detected only at 2.5 min in the absence of the PMVs

genistein, and drugs were removed before cell stimuli (60 min at 37°C) with parasites (5.0×10^6). PMVs released in the supernatant were analyzed by flow cytometry. The data shown in *A*, *B*, and *D*–*J* represent the mean ± SD from at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
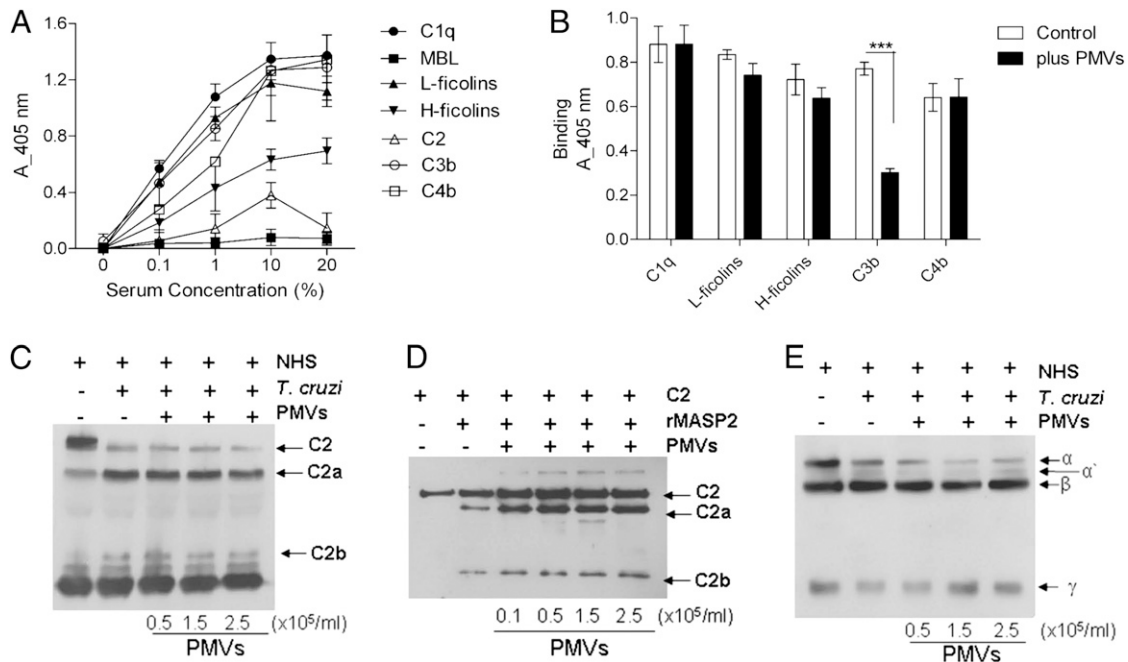
FIGURE 3. PMVs inhibit C3 deposition on the *T. cruzi* surface without affecting C2 or C4 cleavage. *A*, PMVs (adsorbed on ELISA plates) were incubated with increasing concentrations of NHS (0.1–20%) for 1 h at 37˚C. Abs against complement factors were used for detection by ELISA. *B*, PMVs inhibit binding of C3 to *T. cruzi*. PMVs (1.5×10^5/ml) were added to 1% NHS and incubated for 1 h at 37˚C with metacyclic trypomastigotes (previously adsorbed on ELISA plates). Detection was performed by ELISA using polyclonal Abs against complement factors. *C*, PMVs were added to 10% NHS and incubated with metacyclic trypomastigotes for 10 min at 37˚C. Parasites were harvested, and supernatants containing NHS were analyzed for C2 cleavage by Western blotting with polyclonal anti-C2 Abs. *D*, PMVs were incubated with rMASP-2 (CCP1-CCP2-SP) and C2 for 1 h at 37˚C. Reactions were analyzed for C2 cleavage by Western blotting, as in *C*. *E*, Assays were performed as in *C*, and supernatants were analyzed for C4 cleavage by Western blotting with anti-C4 Abs. The data shown in *A* and *B* represent the mean ± SD from at least three independent experiments. Experiments from *C* and *D* are representative of at least three independent experiments. ***$p < 0.001$.

(as expected, because of the rapid dissociation of C2a). These results indicate that PMVs interact with the C3 convertase C4b2a and delay its dissociation from the *T. cruzi* surface.

We hypothesized that PMVs inhibit surface-bound C3 convertase (C4b2a) from cleaving C3, and therefore analyzed purified C3 cleavage after formation of C3 convertase (C4b2a) on the *T. cruzi* surface. C4b-opsonized *T. cruzi* was incubated with purified C2 and active rMASP-2 to allow C3 convertase formation (C4b2a) in the presence or absence of PMVs. Purified C3 was added, and C3b deposition on the *T. cruzi* surface was analyzed. PMVs inhibited C3b deposition on *T. cruzi* (Fig. 4*D*), confirming that PMVs inhibit C4b2a from cleaving C3.

TGF-β–bearing PMVs on the T. cruzi *surface mediate eukaryotic cell invasion*

Besides escaping complement-mediated lysis, *T. cruzi* metacyclic trypomastigotes must invade host cells to differentiate and multiply. To investigate whether PMVs play a role in *T. cruzi* invasion, we preincubated Vero cells with PMVs for 30 min at 37˚C and performed invasion assays with metacyclic trypomastigotes. Addition of THP-1–derived PMVs increased *T. cruzi* invasion (Fig. 5*A*), and similar results were obtained with PBMC-derived PMVs (Supplemental Fig. 4). Furthermore, the invasion was dose dependent, nonspecific for parasite strains or eukaryotic cell lines, and dependent on the parasite infective stage (Supplemental Fig. 4). PMVs originating from different cell lines were analyzed to determine whether their effect on invasion was dependent on a molecule produced by the cell of origin. Interestingly, PMVs from THP-1 and Jurkat cells, and to a lesser extent those from HepG2 cells, increased parasite invasion (Fig. 5*B*). Measurements of cytokines carried by these PMVs revealed a striking correlation

between the levels of TGF-β and the extent of invasion (Fig. 5*C*, compare with Fig. 5*B*). Previous works have shown that TGF-β induces invasion of *T. cruzi* in several cell lines (32, 33). Because PMVs bind to the surface of *T. cruzi* (Fig. 4*B*), and it remains bound to the parasite surface for as long as 1 h (Supplemental Fig. 3), we reasoned that TGF-β–bearing PMVs on the *T. cruzi* surface could be mediating enhanced invasion. To investigate this, parasites were preincubated with PMVs prior to invasion assays. Increased invasion was observed (Fig. 5*D*, Supplemental Fig. 4); this increase was inhibited by anti–TGF-β Abs and the TGF-β receptor antagonist, SB-431542 (Fig. 5*D*, Supplemental Fig. 4). Consistently, about one third of the parasites incubated with PMVs were positive for TGF-β when analyzed by flow cytometry (Fig. 5*E*, *right*) in agreement with TGF-β being carried on PMV surface (Fig. 5*E*, *left*, Supplemental Fig. 4). It is noteworthy that PMVs increased the number of intracellular parasites per cell in addition to an increase in infection (Fig. 5*F*), indicating that invading *T. cruzi* carry PMVs to cells and thereby make them more susceptible to additional invasions.

To determine whether PMVs released from THP-1 cells after *T. cruzi* stimulation could simultaneously increase invasion of neighboring cells, metacyclic trypomastigotes were incubated either in the upper chamber of a Transwell plate with THP-1 cells or in a lower chamber containing Vero cells. Addition of *T. cruzi* to the THP-1 cell chamber increased invasion of Vero cells (Fig. 5*G*), indicating that PMVs released by THP-1 cells could simultaneously induce an increase in Vero cell invasion. Furthermore, addition of SB-431542 to the Vero cells ablated the enhancement in invasion (Fig. 5*G*). Together, these results confirm that *T. cruzi* carry TGF-β–bearing PMVs on their surface, thereby promoting enhanced invasion.
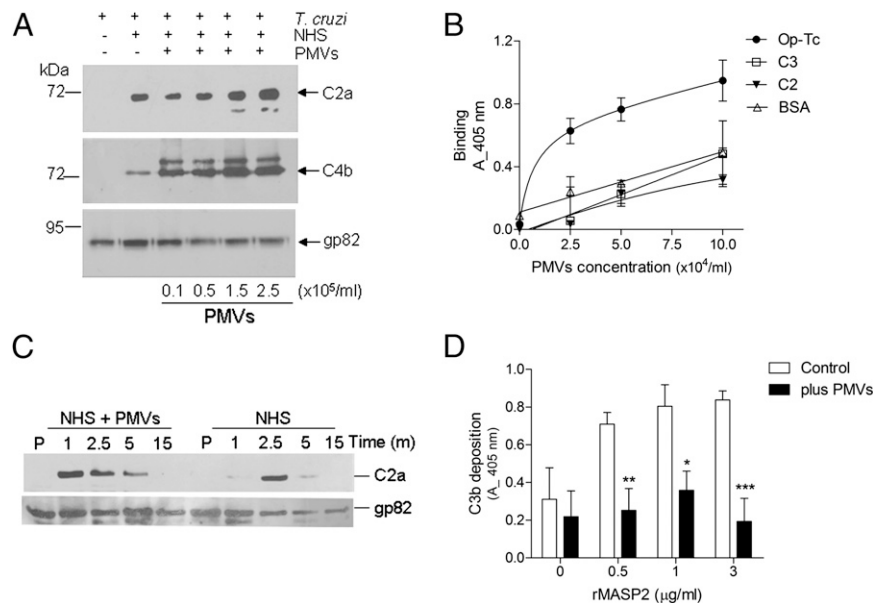
FIGURE 4. PMVs bind to C4b2a and inhibit the cleavage of C3. *A*, PMVs were added to 10% NHS and incubated with parasites for 5 min at 37°C. Parasites were harvested (to remove NHS), and their protein extracts were analyzed by Western blotting with Abs against C2 and C4. The membrane was stripped and reprobed with anti-3F6 mAbs to detect gp82. *B*, Purified C2, C3, BSA, or *T. cruzi* opsonized with NHS (Op-Tc) were adsorbed on ELISA plates and incubated with biotinylated PMVs for 2 h at 37°C. Binding of PMVs was detected with streptavidin-HRP. *C*, PMVs (1.5×10^5/ml) were added to 10% NHS and incubated with parasites at 37°C for 1–15 min. Parasites were harvested (to remove NHS), and their protein extracts were analyzed by Western blotting with anti-C2 Abs. The membrane was stripped and reprobed with anti-3F6 Abs. P, parasites with or without PMVs. *D*, PMV inhibits the activity of C3 convertase assembled on metacyclic trypomastigote surface. C4b-opsonized *T. cruzi* were incubated with fixed concentrations of C2 (1 μg/ml) and C3 (5 μg/ml). Increasing concentrations of rMASP-2 (0.5–3 μg/ml) were added and incubated for 90 min (for C3 convertase formation) with or without PMVs (1.5×10^5/ml). C3b deposition on parasites was analyzed with anti-C3 Abs by ELISA. Note that addition of rMASP-2 results in cleavage of C2, which leads to C3 convertase formation (C4b2a) through the binding of C2a fragments to C4b (previously bound to the parasite surface). The C3 convertase (C4b2a) cleaves C3, forming C3a and C3b; the latter bind to the parasite surface. The cleavage is monitored, analyzing the deposition of the cleavage product C3b on the parasite surface using anti-C3b Abs. The data shown in *B* and *D* represent the mean ± SD from four independent experiments. Experiments in *A* and *C* are representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

PMVs increase T. cruzi *invasion of eukaryotic cells via the lysosome-independent pathway*

Invasion of nonprofessional phagocytic cells by *T. cruzi* has been reported to occur either via a lysosome-dependent route (20–30%), or via plasma membrane invagination (70–80%) (34). The latter is followed by late lysosome migration and fusion with the host plasma membrane, forming the phagolysosome (34). To determine by which pathway PMVs induce *T. cruzi* entry, Vero cells were pretreated with thapsigargin (which inhibits invasion by depleting intracellular Ca²⁺) (35) and/or wortmannin (which inhibits specifically the lysosome-dependent pathway) (34) and then infected with metacyclic trypomastigotes. Addition of thapsigargin inhibited the PMV enhancement of *T. cruzi* invasion (Fig. 6A). However, addition of wortmannin in the presence of PMVs caused only a slight reduction of *T. cruzi* invasion, suggesting that parasite entry occurs by the lysosome-independent pathway (Fig. 6A). To confirm this result, Vero cells expressing GFP-tagged lysosomal-associated membrane protein-1 (LAMP-1) were used to monitor lysosome-dependent entry. Addition of PMVs caused an increase of parasite invasion independent of LAMP-1 association (Fig. 6B). The number of parasites associated with lysosomes was constant, but an increase in unmarked parasites was detected (as depicted in Fig. 6B, 6C), arguing that PMVs induce *T. cruzi* entry in non-professional phagocytic cells by a lysosome-independent route.

T. cruzi *induce the release of TGF-β–bearing PMVs from blood cells in vivo*

To address whether *T. cruzi* induce PMV release from blood cells in vivo, mice were infected with metacyclic trypomastigotes and plasma-circulating PMVs were analyzed (detail exemplified in Supplemental Fig. 1). A ~40% increase in the number of annexin V-positive PMVs was detected at 30 min postinfection (Fig. 7A). This amount was maintained at 4 and 24 h and even 8 d postinfection (Fig. 7B), indicating that *T. cruzi* induces PMV release in vivo. The stable level of PMVs over time in infected mice suggests that they might engage a mechanism of controlling plasma PMV levels, such as phagocytosis by macrophages (36). A 48% increase of TGF-β1 in PMVs from infected mice was also detected at 24 h (Fig. 7C). Indeed, 35% of the annexin V-positive PMVs from infected mice contained TGF-β at the peak of the parasitemia (Fig. 7D), confirming the release of TGF-β–bearing PMVs during the infection. Annexin V-positive PMVs containing CD184 (CXCR4) also increased 68% in infected mice (Fig. 7D). CD184 is a molecule present in monocytes, T subset cells, B cells, dendritic cells, and endothelial cells, suggesting that *T. cruzi* could be inducing these cells to release PMVs in vivo. To determine whether the PMVs could promote an increase in *T. cruzi* infection in vivo, mice were infected in the presence of THP-1–derived PMVs. Parasitemia was higher in mice infected in the presence of PMVs than in control mice (infected without PMVs) (Fig. 7E). At the parasitemia peak, there were 7 times more parasites in mice infected in the presence of PMVs than in the control (Fig. 7E); however, parasitemias were equal at day 20 postinfection. Taken together, these results suggest that *T. cruzi* metacyclic trypomastigotes induce in vivo the release of PMVs from blood cells early in the infection, contributing to the parasite's escape from the complement attack. Furthermore, the release of TGF-β–bearing PMVs could also favor parasite invasion in the course of infection.
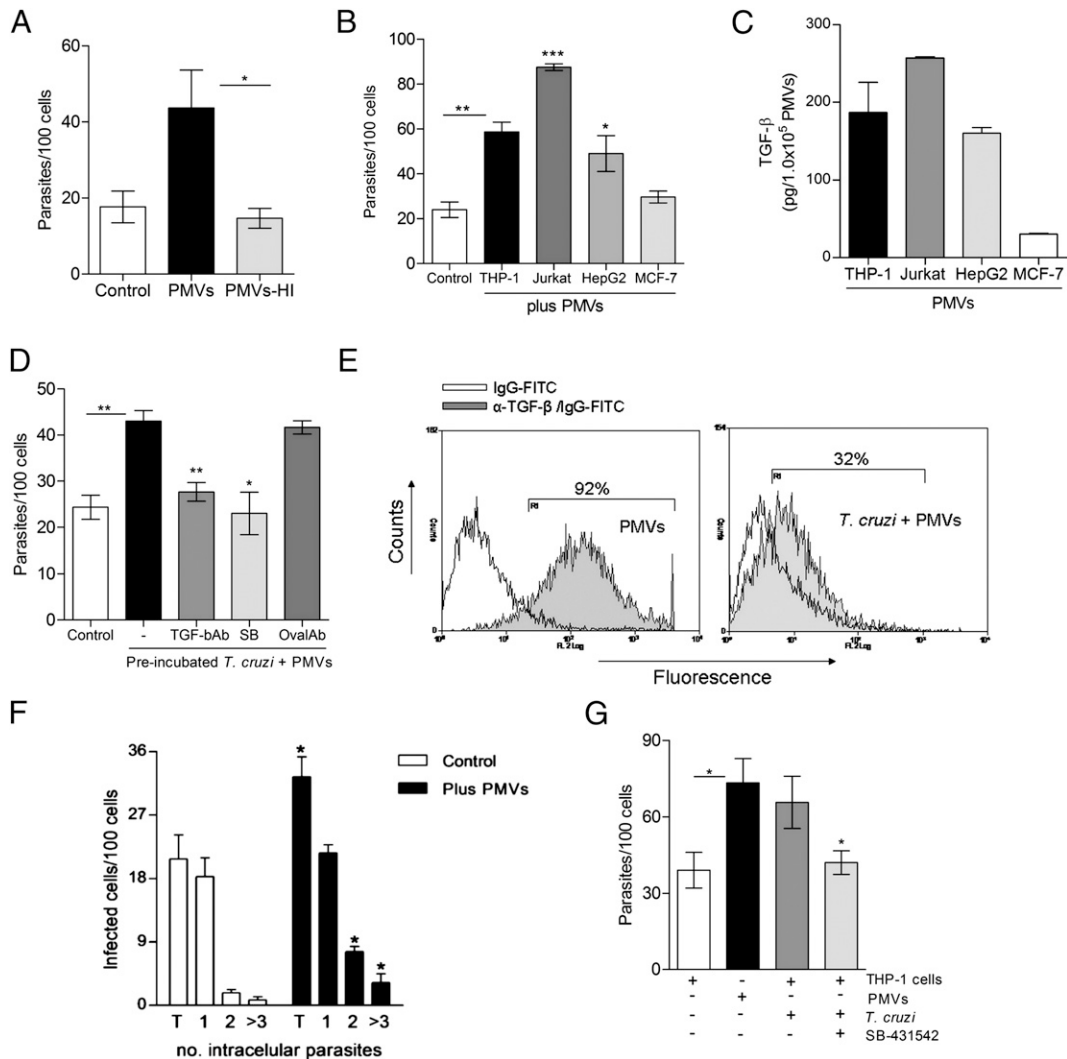
FIGURE 5. TGF-β–bearing PMVs bound to *T. cruzi* surface mediate cell invasion. *A*, THP-1–derived PMVs (1.0 × 10^5/ml) induce increased metacyclic trypomastigote invasion of Vero cells. Heat-inactivated PMVs (PMVs-HI) were used as control. Invasion assays were performed at 37°C for 3 h, and intracellular parasites were quantified. Ratio = 10:1 (parasites/cell). *B*, PMVs (1.0 × 10^5/ml) were obtained from THP-1, Jurkat, HepG2, and MCF-7 cells and used for Vero cell invasion assays with metacyclic trypomastigotes (as described in *A*). *C*, TGF-β levels in PMVs from THP-1, Jurkat, HepG2, and MCF-7 cells (used in the experiment in *B*) measured by ELISA. *D*, *T. cruzi* preincubated with PMVs (30 min at 37°C) were either added to the Vero cells (-) or incubated with Abs against TGF-β (TGF-βAb) or egg-OVA (OvalAb) before invasion assays. Vero cells were also treated with SB431542 (SB) before invasion assays. Invasion assays were performed for 3 h at 37°C, and intracellular parasites were quantified. Ratio = 10:1 (parasites/cells). *E*, Flow cytometry detection of TGF-β on PMVs (*left panel*). TGF-β detection on metacyclic trypomastigotes preincubated with PMVs (*right panel*). PMVs were incubated at 37°C for 30 min with metacyclic trypomastigotes (ratio = 5:1, PMV/parasites). Afterward, parasites were washed for removal of unbound PMVs and analyzed by flow cytometry with polyclonal anti–TGF-β Abs. *F*, PMVs induce an increase of infected cells containing 2, 3, or >3 parasites/cell. Invasion assays were performed with or without PMVs for 3 h at 37°C, and the amount of intracellular parasites per infected cells was quantified. *G*, PMVs released by THP-1 cells induce simultaneous *T. cruzi* invasion. Vero cells were seeded in the lower chamber of a Transwell plate, whereas THP-1 cells were incubated in the upper chamber. *T. cruzi* were added on both chambers (with Vero cells for invasion assay; and with THP-1 for PMV induction). PMVs (1.0 × 10^5/ml) were added in the upper chamber, and 10 μM SB431542 was preincubated with Vero cells before parasite addition (see figure). For invasion assay, a ratio of 10:1 was used, and for PMV induction, a ratio of 5:1 was used, parasites/cells. The data shown from *A–D* and *F–G* represent the mean ± SD from four independent experiments. Experiment in *E* is representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

We report in this study a novel mechanism used by *T. cruzi* to evade the host innate immune system. *T. cruzi* induce host blood cells to release PMVs, which are involved in both inhibiting complement-mediated lysis and also in aiding host cell invasion. First, we showed that *T. cruzi* induce PMV release from blood cells in vitro and in vivo. Induction of PMV release from blood cells is specifically stimulated by the *T. cruzi* infective stage, and is dose dependent on parasite number. This induction occurs rapidly, only a few minutes after parasite/cell contact, and is dependent on Ca^{2+}. Because it has been shown that the *T. cruzi* molecules gp82 and

oligopeptidase B can induce a transient increase of intracellular Ca^{2+} in host cells (37–39), it is possible that they could be involved in inducing PMV release. Our finding that *T. cruzi* induce PMV release from blood cells is consistent with observations that conditions of disease or stress result in increased PMV levels (15, 16, 18). For example, high plasma levels of PMVs have been observed during malaria infection (20), pregnancy (40), thrombosis (41), and cancer (18, 40). We and others have shown that PMVs can be induced from PBMCs (41, 42). Pathogens have most likely evolved this strategy because PBMCs migrate to infection sites, thereby creating a high concentration of PMVs at the site of invasion.
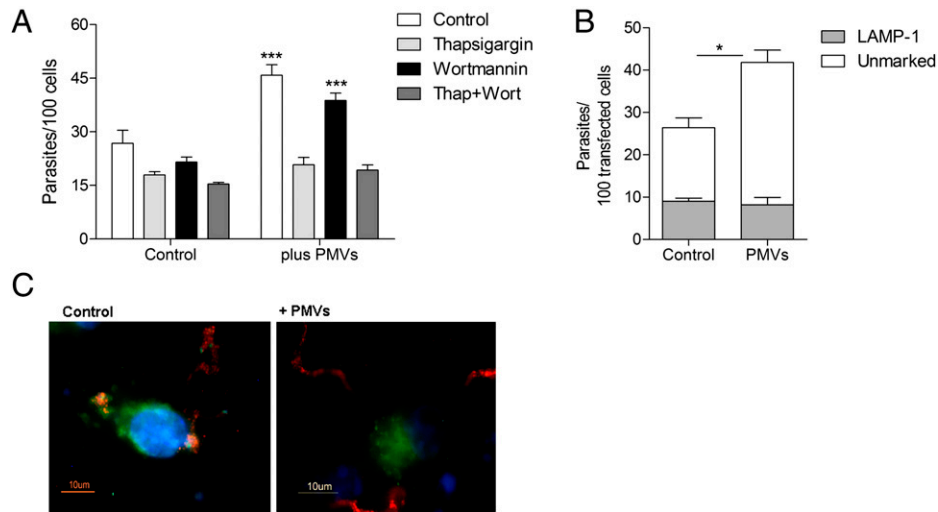
FIGURE 6. PMVs induce *T. cruzi* invasion of eukaryotic cells by the lysosome-independent pathway. *A*, Vero cells were pretreated with 1.5 μM thapsigargin, 100 nm wortmannin, or both together (for 15 min at 37°C) before invasion assays. Cells were washed and incubated with metacyclic trypomastigotes (ratio = 20:1, parasites/cells) for 30 min at 37°C, and intracellular parasites were quantified. *B*, Vero cells expressing GFP-tagged LAMP-1 were used for invasion assays. Cells were incubated with metacyclic trypomastigotes (ratio = 20:1, parasites/cells) for 30 min at 37°C, and intracellular parasites were quantified. *C*, Immunofluorescence of Vero cells expressing GFP-tagged LAMP-1 (green) infected with *T. cruzi* (red, detected with anti-3F6 Abs) from experiment in *B*. Nuclei were stained with DAPI (blue). The data shown in *A* and *B* represent the mean ± SD from four independent experiments. $*p < 0.05$, $***p < 0.001$.

We have also found that PMVs aid parasite immune invasion by inhibiting complement-mediated lysis of *T. cruzi*. PMVs inhibited the classical and lectin pathways through their binding to the complement C3 convertase (C4b2a), a key complex in the com- plement cascade. It is noteworthy that *T. cruzi* strongly activate the lectin pathway in nonimmune serum (7, 23) [as well as the clas- sical pathway in the presence of specific Abs (4)]; however, there are only two molecules described to date involved in controlling
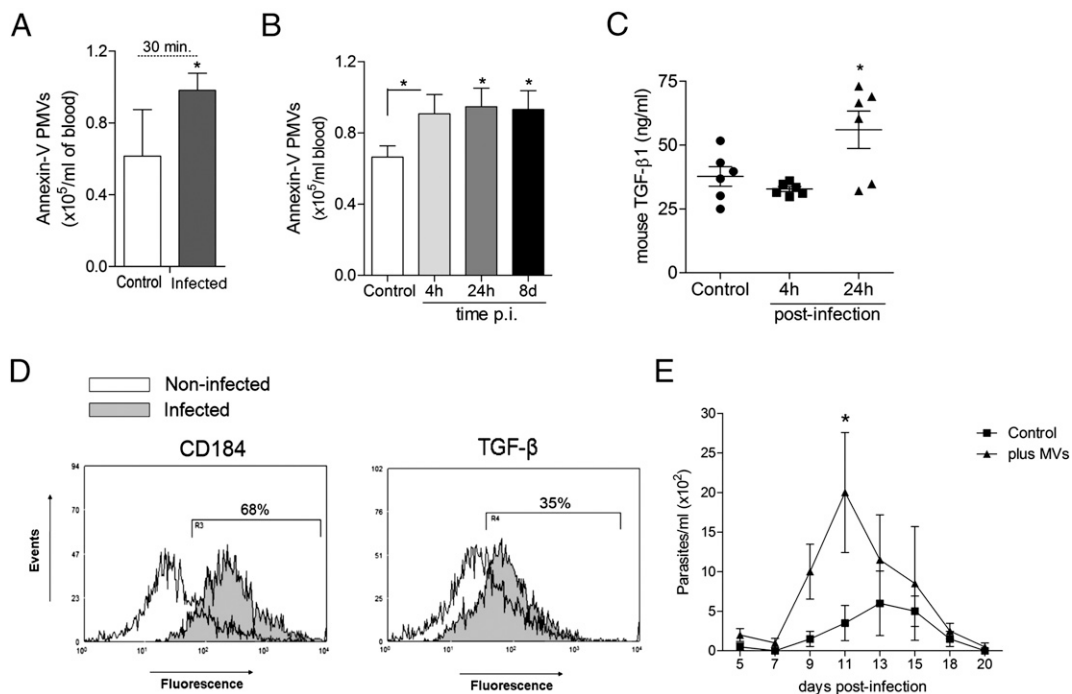
FIGURE 7. *T. cruzi* induce PMV release in vivo. *A*, *T. cruzi* induce PMV release in mice early during infection. Mice were infected with metacyclic trypomastigotes (5.0×10^5) or PBS by i.p. injection, and, after 30 min, the plasma was collected by cardiac puncture. PMVs were obtained from plasma by differential centrifugation, labeled with annexin V-FITC, and quantified by flow cytometry. $n = 3$ for each treatment. *B*, As in *A*, except that the plasma was collected after 4 h, 24 h, or 8 d postinfection. $n = 5$ for each treatment. *C*, PMVs obtained from mice (as in the experiment in *A*) were analyzed by ELISA for mouse TGF-β1 detection. The experiment was performed three times in duplicate each. *D*, Mice were infected with metacyclic trypomastigotes (5.0×10^5) by i.p. injection, and 11 d postinfection (parasitemia peak) the plasma was collected by cardiac puncture. Noninfected mice received PBS only. PMVs were obtained by differential centrifugation, and annexin V-FITC–positive PMVs were analyzed by flow cytometry with anti-CD184 and anti–TGF-β. Result shown is a representative experiment of three single experiments. *E*, Mice were infected with metacyclic trypomastigotes (5.0×10^5) with or without THP-1–derived PMVs (1.5×10^5) by i.p. injection, and parasitemias were analyzed every 2 d postinfection by tail pick. $n = 4$ for each treatment. $*p < 0.05$.

complement activation by these pathways, named CRIT (a complement C2 receptor) and calreticulin (which bind C1q) (23, 27, 43), strengthening the idea that *T. cruzi* uses PMV as an additional factor to inhibit the activation of these complement pathways. In contrast, the alternative pathway is slowly activated by *T. cruzi* because of the high amount of sialic acid on the surface mucins (44, 45). It has been shown that the sialic acid on *T. cruzi* surface binds to factor H, causing C3 inactivation to iC3b, resulting in reduction of the alternative pathway activation (44, 46). The mechanism of recruiting complement regulators (including factor H) by surface carbohydrates has also been reported in other pathogens, such as *Neisseria sp* (47). A slow activation of the alternative pathway by other trypanosomatids has also been observed (48). In contrast to few molecules involved in evasion of the classical and lectin pathways, several molecules have been reported to specifically inhibit the alternative pathway (2, 45). Altogether, it indicates that *T. cruzi* evolved a specific mechanism to compensate the absence of complement receptors to control the classical and lectin pathway C3 convertases. The binding of PMVs to the C3 convertase assembled on the parasite surface causes a delay in its dissociation, as well as affecting its activity by inhibiting C3 cleavage. Inhibition of C3 cleavage has several biological significances, as follows: 1) C3 is necessary for complement lysis by all pathways (2); 2) it is required to generate the anaphylatoxin C3a and C5a, important in the recruitment of cell response against the pathogen (49); and 3) it is also involved in opsonization, which mediates phagocytosis of pathogens during the infection (2). It is noteworthy that PMVs interact with complement factors and mediate immune adherence to erythrocytes (16, 42, 50). Indeed, vesicles exposing phosphatidylserine have been shown to inhibit the complement system (51). Altogether, these data suggest that PMVs interact with the complement system inhibiting parasite clearance early during the infection, thereby increasing the chance of parasites succeeding in infection.

Recently, another complement evasion mechanism inhibiting the C3 convertase was described in *Staphylococcus aureus* (52). The molecule staphylococcal complement inhibitor stabilizes the C3 convertases and inhibits their catalytic activity (52), showing that inhibition of C3 convertase dissociation and activity is a mechanism also used by other pathogens. We still do not know which molecules on the PMVs interact with C3 convertases. However, further investigation has been taken to identify the molecules involved in this mechanism.

An important feature of PMVs is their capacity to carry molecules from the cell of origin (10–12). Consistent with others (17, 53), we have found that PMVs carry TGF-β, a cytokine proposed to increase *T. cruzi* invasion of epithelial and cardiac cells (32, 54), aid the intracellular parasite cycle (55), and contribute to fibrosis during acute and chronic Chagas disease (56). The effect of PMVs on *T. cruzi* invasion was inhibited by using neutralizing anti–TGF-β Abs, as well as by treating the cells with TGF-β receptor antagonist, arguing strongly that the increase in parasite invasion relied upon TGF-β. Consistent with our finding that *T. cruzi* induces the release of PMVs carrying TGF-β, chronic-phase Chagas disease patients have been shown to have elevated levels of circulating TGF-β (54). Furthermore, in mouse models, the levels of TGF-β increase with acute *T. cruzi* infection (57), and TGF-β is activated by *T. cruzi* during cell invasion (58). Our results are in agreement with these reports, because the levels of TGF-β–bearing PMVs increased 24 h postinfection and persisted for several days. Indeed, we found that PMVs carry a high amount of TGF-β (~35–55 ng/ml plasma), and PMVs released by monocytes and lymphocytes can simultaneously promote *T. cruzi* invasion. Because many nonprofessional phagocytic cells synthesize TGF-β either at low levels or

not at all, the binding of TGF-β–bearing PMVs to the surface of *T. cruzi* provides an important mechanism of targeting the cytokine to the site of invasion. We also observed that the increase of *T. cruzi* invasion in the presence of PMVs consisted of an increase of the number of intracellular parasites per cell in addition to an increase in the total number of infected cells. This result suggests that once a cell is infected, it is more susceptible to a second round of infection. We speculate that the initial invading parasite carries PMVs to the host cell it invades, which activate signaling cascades that weaken the cell's defenses. Furthermore, PMVs induced *T. cruzi* cell invasion specifically through the lysosome-independent route, providing further evidence that PMVs promote particular cellular cascades. It is therefore likely that TGF-β–bearing PMVs activate the TGF-β signaling pathway to promote *T. cruzi* invasion. It is noteworthy that PMV enhancements in cell invasion also contribute to parasites escaping the complement attack, as well as increase the number of parasites that progress to their life cycle, what can be an initial determinant in the success of infection.

Finally, we showed that *T. cruzi* parasitemia increases in mice infected in the presence of PMVs, which corroborates our in vitro experiments. The increased parasitemia in the presence of PMVs could be interpreted as a consequence of two factors, as follows: 1) the effect of PMVs on the inhibition of *T. cruzi* complement-mediated lysis; and 2) the effect of TGF-β on *T. cruzi* cell invasion. Therefore, we establish in this work that *T. cruzi* induce blood cells to release PMVs, which act as a host factor contributing to parasite immune evasion.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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