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Differential ability to resist to complement lysis and invade host cells mediated by MBL in R4 and 860 strains of *Trypanosoma cruzi*

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ABSTRACT

To produce an infection *Trypanosoma cruzi* must evade lysis by the complement system. During early stages of infection, the lectin pathway plays an important role in host defense and can be activated by binding of mannan-binding lectin (MBL) to carbohydrates on the surface of pathogens. We hypothesized that MBL has a dual role during parasite-host cell interaction as lectin complement pathway activator and as binding molecule to invade the host cell. We used two polarized strains of *T. cruzi*, R4 (susceptible) and 860 (resistant) strains, to investigate the role of MBL in complement-mediated lysis. Interestingly R4, but not 860 metacyclic strain, markedly increases the invasion of host cells, suggesting that MBL drives the invasion process while the parasite deactivates the Lectin complement pathway.

1. Introduction

Chagas' disease (CD) affects several million people worldwide, mostly in Latin America, being considered one of the most important neglected tropical diseases and a global health problem [1]. This disease is caused by an obligate intracellular protozoan parasite, *Trypanosoma cruzi*, transmitted mainly by triatomine bugs. The parasite replicates inside host cells and evades immune system recognition, causing a life-longing disease. Clinical manifestations de CD include digestive, neurologic and especially cardiac symptoms, which may progress from arrhythmia to heart failure [2].

The complement system is one of the first mechanisms of innate immunity against pathogen infection, which culminates with the formation of a membrane attack complex leading to pathogen lysis. There are three pathways of complement activation: the classical, the alternative and the lectin pathway. At early stages of infection, the lectin pathway plays an important role in the host defense and can be activated by the binding of mannan-binding lectin (MBL), L-Ficolin, H-Ficolin and M-Ficolin to carbohydrates on pathogens surface [3,4]. In order to succeed the infection, *T. cruzi* needs to circumvent complement activation by producing a series of inhibitory proteins such as Crit [5], CRP [6] and Calreticulin [7].

MBL is a recognition molecule of the collectin family that has a central role in the activation of the lectin pathway. MBL forms complexes with MBL-associated serine proteases (MASPs) and recognizes the terminal mannose or N-acetylglucosamine (GlcNAc) residues on glycoproteins or carbohydrate polymers on pathogen surface, in a Ca-dependent manner, resulting in pathogen opsonization and complement activation [8].

MBL deficiency is the most common form of congenital immune deficiency, with a prevalence of approximately 10% in European populations. Clinically significant defects in MBL serum level were associated with recurrent bacterial infection. Although MBL levels may differ between individuals with the same MBL2 genotypes, MBL levels are mostly genetically determined. Increased MBL levels are associated with high secretor MBL genotypes, related to MBL2 gene polymorphisms in both promoter and structural (exon 1) regions [9]. On the other hand, null and low producing MBL haplotypes are to be found in 22% and 13% of the world's population, respectively [10].

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MBL deficient mice were shown to have increased susceptibility to *T. cruzi* infection, with higher parasite load, cardiomyopathy and fibrosis when compared to normal mice [11]. MBL deficiency was also associated with infection by the enteric protozoa Cryptosporidium in children [12]. MBL levels were also correlated with clinical outcome of CD patients, with acute symptoms related to increased MBL levels in serum in comparison to asymptomatic and indeterminate forms [11]. In addition, high levels of MBL (>1000 ng/ml) were associated with the severity of cardiomyopathy in CD, which may be related to the pro-inflammatory role of MBL [13].

Other components of the lectin pathway were also shown to be associated with susceptibility to CD and disease outcome. Lower L-ficolin levels, correlated to 258S variant, were associated with CD susceptibility and severity of the disease [14]. Furthermore, MASP2*CD genotypes resulting in low MASP-2 levels were related to increased risk of chagasic cardiomyopathy. Low levels of MASP-2 could result in inefficient lectin pathway activation and therefore favor parasite infection and disease progression [15]. *T. cruzi* activates the lectin pathway through the rapid binding of MBL, L-Ficolin and H-Ficolin to glycosylated molecules in the surface of metacyclic trypomastigotes forms. Complement activation using human serum depleted of MBL and ficolins resulted in almost 70% decrease of C3b and C4b deposition on parasite surface and complement-mediated lysis. The ability to resist complement system killing and to invade the host cells to progress in infection is known to differ among *T. cruzi* strains [16,17].

It has been reported that *T. cruzi* uses a complex repertoire of molecules and mechanisms to invade host cells [18,19]. Here, we hypothesize that some strains of *T. cruzi* use MBL to invade host cells before complete activation of complement through the lectin pathway; while other strains may activate mechanisms to inhibit the efficient MBL-dependent infection.

2. Methods

2.1. Parasites and cells

T. cruzi epimastigote forms from 860 and R4 strains were cultivated in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum at 27 °C [20]. Metacyclic trypomastigotes were obtained from grown epimastigote cultures cultivated in GRACE medium (Sigma–Aldrich, USA) for 10–15 days and purified using DEAE-cellulose columns in ice-cold PSG buffer (3.8 mM KH_2PO_4, 57.2 mM Na_2HPO_4, 44.5 mM NaCl, 55.5 mM $C_6H_{12}O_6$, pH 8.0).

Vero cells were grown in RPMI 1640 (Sigma–Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine, 100 g/mL streptomycin and 100 U/mL penicilin in a humidified incubator at 37 °C and 5% CO_2.

2.2. Human serum

Normal human serum (NHS; MBL > 1000 ng/mL) and MBL deficient human serum (ΔMBL > 100 ng/mL) were obtained from voluntary donors. MBL levels were previously determined using ELISA kit (Antibodyshop, Denmark). Recombinat MBL (rMBL) (R&D systems) was used in the complement-mediated lysis and cell invasion essays.

2.3. Complement mediated-lysis assay

Complement assays were carried out as previously described [5]. In brief, *T. cruzi* epimastigotes or metacyclic trypomastigotes at 5.0×10^6 parasites/100 μL in serum-free RPMI, were incubated with 100 μL of 25% or 50% NHS, ΔMBL or RPMI only (as negative control) for 15 and 30 min at 37 °C. Reactions were stopped upon the addition of 800 μL ice-cold RPMI.

In assays blocking classical and lectin pathways activation, epimastigote forms (5.0×10^5 in 100 μL) resuspended in serum-free RPMI were incubated with both 100 μL of 10% NHS or 10% ΔMBL with 10 mM ethylene glycol tetraacetic acid (EGTA) for 15 or 30 min at 37 °C. Reactions were stopped as described above.

Viable parasites were then counted in a Neubauer chamber under light microscopy.

2.4. Western blotting

Parasites from both 860 and R4 strains of *T. cruzi* epimastigote and metacyclic trypomastigote forms were treated with 10% NHS during 30 min. Protein extracts of parasites were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and after transferred to nitrocellulose membrane. The membrane was then incubated with monoclonal mouse anti-human MBL (1:1000, Antibodyshop, Denmark) and secondly with goat anti-mouse IgG conjugated with HRP (1:500) (Santa Cruz Biotech) as described elsewhere [21].

2.5. Parasite invasion assays

The growth of Vero cells and the metacyclic trypomastigote invasion assays were carried out as described previously [16] with minor modifications. Briefly, metacyclic trypomastigotes were incubated with Vero cells in either 10% NHS, 10% ΔMBL, 1 μg/mL of recombinant MBL or serum-free RPMI for three hours at 37 °C. The parasite/cell ratio was 10:1 in all experiments. The rate of infection was determined by counting the number of intracellular parasites in 500 cells stained by Giemsa.

2.6. Fluorescence-activated cell sorter (FACS) analysis

FACS analyses were carried out as described previously [5]. Briefly, *T. cruzi* (1.0×10^6 parasites) were incubated with NHS at 10% final concentration for 1 h at 4 °C, washed in PBS, and incubated with anti-MBL (1:1000) in PBS 3% BSA for 1 h at 4 °C. Parasites were then incubated with anti-mouse IgG-FITC (1:500) antibodies in PBS 3% BSA for 1 h at 4 °C and washed in PBS. We have established gating on live cells on forward (FSC) v/s side scatter (SSC) plots, where we have discriminated live cells gate than dead and debris. We also have used single-stained BD™ compbead particles as compensation controls. After reaction, the parasites were analyzed with Accuri C6 flow cytometer using CFlow Plus software (Accuri, Ann Arbor, MI, USA).

2.7. Statistical analysis

All experiments were performed in triplicates. Values are shown as mean ± 2S.D. Comparisons were made using the unpaired *t*-test for repeated measures using GraphPad Prism version 5.04 software (San Diego, California, USA). $P < 0.05$ was considered significant.

3. Results and discussion

In this study, we defined the susceptibility to complement-mediated lysis of R4 and 860 strains of *T. cruzi*. We hypothesized that strains of *T. cruzi* display MBL acceptors at the plasmatic membrane and use the high MBL concentration in serum to rapidly invade host cells, avoiding successful complement activation.

In our assays we used a pool of ΔMBL serum and a pool of NHS to investigate the involvement of MBL in the invasion of metacyclic tripomastigote forms of *T. cruzi* to eukaryotic cells and to evaluate the activation of the lectin complement pathway.

First of all, we analyzed the ability of the different sera to lyse the epimastigote forms of R4 and 860 strains of *T. cruzi*. We observed a complete inefficiency of ΔMBL serum to lyse 860 strain epimastigote forms when compared with the NHS. However, such strong influence of MBL at the complement activation was not observed in the lysis of the R4 strain. ΔMBL serum lysed more than 80% of the R4 parasites in 30 min, which was similar to the lysis obtained with NHS (85%). These results suggests the involvement of alternative and classic complement pathways in R4 parasite lysis (Fig. 1A). Taken together, these results of complement-mediated lysis indicate a possible important role of MBL in the lysis of *T. cruzi* 860 strain.

To better understand MBL participation in complement-mediated lysis of 860 strain, we investigated the role of alternative pathway by inhibiting lectin and classic complement pathways using EGTA-treated serum. Interestingly, the EGTA-treated serum was not able to lyse parasites at the first 15 min and only less than 10% of the parasites were lysed after 30 min, indicating a minor role of the alternative pathway in the 860 complement mediated lysis (Fig. 1B). To confirm the role of MBL in the complement-mediated lysis of 860 strain we evaluated the ability of MBL to restore the lytic effect of complement in ΔMBL serum. A remarkable restitution of the complement efficiency to lyse the parasites was obtained by the addition of rMBL to the ΔMBL serum, thus restoring the function of the lectin pathway activation. We observed a dose dependent effect by adding 1 and 2 μg/mL rMBL to the ΔMBL serum, which resulted in 25–50% of lysis of epimastigote forms of 860 strain (Fig. 1C). Once we defined the effect of the complement system in epimastigote forms of both strains, we investigated the complement-mediated lysis of metacyclic trypomastigote forms. Again, the sensitivity to complement-mediated lysis of metacyclic trypomastigote differed between R4 and 860 strains. Metacyclic forms of R4 strain presented high sensitivity to complement lysis (Fig. 1D). By analyzing serial dilutions of NHS and ΔMBL serum using R4 strain, we observed a dose dependent effect ranging from 40% to 60% of parasite lysis at 30 min for both serum conditions (Fig. 1D). However, no lysis was detected in any condition for the 860 strain.

We have already reported empirically that some strains are resistant to complement lysis and other are sensitive [16] and that could be crucial to proceed with the infection. Several evasion mechanisms to the innate immunity have been described for *T. cruzi* before, the more effective being the ability to invade host cells before parasite elimination by the complement system [21].

In order to evaluate whether eukaryotic cell invasion was influenced by the differential susceptibility to complement-mediated lysis seen for R4 and 860 strains, metacyclic tripomastigote forms of the parasites were tested in vitro invasion assays using Vero cells.

The R4 metacyclic invasion was significantly increased (3-fold) when the cells were incubated with 10% NHS, in comparison to the infection obtained when cells were incubated with ΔMBL serum or

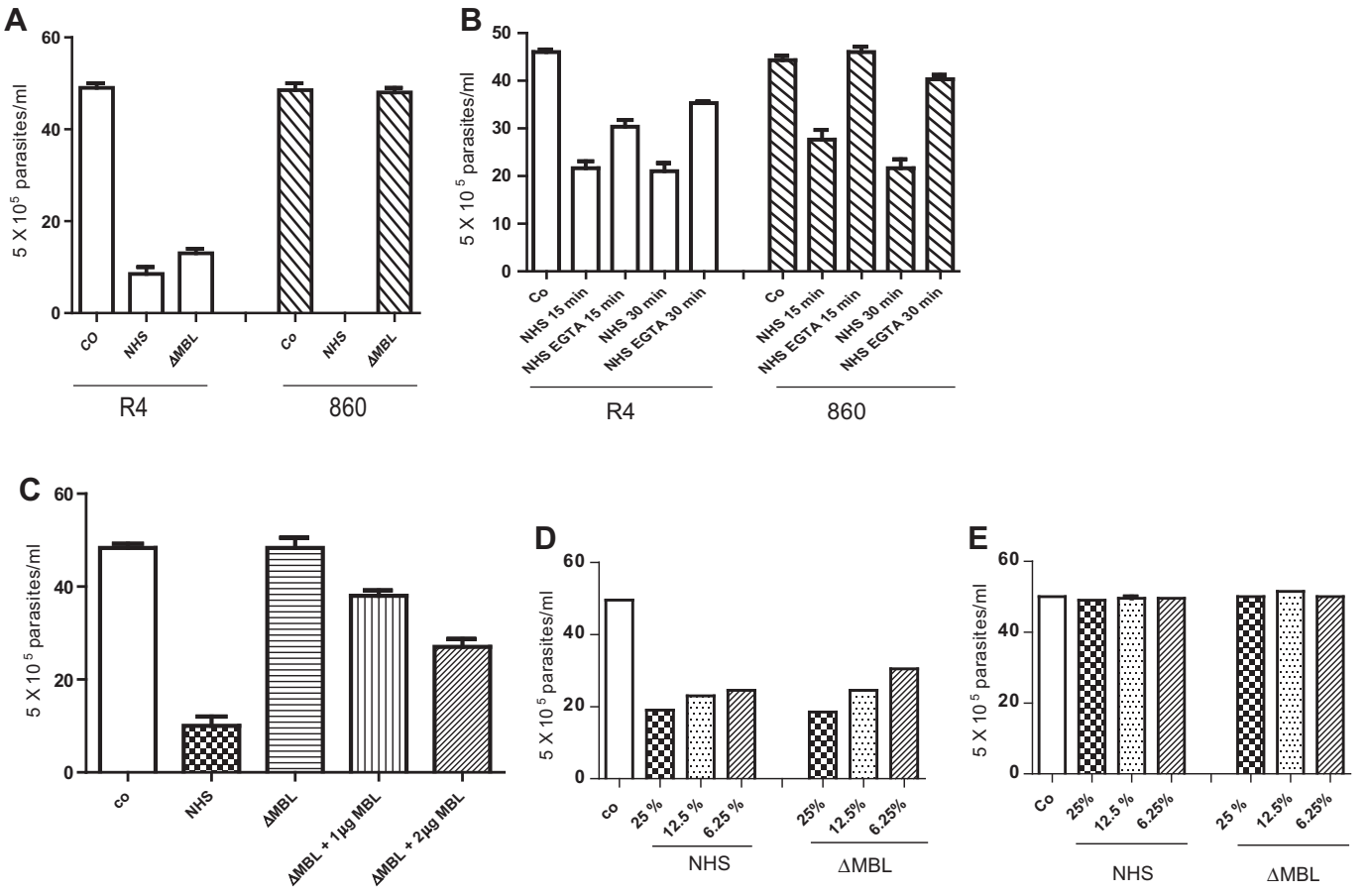

Fig. 1. R4 and 860 *T. cruzi* strains differ in the susceptibility to complement mediated lysis. (A) Lysis of epimastigote forms. 860 and R4 strains were incubated with 50% normal or ΔMBL serum for 30 min. (B) Alternative complement pathway lysis. Epimastigote forms of 860 and R4 strains were incubated with NHS treated with 10 mM EGTA and 7 mM Mg (to inhibit lectin and classical complement pathways). (C) Induction of complement lectin pathway activation using ΔMBL serum restored with addition of rMBL. Epimastigote forms of 860 strain were incubated with 12% ΔMBL serum treated with 1 or 2 μg/mL rMBL. (D) Metacyclic trypomastigote forms of R4 strain are sensitive to complement-mediated lysis and this is not dependent on the lectin pathway lysis. (E) Metacyclic trypomastigote forms of 860 strain are resistant to lysis of all complement pathway.

with RPMI alone (Fig. 2C).Inversely, the invasion rate of 860 strain was not significantly increased when the metacyclic tripomastigotes were opsonized with NHS (Fig. 2C).

These results suggest that components from NHS are stimulating the R4 parasite invasion into Vero cells, including the deposition of MBL at the surface of metacyclic tripomastigote forms. This ability of the parasite surface to bind MBL unveils a fine balance between the complement activation and the opsonization effect during the invasion of eukaryotic cells.

The ability of both strains R4 and 860 to bind MBL were investigated by flow cytometry (Fig. 2A) and by western blotting (Fig. 2B). We observed a higher deposition of MBL in R4 metacyclic forms in comparison to 860 strain. This is in agreement with the interesting pattern of MBL acceptors observed when the parasites were incubated with NHS and analyzed by western blotting with anti-human MBL. Fig. 2B shows a band ranking over 100 kDa in both epimastigote and metacyclic tripomastigote forms of R4 but not in 860 strain. Indeed, a band about 60 kDa was observed in both metacyclic strains R4 and 860 and not in epimastigote forms, suggesting that different MBL acceptors are probably involved in complement activation or in the invasion process depending on the strain and evolutive forms of the parasite. This difference was also observed in flow cytometry where a higher deposition of MBL at the membrane surface of R4 was observed in comparison to 860 strain (Fig. 2A). Moreover we have neutralized the effect of MBL at the R4 invasion and at the Lectin complement pathway activation pre-treating the NHS with N-acetyl glicosamine and Mannose, blocking invasion and avoiding lysis of the parasite (Fig. 2D and E).

In order to illustrated our hypothesis we have summarized the effect of our observations in the Fig. 3. In conclusion, NHS revealed an increased ability of R4 parasites to invade host cells, thus subverting the efficiency of complement system in the infection clearance. Moreover, ΔMBL serum decreased the infection of R4 strain in eukaryotic cells, suggesting a role of MBL in this process (Fig. 2A). On the other hand, epimastigote forms of 860 strain were sensitive to complement-mediated lysis but when differentiate to metacyclic, they acquired resistance to complement-lysis (Fig. 1B and D). In addition, NHS and ΔMBL serum did not influence the invasion of 860 strain to host cells. Probably other mechanisms of complement evasion are working fulltime, avoiding lysis and influencing the invasion process (Fig. 3).

The observed resistance to complement lysis of 860 strain may be very important at the beginning of the infection, where the parasites could induce different evasion mechanisms to avoid innate immunity response and allowing the invasion to eukaryotic cells. However, R4 strain does not display this kind of evasion mechanism. Differences at the invasion process of R4 strain could be related to different invasion mechanism. *T. cruzi* utilize a complex route of entry into eukaryotic cells involving a wide range of interactions between parasite surface proteins and eukaryotic cell receptors. The main surface molecules of parasites are glycosilated and is essential for parasite entry. Moreover, MBL can bind selectively to mannose, glucose, L-fucose and N-acetylglucosamine. Functional MBL circulate as higher order multimers (tetramer, pentamer and hexamers) of the structural unit. These structures allow high affinity interaction between MBL Carbohydrate recognition domains (CRDs) and parasites oligosaccharides.

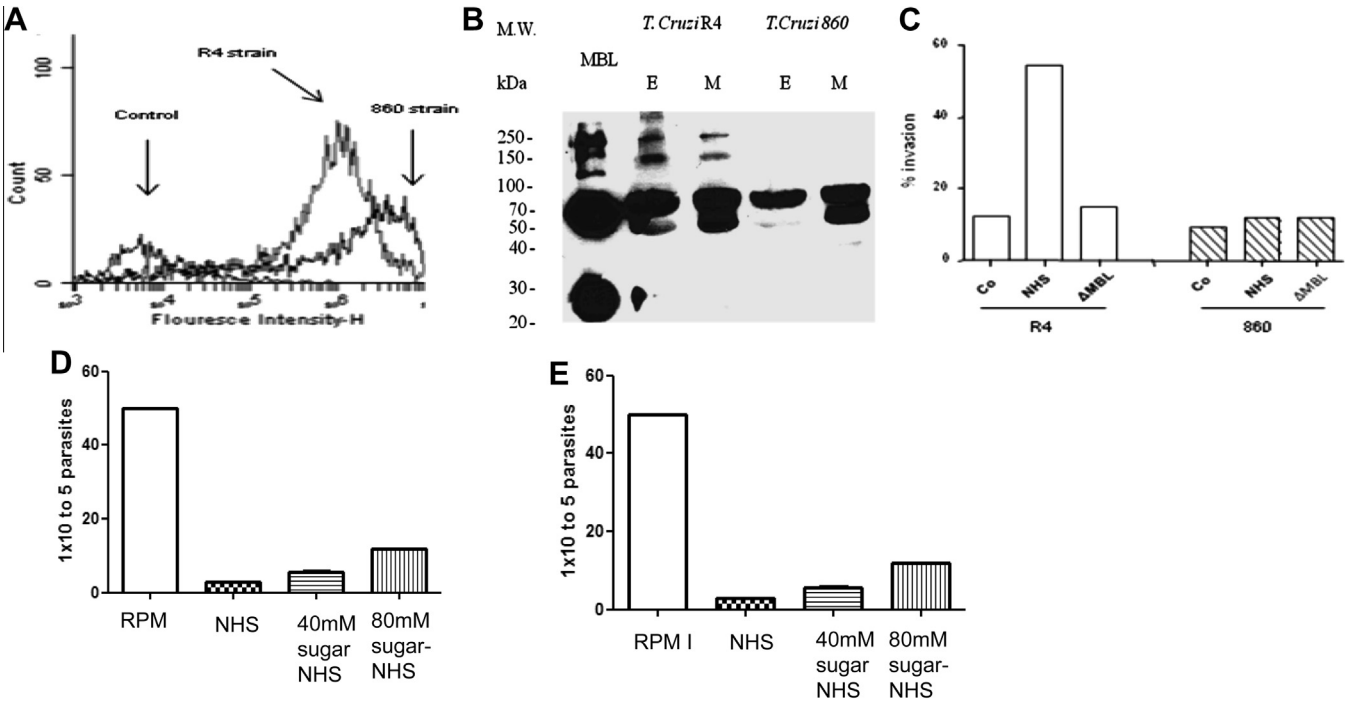

Fig. 2. (A) Flow cytometry analysis of metacyclic trypomastigotes shows similar MBL binding on both R4 and 860 strains. Parasites (2.0×10^5) were incubated with NHS for 1 h at 4 °C followed by incubation with anti-human MBL and then with fluorescein isothiocyanate (FITC) conjugated antibody. IgG-FITC was used as an isotype control. (B) Western blot analysis shows differential MBL deposition between R4 and 860 strains. Epimastigotes and metacyclic trypomastigotes from the two strains (1.0×10^7) were incubated in 100 μL of 10% NHS for 30 min at 4 °C. Parasite protein extracts were obtained and analyzed by SDS–PAGE and Western blotting with anti-human MBL antibody. Recombinant MBL is shown as a loading control. (C) NHS increased dramatically the invasion of R4 metacyclic trypomastigote forms to host cells, and have no effects over 860 strain. Parasites of 860 or R4 strain (1.0×10^6) were incubated with Vero cells (1.0×10^5) in 10% NHS or 10% ΔMBL serum for 3 h at 37 °C. As control, parasites and cells (ratio 10:1) were incubated in serum-free RPMI. Intracellular parasites in at least 500 cells were counted. (D) NHS treated with N-acetylglicosamine and mannose (40–80 mM) block the MBL effect in invasion. R4 strain (1.0×10^6) were incubated with Vero cells (1.0×10^5) in 10% NHS or 10% sugar treated-NHS (Mannose and GluNac 40–80 mM) for 3 h at 37 °C. As control, parasites and cells (ratio 10:1) were incubated in serum-free RPMI. Intracellular parasites in at least 500 cells were counted. (E) R4 epimastigote forms are more resistant to lyse in sugar-treated NHS. 1×10^5 R4 epimastigotes forms were incubated with 25% NHS or 25% NHS pre-treated with N-acetylglicosamine and mannose (40–80 mM) for 15 min at 37 °C.

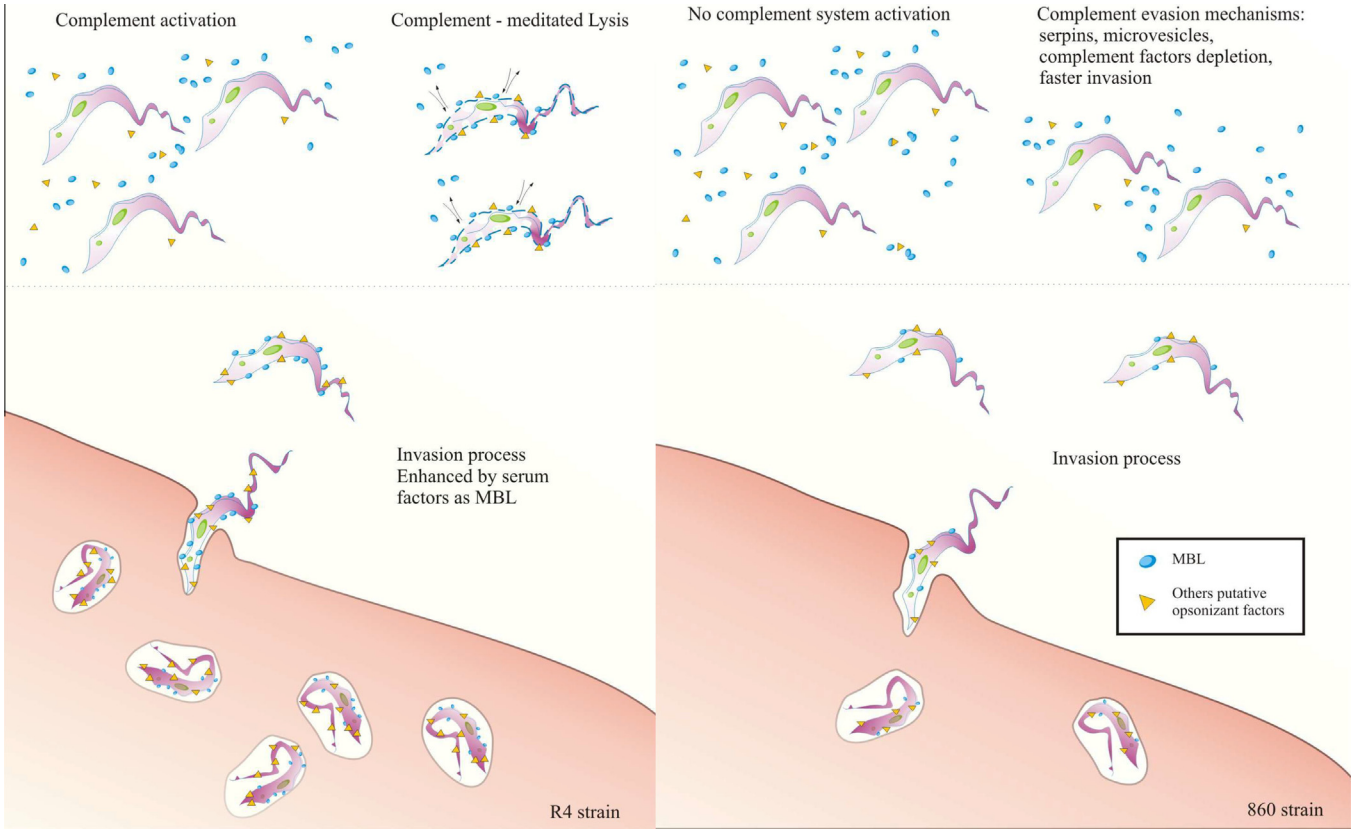

Fig. 3. Dual function of MBL during parasite–host cell interaction. (A) R4 metacyclic strain is sensitive to complement-mediated lysis, however the parasite uses MBL and serum factors to invade eukaryotic cells before complement lysis. (B) 860 metacyclic strain displays evasion mechanisms and are resistant to complement-mediated lysis and invade host cells with no apparent benefit from NHS.

The MBL interaction open the possibility to in same cases neutralize the invasion process blocking antigen-receptor binding. Similar observation were reported in invasion of intestinal mucosa by *Shigella Flexneri* [22] and in the endovascular trophoblast invasion in pre-eclampsia [23].In other cases MBL bind directly to receptor or as co-factor changing the topology of the membrane surface by exposing new molecules that facilitate antigen-receptor contact. Then, signal transduction pathways leading to elevation in cytosolic Ca^{2+} concentration in both cells. Disruption of host cell actin cytoskeleton that follows the interaction with *T. cruzi* mobilizate lysosomes to the cell periphery contributing for the parasitophorus vacuol formation, parasite internalization and in fact, increasing the invasion [18].

The dual function of MBL during the parasite-host cell interaction seems to be a novel evasion mechanism of pathogens in order to subvert innate immunity and increase invasion of eukaryotic cells.

Differential role of MBL with different *T. Cruzi* strains may help better understand the mechanism involved in the interaction between the host and parasite. Overall our study provides a better understanding on the underlying mechanism of MBL in establishment of CD.

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