

Research Article

Leishmania Donovanii Cell Surface Sialoglycans Regulate Susceptibility for Siglec Mediated Macrophage Invasion and Parasite Survival

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Abstract

Glycoconjugates play a pivotal role in the survival of *Leishmania* parasites in destructive surroundings. An important constituent present on many glycoconjugates is sialic acid. By virtue of their peripheral position on oligosaccharide chains of glycoconjugates, sialic acids are well suited as molecular determinants of specific biological processes, including the interaction of pathogenic microorganisms with sialylated cellular receptors. Differences in α 2,3- and α 2,6-sialoglycan patterns detected in clonal virulent *Leishmania donovani* promastigotes, correlated with the level of α 2,3- and α 2,6-sialyltransferase activity present in these parasites. The role of macrophage sialic acid-receptors in uptake and survival of *L.donovani* was studied in

the murine macrophage cell line raw 264.7. Macrophage invasion was dependent on the binding to Siglec-1, while suppression of MAPK signaling was mediated through Siglec-5. Sialic acid removal by neuraminidase treatment reduced parasite infectivity. The presence of trypsin resistant sialic acid residues in the neuraminidase treated parasites grown in a serum free medium in presence of sialoglycoconjugates indicated that the parasites could salvage sialic acid from exogenous sialoglycans and reutilize it for *de novo* glycoprotein sialylation in *L.donovani* parasites. Thus, our results demonstrate the involvement of sialoglycans in the invasion as well as the survival process of *L.donovani* parasites.

Introduction

Leishmania are protozoan parasites that cause severe diseases in human and animals. The *Leishmania* parasites are enveloped by a dense glycocalyx constituted of the glycosylphosphatidylinositol-anchored glycoproteins, family of glycoinositol-phospholipids, the lipophosphoglycans, the glycolipids, and the proteoglycans (Ferguson 1997). Together they have been implicated in a surprisingly large number of functions for the parasites throughout their life cycle and, therefore, are key players in their pathogenesis (Guhaniyogi *et al.* 2001, Kamhawi 2006).

Due to their exposed terminal position on glycoconjugates, sialic acids (Sia) have been implicated in a variety of biological processes, including cell adhesion and immune recognition (Crocker *et al.* 2001, Schauer & Kamerling 1997). The biosynthesis of sialoglycans comprises of a chemically and topologically

complex set of events involving CMP-Sia, CMP-Sia transporter and the sialyltransferases. The sialoglycan is finally secreted or delivered to the plasma membrane by the secretory machinery (Jacobs *et al.* 2001). The presence of Sia on the promastigotes of *Leishmania donovani* and *Trypanosoma* species has been shown previously (Chang 1981, Chatterjee *et al.* 2003, Schauer *et al.* 1983). Unable to synthesize Sia, *T.cruzi* trypomastigotes acquire Sia from host glycoconjugates by means of a plasma membrane-associated transsialidase (Previato *et al.* 1985) that has the unique ability to efficiently carry out a sialyltransferase reaction using preformed glycoconjugates (Buschiazzo *et al.* 2000). This multifunctionality is a common feature of many bacterial sialyltransferases (Cheng *et al.* 2005, Cheng *et al.* 2010, Hollister & Jarvis 2001, Hollister *et al.* 2002, Yu *et al.* 2005). Though the machinery that incorporates Sia to the parasite surface is present in parasites like *Trypanosoma* sps (Buschiazzo *et al.*

2000), they are still not reported in *Leishmania*. Of note, two protein coding putative Sia transporters LINJ_24_0350 and LINJ_24_0360 located at chromosome Chromosome 24, NC_009408.2 (110565..112238) and chromosome Chromosome 24, NC_009408.2 (118081..119703) respectively have been reported in the *L.infantum* data base.

Here we report for the first time the presence of sialyltransferases in *L.donovani* suggesting that these sialyltransferases may play an important role in the acquisition of Sia on *Leishmania* surface. Our results further suggest that, these sialoglycans participate in the invasion process and contribute to macrophage (M ϕ) deactivation and to disease pathogenesis.

Materials and Methods

Infection

Virulent *L.donovani* (MHOM/IN/83/AG83) parasite clones used in this study were isolated from a mixed population of 7th passage AG83 promastigotes as described (Bhaumik *et al.* 2008). *In vitro* infection experiments were carried out with macrophages using stationary phase *L.donovani* clonal promastigotes at a 20:1 parasite/macrophage ratio as described with slight modifications (Karmakar *et al.* 2011). Bone marrow derived macrophages were replaced by murine M ϕ cell line RAW 264.7. Cultures without added parasites were run in parallel.

Neuraminidase treatment of *Leishmania* promastigotes

Promastigotes (1×10^8) were treated with 25 units of *Vibrio cholerae* neuraminidase in RPMI-HEPES for 1h at 37°C with slow shaking. Subsequently parasites were washed 3 times in PBS and re-suspended in serum free medium. Control cells were washed in the same manner as enzyme-treated cells. The efficacy of neuraminidase treatment was determined by using *Maackia amurensis* agglutinin (MAA).

Use of 1,2-Diamino-4,5-methylene dioxybenzene (DMB)-HPLC analysis of Sia Promastigotes were washed in PBS and suspended in 1 mL PBS. Cells were lysed by sonication (5 pulses of 10s each). Glycoconjugates were subjected to hydrolysis with 2M propionic acid for 4h at 80°C (Kamerling & Gerwig, 2006). Controls included treatment with neuraminidase in RPMI-10% FCS for 1h at 37°C (2) and N-acetyl neuraminase pyruvate lyase (EC 4.1.3.3) treatment at pH 7.2 for 2h at 37°C. Released Sias were derivatized with DMB and resolved by reverse-phase HPLC (Manzi *et al.* 1990). Sia standards were isolated from bovine submaxillary mucin (Varki & Diaz 1984).

Biotinylation

Proteins (1mg/ml in PBS) were directly labeled with biotin using the Pierce EZ-Link Sulfo-NHS-Biotin Labeling Kit.

FACS analysis of binding of MAA and SNA to *L.donovani* promastigotes

A fixed number of *L.donovani* clonal promastigotes (1×10^6) were suspended in PBS containing 5% BSA (PBSB). Biotinylated MAA or SNA (0–50 μ g/ml) was added to the cell suspension and incubated at room temperature for 30 min. Cells were washed in PBSB thrice and incubated with FITC-conjugated streptavidin for 10 min. Cells were washed in PBSB thrice and fixed in paraformaldehyde (2%). Fluorescence of the cells were analyzed using a FACS Calibur (BD Biosciences) using CELL QUEST software. Unstained cells, or cells stained with FITC-BSA or streptavidin-FITC served as controls.

Western blotting analysis

Sialoglycan profiles of *L.donovani* CSA (25 mg/lane) was analyzed before and after sialidase treatment by Western blot using biotin-labeled MAA and *Sambucus nigra* agglutinin (SNA) (10 μ g/ml). After washing, the blots were incubated with horseradish peroxidase-avidin complex (Vector, ABC Reagent) and developed using an ECL detection system (Thermo Fisher Scientific, Rockford, IL, USA).

For MAPK activation studies, cells were lysed in ice-cold lysis buffer (100 mM Tris, pH 8, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 containing complete EDTA-free protease inhibitors from Roche Diagnostics, which included 5 mM sodium vanadate, 10 mM sodium fluoride, 10 mM b-glycerophosphate sodium, and 5 mM sodium pyrophosphate). Equal amounts of protein were loaded onto 12.5% SDS-polyacrylamide gels, and then transferred to PVDF membranes. The membranes were blocked with 5% BSA in wash buffer (TBS/0.1% Tween 20) for 1 h at room temperature and probed with primary antibody overnight at 4°C at a dilution recommended by the suppliers. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were washed three times with wash buffer and then incubated with HRP-conjugated secondary antibody and detected by the ECL detection system (Thermo Fisher Scientific, Rockford, IL, USA), according to the manufacturer's instructions.

Knockdown of Siglec-1 and Siglec-5 by siRNA in RAW 264.7 cells

RAW264.7 cells cultured in DMEM with 10% fetal bovine serum were transfected with the siRNA for

Siglec-1, Siglec-5 or the scrambled control (40 nM, Santacruz Biotechnology, USA) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were collected and analyzed for Siglec-1 and Siglec-5 proteins by western blotting using the Siglec-1 and Siglec-5 specific polyclonal antibodies (N-20 and M-58 respectively).

Fluorescence labeling of parasites

FITC-labeling of parasites was carried out as described (Butcher *et al.* 1992), except that labeling was carried out at 37°C in PBS. Promastigotes (10^6 /ml in PBS) were incubated for 15 min at 37°C with 30 µg/ml FITC. After incubation, parasites were washed twice with PBS to remove excess ligand and analyzed for fluorescence with a Becton-Dickinson FACS Calibur flow cytometer (San Jose, CA).

L. donovani-Siglec Binding assays

Binding studies were performed with a fixed number of clonal promastigotes (1×10^5) suspended in PBSB. Siglecs (0-10 µg/ml) diluted in 1% BSA/PBS were added to the cell suspension and incubated at 4°C for 2h in a total volume of 0.5ml. Cells were washed in PBSB thrice and incubated with biotinylated Siglec antibody (10 ng/ml in 1% BSA/PBS) for 1h at 4°C. The primary solution was discarded, and the cells were washed with cold PBS three times. Streptavidin coupled to horseradish peroxidase (200 ng/ml in PBS/1% BSA) was added to the cells and incubated for 1h at 4°C. Hundred µl freshly prepared staining solution (2% w/v ABTS [2,2'-azinodi(3-ethyl-benzthiazoline-6-sulfonate)] in 100 mM sodium acetate buffer, pH 4.2, and 0.001% v/v H₂O₂) was added and the reaction was stopped after 15 min by the addition of 0.6% (w/v) SDS and absorbance measured at 405 nm.

Promastigote binding studies

For promastigote binding studies, RAW 264.7 cells were washed twice with serum-free DMEM and incubated with stationary phase FITC-labeled parasites re-suspended in serum-free DMEM at a parasite to macrophage ratio of 5:1 for 30 min at 37°C. Non-adherent parasites were removed by five washings with PBS, followed by a fixation step (0.1 M PIPES-NaOH, pH 7.2; 2% para-formaldehyde, 0.05% glutaraldehyde, 30 min, 22°C). After five washes with PBS and a 20-40 min incubation with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS, 2% BSA, 50 mM NH₄Cl, followed by an additional three washes with PBS, the coverslips were embedded in Mowiol containing 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO, Sigma). Macrophages with bound parasites and the total number of bound parasites were counted

by inspection with a fluorescence microscope.

SHP-1 immunoprecipitation and phosphatase activity

To specifically measure SHP-1 activity, 100 mg of total Mφ protein lysate was subjected to immunoprecipitation using 20 ml anti-SHP-1 SH2 antibody (C14H6, Cell signaling) as reported (Blanchette *et al.* 1999). The immunoprecipitates were isolated using protein A-Sepharose and a fraction (20 mL) of the immunoprecipitate was used to perform SHP-1 phosphatase activity measurement using 2 mM p-nitrophenyl phosphate as substrate (Sigma Chemical Co., St. Louis, MO, USA).

Sialoglycan feeding studies

Gangliosides GQ1b, GT1b, GD1a, GD1b, GM1, and GM2, in chloroform:methanol 1:1 were dried by rotary evaporation, re-suspended in serum-free medium, and briefly sonicated to ensure proper micellar suspension. Ganglioside containing medium was filter sterilized (0.22-µm) and diluted with fresh medium to a final 50 µM concentration. Fetuin (50 µM) in serum free medium was passed through a 0.45-µm sterilizing filter. Sia was removed from clonal parasites by neuraminidase treatment as mentioned earlier. Asialo parasites (2×10^6 parasites/200µL medium) were incubated in ganglioside- or fetuin-containing serum free medium for 12h at 22 °C. Parasites were thoroughly washed and divided into two parts. One part was used to infect RAW 264.7 cells as mentioned earlier. The other part (1×10^6 parasites) was used to analyze the Sia content by DMB derivatization and liquid chromatography analysis as described later.

Preparation of α2-3- and α2-6-sialylated Fetuin, α₁Acid glycoprotein (AGP) and Complete Soluble Antigens (CSA) acceptor substrates

CSA was prepared from *L. donovani* promastigotes in presence of 0.04% Non-idet P-40 essentially as described previously (De & Roy, 1999). Asialo CSA, asialo Fetuin and asialo α₁ Acid glycoprotein (AGP) was prepared from CSA, Fetuin, and AGP respectively by incubation with 200mU *Clostridium perfringens* α2-3,6-Neuraminidase/mg protein in PBS (pH 6.4) at 37°C for 4h. α2-3-sialylated Fetuin, and AGP were prepared from the respective asialo-glycoproteins as described with slight modifications (Azuma *et al.* 2001). Fetuin and AGP was treated with 100 µU of α1,3/4 Fucosidase (*Streptomyces sp.* 142, EC 3.2.1.51, Takara Shuzo Co. Berkeley, USA) per mg protein. The purified de-fucosylated asialo glycoproteins were incubated with 5 mU α2,3-sialyltransferase (EMD4Biosciences, USA) in 0.1M HEPES buffer (pH

7.0) containing 1 mM CMP-NeuAc and 10 mM MgCl₂ for 48 h at 37°C. The reaction mixture was spotted on Whatman 3 MM paper. Ascending chromatograms were developed in 1% sodium tetraborate and the enzymatic product (α 2,3 sialylated glycoprotein) that remained at the origin was isolated from the papers. Strips from the origin were cut, washed with PBS, containing 0.04% non-idet P-40. Detergent was removed by SM-2 bio-beads and the product (further purified on MAA lectin affinity columns) was used as a substrate for the α 2-6 sialyltransferase assay. α 2-6-sialylated Fetuin, AGP and CSA were prepared from the respective glycoproteins by incubation with 5 U *Streptococcus pneumoniae* recombinant α 2,3-neuraminidase/mg protein at 37°C for 4h. The products were run on SDS-PAGE, blotted onto nitrocellulose and probed with MAA to check the extent of hydrolysis.

Localization of α 2,3- and α 2,6-silyltransferase enzymes by confocal microscopy

α 2,3- and α 2,6-silyltransferase antibody was directly labeled with FITC using the Pierce EZ-Label™-FITC Protein Labeling Kit. The Golgi of live promastigotes was visualized with the Golgi marker BODIPY-TR and ER was visualized with BiP. α 2,3- and α 2,6-silyltransferase was visualized with anti ST3Gal4 (Sigma Chemical Co., St. Louis, MO, USA) and anti CD-75 (H-70) (Santacruz Biotechnology, USA) antibodies respectively. Parasites were stained with the antibodies by using standard procedures. BiP antibody was visualized with PE-conjugated secondary antibody. Imaging was performed with the Nikon A1R live cell confocal imaging system.

Results and Discussions

Due to their exposed position and physicochemical properties, Sias play important roles in cellular interactions and adhesion processes. Sia is a receptor determinant for infection of cells by virions (Winter *et al.* 2006) and protozoa (Chitnis 2001). It has been suggested that terminal Sia residues may represent a shared but tailored invasion pathway among apicomplexan parasites (Hager & Carruthers 2008). *Plasmodium falciparum* strain W2mef depends on Sia for invasion of erythrocytes (Durasingsh *et al.* 2003). Sia associated with the host cell contributes to invasion by *T. cruzi* (Schenkman *et al.* 2003) and *Toxoplasma gondii* (Monteiro *et al.* 1998). The occurrence of Sia has been reported in a broad range of trypanosomatids (d'Avila-Levy *et al.* 2004). Though sialoglycans have been reported in *Leishmania* species (Chatterjee *et al.* 2003; Vannier-Santos *et al.* 1991, Dwyer 1977, Ghoshal *et al.* 2010, Iovannisci *et al.* 2010), the impact of

sialoglycans in leishmaniasis still remains a poorly understood area. Recently Sia has been implicated in nitric oxide mediated host resistance in leishmaniasis (Ghoshal *et al.* 2010). This study was undertaken to assess the sialylation pattern of virulent *L. donovani* AG83 clonal promastigote surface glycans and to elucidate their role (a) in the invasion process and (b) in modulating the host cell signaling.

Presence of sialoglycans on *L. donovani* promastigotes

Glycoconjugates have been shown to play important roles in the infectious cycle of *Leishmania* (Guha-Niyogi *et al.* 2001, da Silva *et al.* 2009). We had earlier established that differential expression of galactoterminal glycoconjugates are implicated in virulent attenuation of *L. donovani* promastigotes (Bhaumik *et al.* 2008). The presence of sialic acids on *L. donovani* promastigotes was demonstrated by performing fluorimetric high-performance liquid chromatography (HPLC) of acid hydrolysates of virulent clonal parasites. The chromatogram exhibited a well-resolved peak that coincided with N-acetyl neuraminic acid (Neu5Ac) (Figure 1B). There was about 275 ng of Sia in 1X10⁸ cells corresponding to 5.37 X 10⁶ molecules of Sia per cell (Table 1). Neuraminidase treatment completely removed Sia residues in the virulent promastigotes (Figure 1c). A lectin blot assay was performed using MAA to confirm that the neuraminidase treatment was effective. Diminution of the corresponding peak, following treatment with N-acetyl neuraminate pyruvate lyase confirmed the presence of Sia on these parasites (inset, Figure 1C).

Specificity of sialic acid linkage of *L. donovani* sialoglycans

The cell surface expression of sialoglycans was measured by flow cytometric analysis of the virulent clonal parasites stained with two fluoresceinated plant lectins MAA and SNA with specificity for terminal α 2,3- and α 2,6- linked Sia respectively. Flow cytometric analysis revealed that the amount of the lectin MAA needed for 50% binding was 9µg/ml. At similar concentrations, SNA bound to < 5% of the promastigotes (Figure 2A). In contrast, Chatterjee *et al.* observed that MAA showed a relatively lower binding than SNA (Chatterjee *et al.* 2003). This discrepancy may be explained by the fact that while we are working with virulent clonal parasites, Chatterjee *et al.* were working with a multiclonal population of *L. donovani* promastigotes. In the original paper they have referred to, parasites were sub-cultured every 4-5 days. These cells lose their infectivity to host macrophages after prolonged cultivation in axenic growth media (De & Roy

1999). Presence of predominantly avirulent variants in the mixed *L.donovani* population maintained in prolonged axenic culture would account for the predominance of -2,6 linked Sias that Chatterjee *et al.* (2003) observed, since we too observed a similar higher level of -2,6 linked Sias as compared to -2,3 linked Sias in a multi-clonal *L.donovani* AG83 promastigote population maintained in culture for > 2 weeks (data not shown).

Lectin-blot studies were carried out to characterize the linkage-specificity of sialoglycans present on the virulent clonal parasite surface. The reactivity of MAA and SNA was examined by western blotting. The western blot studies indicated that the level of 2,3-sialoglycans, was higher than the level of 2,6-sialoglycans, (Figure 2B). The population structure of *Leishmania* spp. and other kinetoplastids is mainly clonal and strains isolated from naturally infected hosts are likely composed of multiclonal parasite associations (Garin *et al.* 2001). Working with a multiclonal population consisting of both virulent and attenuated variants of the parasite cells, Chatterjee *et al.* (2003) reported the presence of both -2,3 and -2,6 linked, as well as 9-O-acetyl linked sialoglycans in the *L.donovani* promastigotes. We however could not detect any Neu5,9Ac2 peak in the acid parasite hydrolysates (Figure 1C). It may be mentioned that, Chatterjee *et al.* (2003) did not detect any Neu5,9Ac2 Sia by

mass spectrometry analysis either. Our findings are in agreement to another member of the kinetoplastid family, the trypanosomes, where -2,3 linked Sias predominate (Engstler *et al.* 1995). Pre-treatment of the parasites with *Clostridium perfringens* $\alpha(2-3,6)$ -neuraminidase abolished lectin binding. Prior incubation of the lectins with fetuin plus sialyllactose also abolished binding (Figure 2A, lane3).

Effect of culture conditions on *L.donovani* sialoglycan expression

To rule out the possibility that the sialoglycans were not present as contaminants, cells were grown under different conditions. The binding of SNA or MAA and the total Sia expression on *L.donovani* promastigotes remained unchanged despite alterations in the media, temperature or pH (Table 1). Similarly, cells grown in different batches of FCS for 72h or different concentrations of FCS (M 199 medium, 22°C and pH 7.2) did not show any differences in their lectin binding properties and total Sia expression. The mass spectrometry of the per-trimethylsilyl-derivative of the methyl ester demonstrated the presence of Neu5Ac with fragment ions (m/z) at 668, 624, 478, 400, 317, 298, 205, and 173. Only m/z values over 100 are given.

Binding of Siglecs to *L.donovani* promastigotes

Cumulative evidence indicates that in trypanosomat-

Table 1. Binding of MAA and SNA to virulent *L. donovani* clonal promastigotes.

Virulent *L.donovani* clonal promastigotes were grown under different conditions. The binding of Sia-binding lectins SNA or MAA (50 $\mu\text{g/ml}$) was measured by flow cytometry. A fixed number of *L.donovani* clonal promastigotes (1×10^6) were suspended in PBS containing 5% BSA (PBSB). Biotinylated MAA or SNA (0–50 $\mu\text{g/ml}$) was added to the cell suspension and incubated at room temperature for 30 min. Cells were washed in PBSB thrice and incubated with FITC-conjugated streptavidin for 10 min. Cells were washed in PBSB thrice and fixed in paraformaldehyde (2%). Fluorescence of the cells were analyzed using a FACS Calibur (BD Biosciences) using CELL QUEST software. Unstained cells, or cells stained with FITC-BSA or streptavidin-FITC served as controls. Total Sia content was estimated by the Prozyme rapid sialic acid quantitation kit.

	Medium	Temp. (°C)	pH	FCS Source (10%)	% Lectin Binding		ng acid/10 ⁸ cells/Sialic	Number of Sialic acid Molecules/ Cells
					MAA	SNA		
1	M199	22	7.2	Invitrogen	99 ± 1	31 ± 4	275	5.37 X 10 ⁶
2	RPMI 1640	22	7.2	Invitrogen	97 ± 1	29 ± 3	270	5.26 X 10 ⁶
3	Schneider's Drosophila medium	22	7.2	Invitrogen	97 ± 3	34 ± 2	290	5.65 X 10 ⁶
4	M 199	30	7.2	Invitrogen	96.5 ± 3	32 ± 5	265	5.16 X 10 ⁶
5	M 199	22	6.3	Invitrogen	95 ± 4	31 ± 3	252	4.90 X 10 ⁶
6	M199	22	7.2	Invitrogen (20%)	96 ± 3	30 ± 2	261	5.08 X 10 ⁶
7	M 199	22	7.2	SIGMA	94 ± 4	31 ± 4	289	5.63 X 10 ⁶
8	M 199	22	7.2	PAN Biotech GmbH	95.5 ± 4	29 ± 4	282	5.49 X 10 ⁶
9	M 199	22	7.2	HiMedia (India)	97 ± 1	30 ± 4	280	5.45 X 10 ⁶

ids, glycoconjugates are relevant in several phases of host parasite interactions (De Souza 1995). Microbes use their own pathogen associated molecular patterns to gain entry into cells by exploiting the host pathogen recognition receptor. *Neisseria gonorrhoeae* uses the asialo-lipooligosaccharide receptor on sperms to infect the female reproductive tract (Harvey *et al.* 2000); mannose/fucose containing glycans on microbes such as *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Leishmania* is recognized by the DC-SIGN on dendritic cells (Appelmelk *et al.* 2003). Many human pathogens express Sias that are recognized by several Siglecs. It is possible that Siglec-dependent recognition of these pathogen glycans leads to altered immune responses, either to the advantage or to the detriment of the pathogen (Crocker *et al.* 2007). Basic structures recognized by Siglecs contain the NeuAc-Gal-HexNAc sequence. Human and mouse Sn/Siglec-1

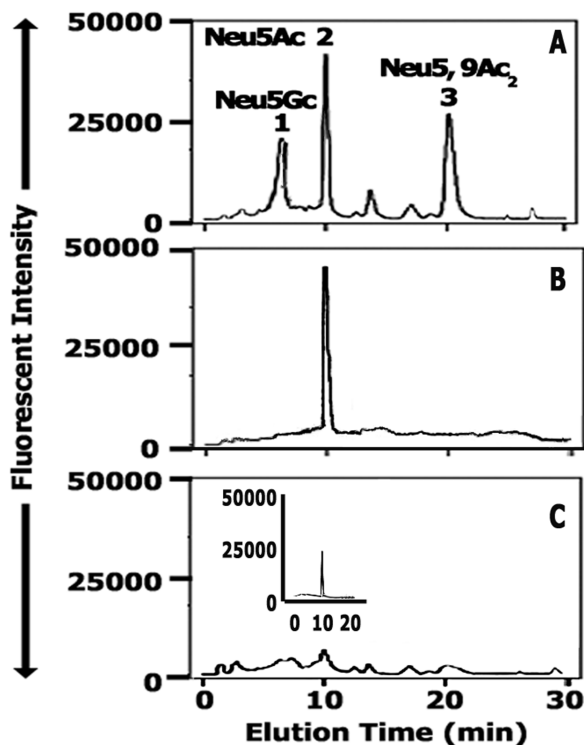


Figure 1. Chromatograms of DMB derivatives of Neu5Ac obtained from virulent *L. donovani*. Representative profile of a HPLC chromatogram of fluorescent derivatives of free sialic acids derived from the standard mixtures of Neu5Ac and Neu5Gc obtained from bovine submaxillary mucins (A). *L. donovani* virulent clonal promastigotes before (B) and after treatment with neuraminidase (C). *L. donovani* virulent clonal promastigotes after pre-incubation with N-acetyl neuraminase pyruvate lyase (C, inset). The fluorescence of DMB derivatives was detected at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. Peak 1, Neu5Gc; peak 2, Neu5Ac; peak 3, Neu5,9 Ac₂. Data are representative of 3 experiments.

preferentially binds NeuAca2-3Gal β 1-, while CD22/Siglec-2 binds NeuAca2-6Gal β 1- (Angata 2006). Hence the question that we sought answer to was- does the parasite utilize Sia for invasion of M ϕ s?

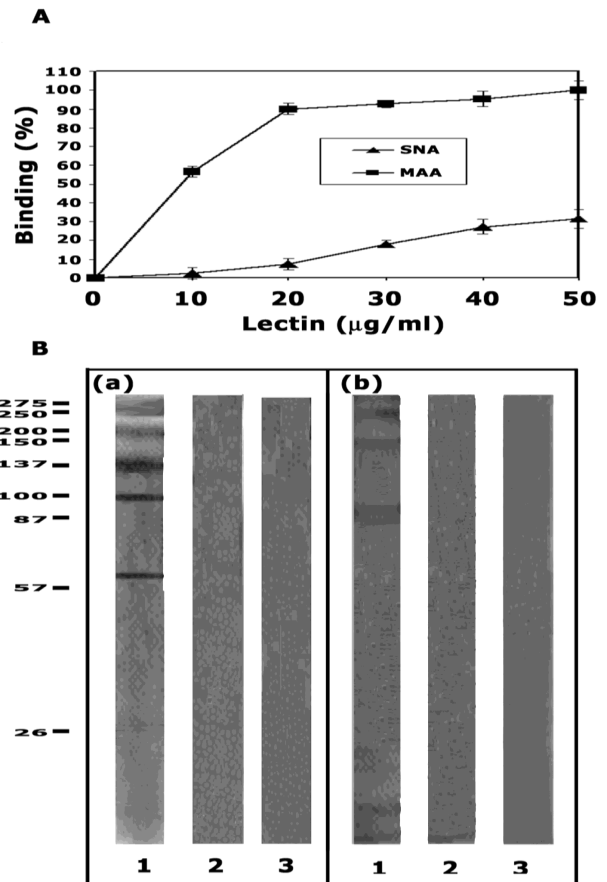


Figure 2. (A) Quantitation of Sia specific lectin binding to virulent clonal promastigote surface with MAA and SNA. The percent of cells with specific Sia expression as determined by flow cytometry using MAA and SNA. A fixed number of clonal promastigotes (1×10^6) were incubated with increasing concentrations of biotinylated-MAA or SNA (0–50 μ g/ml). Binding of biotinylated-lectins to *L. donovani* promastigotes was monitored using FITC-labeled streptavidin followed by flow cytometry of gated populations. FITC-BSA, or streptavidin-FITC were used as control antibodies. The data are means \pm SD of three independent assays. (B) Western blot showing the sialoglycoprotein patterns detected in *L. donovani* virulent clonal promastigotes. Solubilized membrane proteins from neuraminidase untreated (lane 1) and treated (lane 2) promastigotes were resolved by SDS-PAGE under non-reducing conditions (10% gels, 5 μ g protein/lane) and electro-transferred to nitrocellulose. Membrane strips were incubated with biotin-labeled MAA (a) or SNA (b) and developed as indicated in Materials and Methods. Controls (lane 3) were also carried out by incubating the lectins with fetuin plus sialylactose prior to membrane incubation. The numbers on the left indicate the relative position of molecular mass markers expressed in kDa. Images are representative of three independent experiments.

We performed direct binding between *L.donovani* virulent clonal promastigotes and the siglec receptors Sn, Siglec-2, Siglec-3 (CD33), and Siglec-5. Initially it was found that Sn, Siglec-2 and Siglec-5, and not CD33 (Figure 3A) bound to the promastigotes. Unlike human CD33, murine CD33 does not bind to α 2,3- or α 2,6-Sia on lactosamine unit (Brinkman-Van der Linden *et al.* 2008). Removal of α 2,3-Sia with *Streptococcus pneumoniae* sialidase abolished Sn:parasite but not Siglec-2:parasite binding, emphasizing the importance of α 2,3-Sias and α 2,6-Sias in Sn:parasite and Siglec-2:parasite interactions respectively. The more promiscuous sialoglycan recognition molecule Siglec-5, bound to both, clonal promastigotes and NeuAc(α 2,3)⁻clonal promastigotes.

To reiterate Siglec-1:parasite sialoglycan interaction, promastigote binding to M ϕ s was also studied either i) in presence of anti Siglec-1 antibody or ii) by knocking down the expression of M ϕ Siglec-1 by siRNA. Siglec-1 knock down was confirmed by analyzing the whole cell lysates blotted for Siglec-1. Expression of the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Transient transfection of RAW264.7 cells with Siglec-1-specific siRNA reduced Siglec-1 production in these cells (Figure 3B). Siglec-1 suppression either through neutralizing antibody or siRNA knockdown resulted in a reduction in the proportion of M ϕ s with attached promastigotes and also a reduction in the total number of bound parasites (Figure 3C) indicative of an inability of cells lacking Siglec-1 to bind *L.donovani* promastigotes appropriately. Parasite attachment was not reduced in RAW cells expressing a scrambled siRNA (Figure 3C). Replacement of the primary antibody with an equivalent concentration of non-immune rat IgG (used for negative control) did not reduce the binding.

Siglec mediated MAPK signaling in *Leishmania* infected M ϕ s

Glycan binding at the extra-cellular domain of Siglecs and signaling at the cytosolic domain appears to be coupled. The cytoplasmic domains of most Siglecs contain sequence motif(s) involved in signal transduction (Angata & Varki, 2000). *Leishmania* infection induces defects in the host cell signaling pathways that is required to bring the cells to an activated state. *Leishmania* is known to silence the MAPKs (mitogen-activated protein kinase) signaling pathways (Ben-Othman *et al.* 2008). It is suggested that SHP-1 inactivates IRAK-1 kinase (interleukin-1 receptor-associated kinase-1) activity upon *L.donovani* infection through binding to an evolutionary conserved ITIM (immunoreceptor tyrosine-based inhibitory motif) like

motif located within IRAK-1's kinase domain (Abu-Dayyeh *et al.* 2008). Siglec-5, an inhibitory receptor

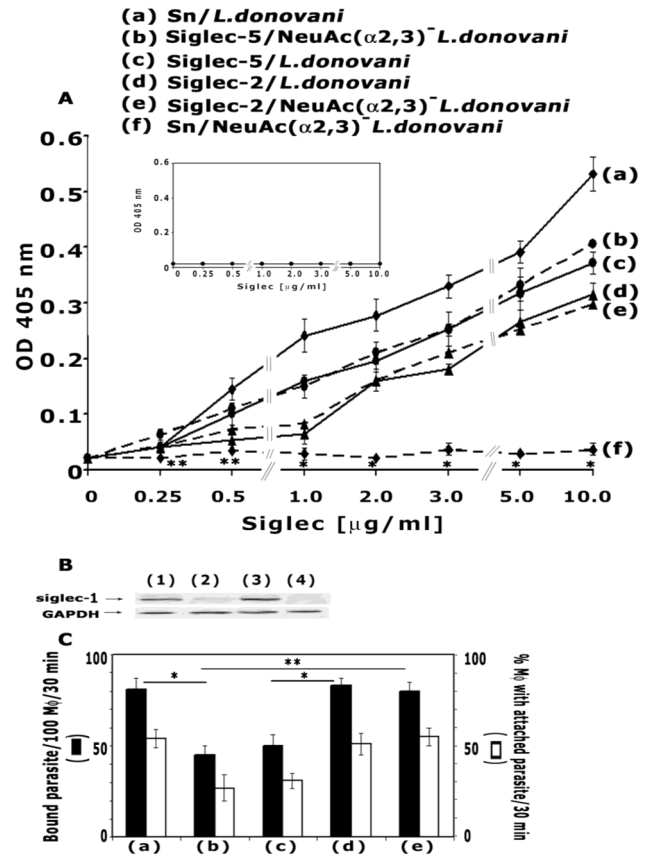


Figure 3. (A) Binding of virulent clonal *L.donovani* or NeuAc(α 2,3) virulent clonal *L.donovani* promastigotes to Siglecs. Clonal promastigotes (1×10^5) were suspended in PBS containing 5% BSA and binding of biotinylated Siglec-1, Siglec-2, Siglec-5, and CD33 (insert) was determined (Materials and Methods). Data shown are mean \pm SD of three experiments performed in triplicate. Sn-*L.donovani* binding was significantly higher than the Sn- NeuAc(α 2,3)⁻ *L.donovani* binding (*, $p < 0.0001$; **, $p < 0.001$); paired two-tailed Student's t-test. (B) Siglec-1 expression by RAW 264.7 cells. Western blotting; RAW cells (Lane 1 and 4), RAW cells transfected with Siglec-1 siRNA (Lane 2), or scrambled siRNA (3) were lysed, electrophoresed, transferred to PVDF membrane and immunostained with anti-Siglec-1 antibody (Lane 1-3) or control antibody (Lane 4). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in total proteins was used as loading controls. Blots are representative of three separate experiments. (C) Binding of virulent clonal *L.donovani* promastigote to RAW cells in (a) absence of anti Siglec-1 antibody, (b) in presence of anti Siglec-1 antibody, (c) transiently transfected with Siglec-1 specific siRNA, (d) transiently transfected with scrambled non-specific siRNA, and (e) in presence of non-immune rat IgG (Materials and Methods). The bars represent the average of three experiments. The standard error is indicated. *, $p < 0.01$; **, $p = 0.001$ versus as indicated; paired two-tailed Student's t-test.

containing an ITIM domain, functions in association with SHP-1 and/or SHP-2 to block survival signaling. The established dogma is that phosphorylation of tyrosine residue in ITIM's during cellular activation results in docking sites for SH2 (Src Homology-2) domain containing phosphatases. The recruited and activated phosphatases can then dephosphorylate relevant substrates in the vicinity and regulate cellular activation.

Experiments were conducted to address the hypothesis that Sia molecules on *L.donovani* surface probably interacts with M ϕ Sia-binding receptors to modulate intracellular signaling. Phosphorylation of p38, JNK and ERK1/2 MAPK was analyzed by Western blot analysis. It was found that the parasite induced suppression of MAPK's signaling was reversed by pre-treatment of the cells with either (i) neuraminidase (data not shown) or (ii) by transiently transfecting RAW 264.7 cells with Siglec-5 specific siRNA. RAW 264.7 cells were transiently transfected with siRNA targeted at Siglec-5. To monitor the down-regulation of Siglec-5 expression, protein expressions were ana-

lyzed. siRNA incubation reduced the protein expression of Siglec-5 (Figure 4A). Down regulation of Siglec-5 resulted in MAPK activation in the parasite infected RAW 264.7 cells. Kinetics of MAPK phosphorylation in *Leishmania* infected RAW264.7 cells from 5 to 120 minutes resulted in the induction of p38 MAPK phosphorylation that peaked at 15 min, followed by dephosphorylation, and an increase in p44/42 MAPK and JNK (c-Jun N-terminal kinase) phosphorylation at 5 min, which was followed by a gradual decrease as infection progressed (Figure 4Ba).

In contrast, activation of p38, p44/42 and JNK MAPK signaling pathways on Siglec-5 siRNA transfection (Figure 4Bb) was accompanied by a reduced rate of parasite survival within these cells (Figure 4D). MAPK phosphorylation was significantly abrogated in cells treated with control siRNA (Figure 4C). Activation of p38, ERK1/2 and JNK MAPK signaling pathways on Siglec-5 siRNA transfection was blocked in presence of MAPK inhibitors SB203580, U0126 and SP600125 (data not shown). Pre-treatment with goat

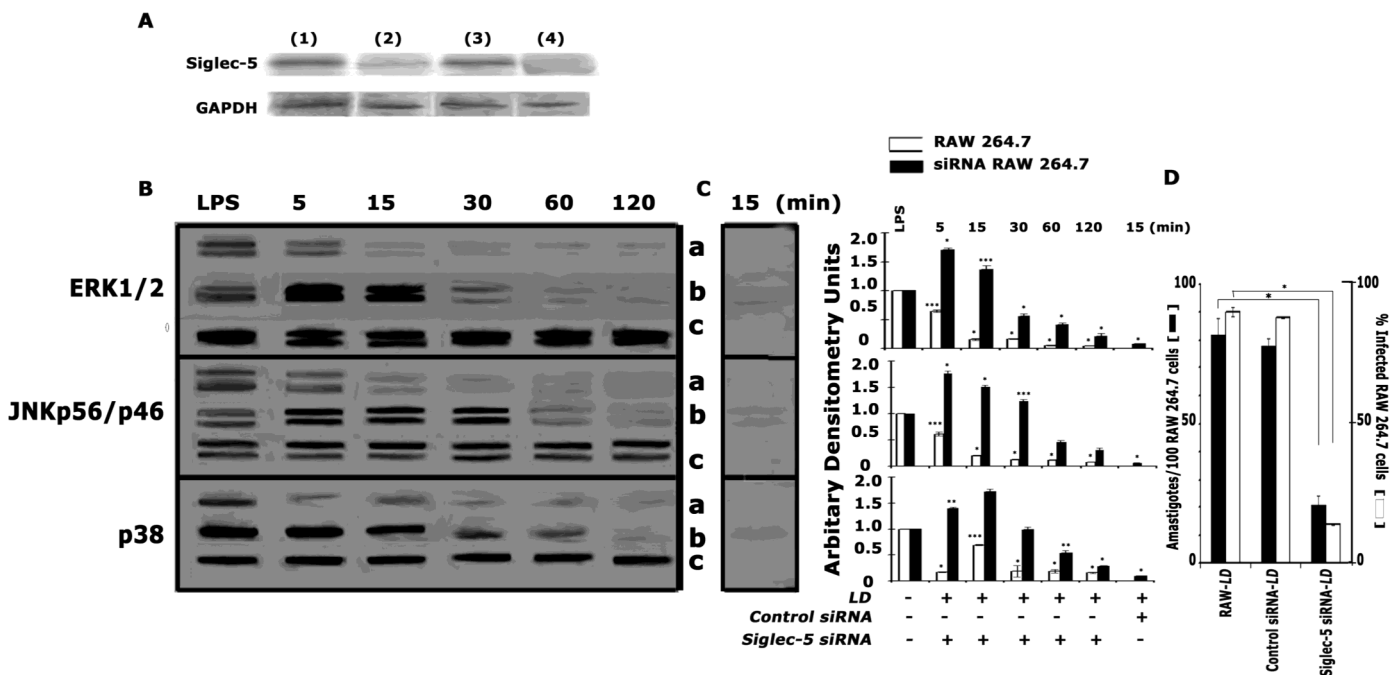


Figure 4. *L.donovani* induced suppression of MAPKs is reversed by blocking Siglec-5. (A) Siglec-5 expression by RAW 264.7 cells. Western blotting; RAW cells (Lane 1 and 4), RAW cells transfected with Siglec-5 siRNA (Lane 2), or scrambled siRNA (3) were lysed, electrophoresed, transferred to PVDF and immunostained with anti-Siglec-5 antibody (Lane 1-3) or control antibody (Lane 4). GAPDH in total proteins was used as loading controls. Blots are representative of three separate experiments. (B) RAW 264.7 cells were exposed to virulent clonal *L.donovani* promastigotes for various times, cell lysates were collected and subjected to Western blot analysis to detect phosphorylated and total forms of p38, ERK1/2 (extracellular-signal-regulated kinase) and JNK. LPS-treated RAW 264.7 cells served as positive controls for MAPK induction. (a,c) RAW 264.7 cells or (b) Siglec-5 si-RNA transfected RAW 274.7 cells infected with *L.donovani* promastigotes. (C) Control siRNA transfected RAW 264.7 cells infected with virulent clonal *L.donovani* promastigotes. Comparable results were obtained in three independent experiments. The data are representative of three independent experiments. Densitometries are shown as bar graphs on the right-hand side of each panel. *Leishmania* infected RAW 264.7 cells (open bar) or si-RNA transfected RAW cells (black bar). The relative phosphorylation of MAPKs in the LPS induced controls was set at 1, and the data represent mean \pm SD of three independent experiments. *, $p < 0.0001$; **, $p < 0.001$; ***, $p < 0.05$ versus LPS stimulated control; paired two-tailed Student's t-test.

IgG failed to activate MAPK signaling (data not shown). LPS (10 ng/ml) treated cells expressed a strong phosphorylation of p38 MAPK, ERK1/2 and JNK that peaked at 15-30 min. Replacement of the primary antibody with an equivalent concentration of rabbit IgG was used for negative controls.

L.donovani sialoglycan induced SHP-1 phosphatase activity

To assess the potential association of *L.donovani* sialoglycans and inhibitory function of Siglec-5, we measured SHP-1 phosphatase activity. Immediate inhibition of SHP-1 activity in RAW 264.7 cells that had been pretreated with anti Siglec-5 confirmed the inhibitory signaling capacity of Siglec-5 (Figure 5). On the other hand, pretreatment of RAW 264.7 cells with Siglec-1 antibody had no significant effect on SHP-1 activity. To further support our data we also checked SHP-1 activity in RAW 264.7 cells after Siglec-1 or Siglec-5 knockdown with siRNA. We observed decreased SHP-1 activity after 24 hours in Siglec-5 siRNA transfected but not Siglec-1 siRNA transfected Mφs. Thus, these results suggest that *L.donovani* parasites can modulate infection via its sialylated glycoprotein antigens and Mφ-Siglec receptors. It may be mentioned that, to date, all Sia-dependent binding of Mφs has been found to be Sn-dependent (Munday *et al.* 1999), suggesting that, on Mφs, Sn is the major Sia binding receptor responsible for mediating cell-cell interactions and that the primary function of other Siglecs may be in signaling. This corroborated with the finding that Siglec-1 suppression either through neutralizing antibody or siRNA knockdown, resulted in a reduction in macrophage *L.donovani* binding (Figure 4C).

Thus it may be concluded from our results that in all probability, following sialoglycan-Sn binding, Siglec-5 dependent inhibition of MAPKs signaling occurred. This study represents a major advance in our understanding of Mφ invasion by *L.donovani*, by delineating sialoglycans as important parasite ligands and the identification of Siglec-1 as its host receptor.

Uptake and incorporation of sialic acid by *L.donovani* promastigotes

The two primary routes to obtain Sia in microbes is either *de novo* biosynthesis or acquisition from the environment (Lauc & Heffer-Lauc 2006). It has been suggested that no *de novo* biosynthetic machinery exists in *L.donovani* (Chatterjee *et al.* 2003). To investigate the possibility that gangliosides and sialoglycoproteins found in serum (Yu & Ledden 1972) may be exploited by the parasites as the supply line for making Sia modified cell surface, parasites were treated with

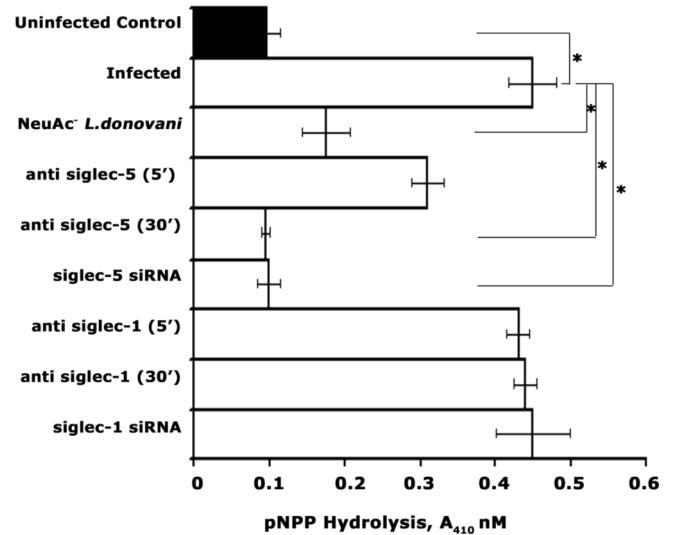


Figure 5. Virulent clonal *L.donovani* induced Mφ SHP-1 activity. Virulent clonal *L.donovani* infected RAW 264.7 cells were incubated in presence of anti Sn and anti Siglec-5 antibodies for the indicated time points. Siglec-1/Siglec-5 siRNA transfected RAW 264.7 cells were infected with *L.donovani* clonal parasites. Cells were lysed, SHP-1 protein immunoprecipitated and used to perform phosphatase assay. Results are the mean \pm SD of three experiments performed in triplicate. *, $p < 0.05$; versus as indicated; paired two-tailed Student's t-test.

sialidase and re-incubated with different sialoglycans in MEM medium containing no serum (serum free medium).

V.cholerae sialidase treatment for 1h resulted in a decrease in parasite infectivity. Incubation of the control parasites in RPMI/10% FCS at 37°C did not affect the viability or infectivity of the parasites. As shown in the Figure 6A incubation of the neuraminidase treated parasites with gangliosides containing the sequence *Neu5Aca(2-3)Galβ(1-3)GalNAcβ-* (GD1a, and GT1b,) partially restored infectivity and Sia content of the parasites ($3-6 \times 10^6$ Sia molecules/cell). The ganglioside GQ1b in which the sequence ends with two Sia in a *NeuAca(2-8)NeuAc* linkage instead of a single Sia as in GD1a and GT1b, was able to fully restore the infectivity and Sia content of the parasites as compared to wild type AG83 promastigotes. Incubation with gangliosides similar in structure to GD1a, GT1b, and GQ1b but lacking the Sia attached to the terminal galactose (i.e., GM1, GM2 and GD1b) had no effect. Incubation with Fetuin, (Sia attached to the terminal galactose), also restored the infectivity and Sia content of the parasites. None of the gangliosides or fetuin affected the viability of the cells; the cell viability was always 92% to 97% of that before culture as examined by a trypan blue exclusion test. The presence of sialoglycans in the re-sialylated parasites were confirmed by FACS analysis using MAA lectin (Figure

6A, hatched bars). Most of the Sia was surface associated since it could be removed by sialidase treatment. Enzymatic treatment did not affect cell morphology as assessed using phase-contrast light microscopy.

Since metacyclic promastigotes do not see serum in the fly and are only briefly exposed to it following animal infection, the kinetics of Sia incorporation was studied with fetuin. The time course of uptake of Sia was essentially linear through 120 min of incubation, but then plateaued (Figure 5B). Our earlier work using radiolabeled-parasites had indicated that the *L. donovani* parasites are cleared from blood within 48 minutes (Bhaumik *et al.* 2008). It has been suggested that the sialoglycans are adsorbed from the serum (Chatterjee *et al.* 2003). Non-specifically adsorbed molecules are trypsin sensitive but once they are inserted into plasma membrane they become trypsin resistant (Schwarzmann *et al.* 1983). The presence of trypsin resistant α 2,3-linked sialoglycans in the asialo-parasites grown overnight in presence of exogenous sialoglycans, in a serum free medium suggests that the

parasite sialylation machinery is active in *L. donovani* promastigotes and that the sialoglycans are not merely adsorbed from the serum.

To note, earlier claim that sialoglycans were merely adsorbed (Chatterjee *et al.* 2003) is in no way supported by the data presented. There is no supporting evidence to show that the sialoglycans were attached to trypsin sensitive membrane components and not inserted into the membrane in the same manner as endogenous sialoglycans. Besides the presence of serum derived sialoglycans in the *L. donovani* membranes, these workers also show the presence of additional α 2,3-linked sialoglycans that are absent in the serum. No explanation for the presence of these additional sialoglycans is presented. The working model of sialylation suggests that cells can salvage terminal Sia from extracellular sialoglycans and process it to CMP-Sia, which can be utilized by the intracellular silyltransferase (Aumiller *et al.* 2003). The ability of virulent clonal promastigotes to produce sialylated glycans in presence of exogenous sialoglycans supports the idea that exogenous sialoglycans can provide a source of Sia for *de novo* glycoprotein sialylation by the parasites. Neuraminidase treated parasites grown overnight in a serum free medium resulted in 52% reduction in infectivity, 55% decrease in percent infected M ϕ s and complete absence of MAA binding.

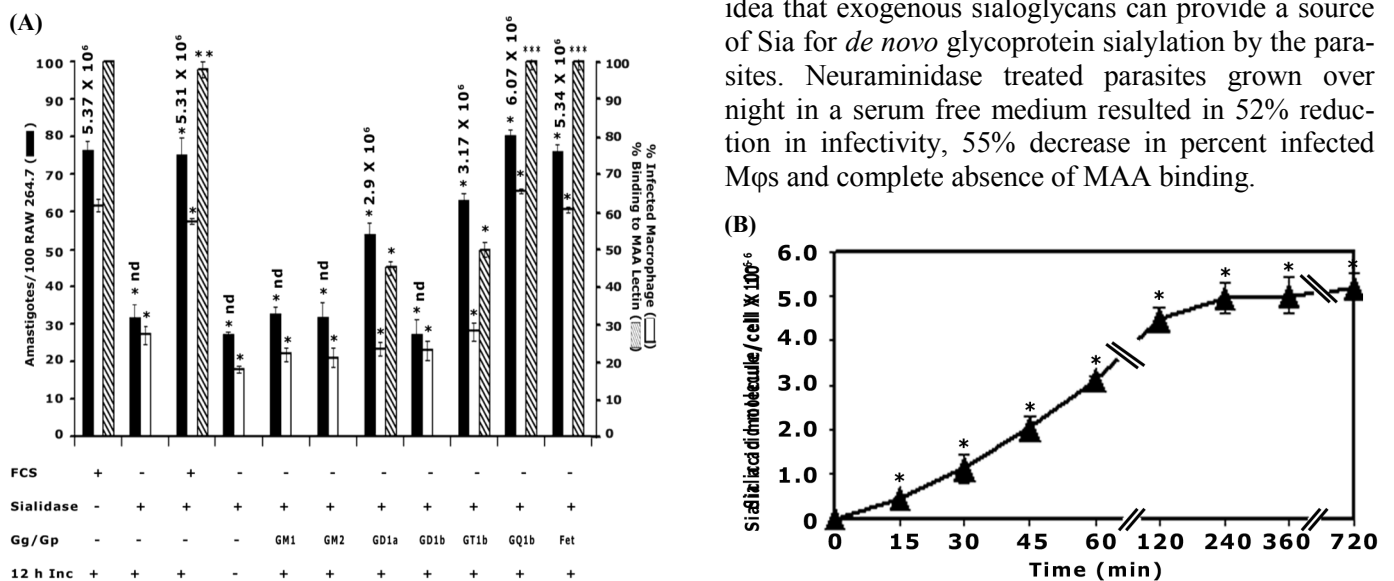


Figure 6. (A) Exogenous resialylation of virulent clonal *L. donovani* parasites. Virulent clonal *L. donovani* parasites, either pre-treated with (+) or without (-) sialidase, were incubated in serum free medium with (+) or without (-) the addition of gangliosides (Gg) or Fetuin (Fet/Gp) and analyzed. RAW 264.7 cells were infected with the resialylated virulent clonal *L. donovani* parasites and the intracellular amastigotes and percent infected M ϕ s were estimated. The percentage of resialylated *Leishmania* promastigotes (1×10^6 promastigotes) binding to Biotinylated MAA lectin ($50 \mu\text{g/ml}$) is shown by the hatched bars. Results are expressed as mean \pm SD for 9 stained coverslips for triplicate experiments. Values (mean of triplicates) at the top indicate sialic acid (Sia) content (number of Sia molecules/parasite). nd=not detected; *, $p < 0.0001$; **, $p < 0.005$; ***, $p < 0.05$ compared to virulent clonal *L. donovani* infected control; paired two-tailed Student's t-test. GM1, $\text{Gal}\beta(1-3)\text{GalNAc}\beta(1-4)[\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-4)\text{Glc}\beta(1-1)\text{Cer}$; GM2, $\text{GalNAc}\beta(1-4)[\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-4)\text{Glc}\beta(1-1)\text{Cer}$; GD1a, $\text{Neu5Aca}(2-3)\text{Gal}\beta(1-3)\text{GalNAc}\beta(1-4)[\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-4)\text{Glc}\beta(1-1)\text{Cer}$; GD1b, $\text{Gal}\beta(1-3)\text{GalNAc}\beta(1-4)[\text{Neu5Aca}(2-8)\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-4)\text{Glc}\beta(1-1)\text{Cer}$; GT1b, $\text{Neu5Aca}(2-3)\text{Gal}\beta(1-3)\text{GalNAc}\beta(1-4)[\text{Neu5Aca}(2-8)\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-4)\text{Glc}\beta(1-1)\text{Cer}$; GQ1b, $\text{Neu5Aca}(2-8)\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-3)\text{GalNAc}\beta(1-4)[\text{Neu5Aca}(2-8)\text{Neu5Aca}(2-3)]\text{Gal}\beta(1-4)\text{Glc}\beta(1-1)\text{Cer}$; Cer, Ceramide. (B) Time course of Sia incorporation. Asialo virulent clonal *L. donovani* parasites (2×10^6 parasites/200 μl medium) were incubated for the indicated times at 22°C in fetuin-containing serum free medium. At the indicated time points, the Sia content of the parasites was analyzed by DMB derivatization and liquid chromatography analysis as described in Materials and Methods. The data are representative of three independent experiments; *, $p < 0.0001$ compared to asialo virulent clonal *L. donovani* parasites; paired two-tailed Student's t-test.

There was a direct correlation between the degree of cell surface sialylation and infectivity of the parasites. The de-sialylated promastigotes in which sialoglycans were absent were found to be less-infective, suggesting that the loss of terminal Sia probably attenuates the promastigotes. This may be due to the exposure of underlying galactose, as it has been established that the presence of galactose terminal glycoconjugates is associated with loss of parasite virulence (Bhaumik *et al.* 2008, Sacks *et al.* 1985).

L. donovani sialyltransferases

In *T. cruzi*, addition of Sia units to glycoconjugates is mediated by a cell surface trans-sialidase and not by an intracellular CMP-Sia dependent transferase (Colli 1993). Till date no sialidase or trans-sialidase has been reported in *Leishmania* parasites. On the other hand, two protein coding putative CMP-Sia transporters LINJ_24_0350 and LINJ_24_0360 have been reported in the *L. infantum* data base. Though it shares 40-50% sequence homology with mammalian UDP-Gal and UDP-GlcNAc transporters, Sia transporters have been shown in a number of systems to only transport CMP-Neu5Ac (Berninsone *et al.* 1997). Gene DB analysis showed that the candidates LINJ_24_0350 and LINJ_24_0360 associated with other transporters from kinetoplastida with specificities for nucleotide sugar transporters as well as CMP-Sia transporters. The cluster contained putative CMP-Sia transporters from

L. major (LMJF_15_1055), *L. braziliensis* (LBRM_24_0440 & _0360); *L. mexicana mexicana* (LMXM_24_0360), *T. brucei* (Tb11.01.8280); *T. brucei gambiense* (Tbg972.11.18660); *T. cruzi* (Tc00.1047053507089.40, Tc00.1047053504085.60); and a putative UDP-Gal transporter from *L. major* (LmjF24.0360).

In the formation of sialoglycans, Golgi sialyltransferases need to have access to their glycoconjugate substrates and nucleotide sugar donor, CMP-Sia; CMP-Sia in turn is transported into the lumen of the Golgi complex through the CMP-Sia transporter (Zhao *et al.* 2006). Aumiller *et al.* (2003) have engineered transgenic insect cell lines to express mammalian glycosyltransferases to show that, despite the fact that these cells have little intracellular Sia and no CMP-Sia, these cells can salvage Sia for *de novo* glycoprotein sialylation from extra-cellular sialoglycoproteins. Searching for an answer to the question how these cell lines sialylate their newly synthesized glycoproteins in absence of CMP-Sia, they discovered that these cell lines can produce sialylated glycoproteins when they expressed a sialyltransferase and were co-cultured in presence of an exogenous source of Sia. Though, the precise mechanism by which extra-cellular sialoglycoproteins effectively support sialylation by the sialyltransferase remains to be determined, it has been suggested that the cells somehow salvaged Sia from extracellular sialoglycans and used them to produce CMP

Table 2. Acceptor specificity of Sialyltransferases of *Leishmania donovani*.

Sialyltransferase was assayed essentially according to the method of Basu *et al.* (Basu *et al.*, 1982). In brief, complete incubation mixture contained the following components (in μ moles) in a final volume of 0.05mL: acceptor glycosphingolipids, 0.025 or glycoprotein, 0.4 mg/ml; Triton X-100, 0.5%; MgCl₂, 0.125; cacodylate-HCl buffer, pH 6.4, 10; EDTA, 0.025; CMP-[¹⁴C] NeuAc, 0.015 (2 X 10⁶ cpm/ μ mole); and enzyme fraction, 100 μ g protein. The mixture was incubated at 37°C for 2h and the reaction was stopped with 3 mmol EDTA (protein) or 10 ml chloroform:methanol (C:M, 2:1, v/v; glycolipid). The reaction mixture was spotted on Whatman SG81 paper. Ascending chromatograms were developed in 1% sodium tetraborate for the protein substrates and in C:M-0.2% CaCl₂ (55:45:10, v/v) for glycolipid substrates. The radioactivity of appropriate areas of each chromatograms were quantitated in cocktail "O" (contains per L, 6g of PPO and 0.2g of POPOP) in the liquid scintillation counter (TRI CARB 2100TR; Packard).

Acceptor substrate	nmol [¹⁴ C]NeuAc Incorporated/ mg protein/hr
	V-LD
asialo Fetuin	5.76
α 2-6 sialylated Fetuin	4.06
α 2-3 sialylated Fetuin	1.71
asialo AGP	5.41
α 2-6 sialylated AGP	4.42
α 2-3 sialylated AGP	1.11
LacCer [Gal β (1-4)Glc β (1-1)Cer]	0.72
nLcOse4Cer [Gal β (1-4)GlcNAc β (1-3) Gal β (1-4)Glc β (1-1)Cer]	1.05
SA ⁻ GM1 [Gal β (1-3)GalNAc β (1-4)Gal β (1-4)Glc β (1-1)Cer]	1.11
GM1 [Gal β (1-3)GalNAc β (1-4)[Neu5Ac α (2-3)]Gal β (1-4)Glc β (1-1)Cer]	0.87
asialo -CSA	6.65

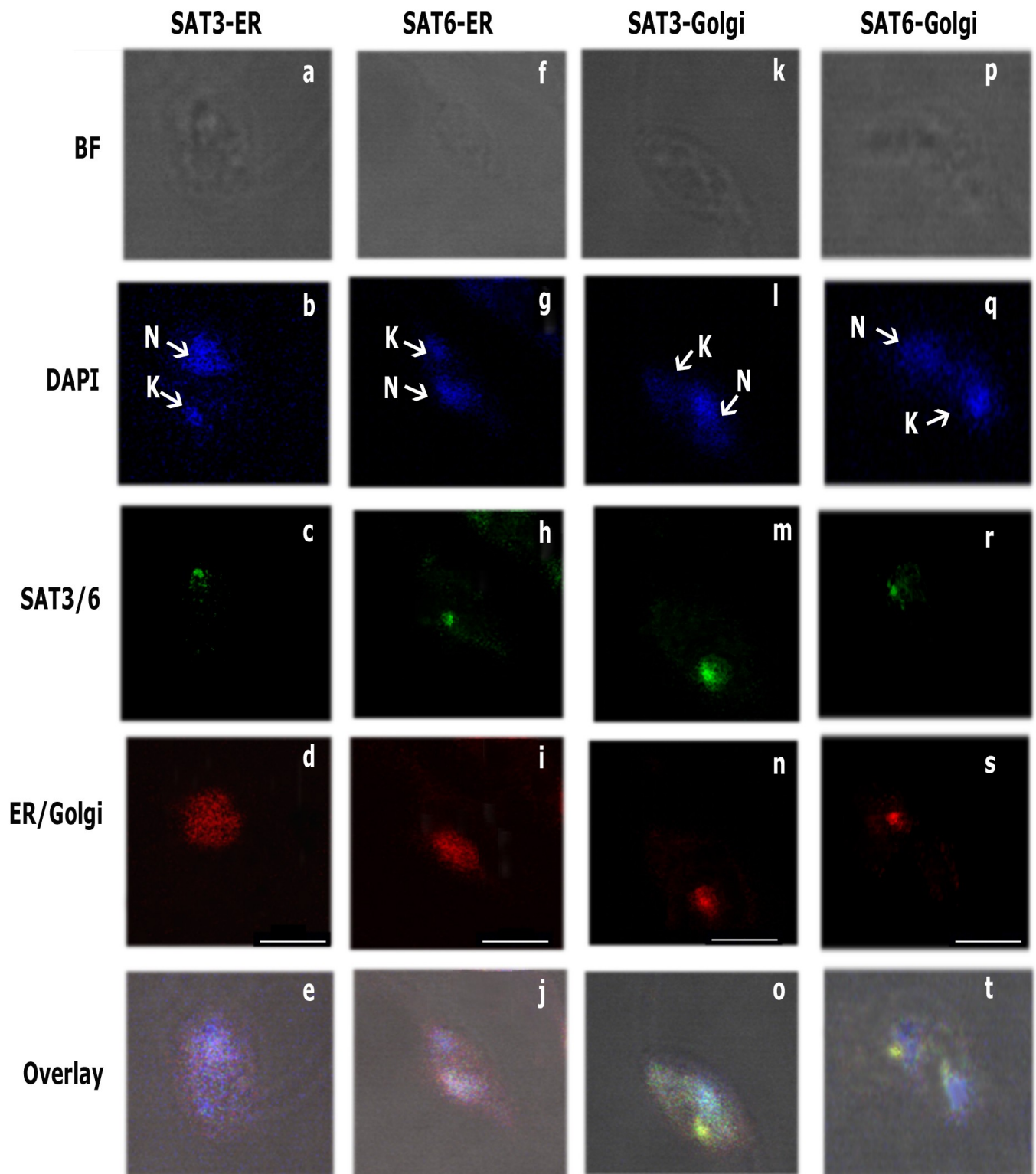


Figure 7. Localization of sialyltransferases. Confocal images of *L. donovani* expressing sialyltransferases. Counter staining is as follows: with DAPI (in blue) for the nuclear DNA and the kinetoplast; with BODIPY TR ceramide (in red) for the Golgi; with BiP (in red) for the ER, with FITC-labeled (green) anti $\alpha 2,3$ - and $\alpha 2,6$ -sialyltransferase antibody for sialyltransferases. Anti BiP was detected using goat anti rat IgG-PE. a,f,k,p, bright field image; b,g,l,q, DAPI; c,m, $\alpha 2,3$ -sialyltransferase; h,r, $\alpha 2,6$ -sialyltransferase; d,i, BiP; n,s, BODIPY TR; e, overlay of b-d showing non-colocalization of ER and $\alpha 2,3$ -sialyltransferase; f, overlay of g-i showing non-colocalization of ER and $\alpha 2,6$ -sialyltransferase; o, overlay of l-n showing colocalization of $\alpha 2,3$ -sialyltransferase and Golgi and t, overlay of q-s showing colocalization of $\alpha 2,6$ -sialyltransferase and Golgi. More than 100 cells were visualized microscopically. Data presented in each panel are representative of at least 90% of the population. SAT3, $\alpha 2,3$ -sialyltransferase; SAT6, $\alpha 2,6$ -sialyltransferase; N, nucleus; K, kinetoplast. Scale bars, 10mm.

-Sia, which was transported into Golgi apparatus and used for *de novo* glycoprotein sialylation by the intracellular sialyltransferase. As already stated, the ability of the promastigotes to produce sialoglycans in the presence of serum or exogenous sialoglycan indicates that the exogenous sialoglycoproteins can provide a source of Sia for the *de novo* glycoprotein sialylation by *L.donovani*.

In view of these observations, we looked for the presence of sialyltransferases in the virulent clonal promastigotes. Using different acceptor molecules, it was found that both α 2,3-sialyltransferase and α 2,6-sialyltransferase enzymatic activities could be detected in the virulent clonal parasites (Table 2). The sialyltransferases in the parasites could transfer [14 C]NeuAc more efficiently to α 2,6-sialylated fetuin and α 2,6-sialylated α 1acid glycoprotein (AGP) as compared to α 2,3-sialylated fetuin and α 2,3-sialylated AGP, indicating an elevated α 2,3-sialyltransferase activity in the virulent parasites. The fact that the enzymes could transfer [14 C]NeuAc to the de-sialylated antigen (asialo-CSA) from the virulent parasites indicated the presence of the natural substrates in these parasites. The sialyltransferases could also transfer [14 C]NeuAc to glycolipid substrates. Therefore, we conclude that differential expression of α 2,3-sialyltransferase and α 2,6-sialyltransferase resulted in the differential expression of α 2,3- and α 2,6-linked sialoglycans in the virulent clonal promastigotes.

The α 2,6-sialyltransferase enzyme, regardless of its species or tissue origin, is very specific for Gal β (1-4)GlcNAc and acts with very low activity on Gal β (1-3)GlcNAc and Gal β 1-3GalNAc, if at all (Weinstein *et al.* 1982). The natural as well as the recombinant form of the α 2,3-sialyltransferase enzyme, however, has a high preference for acceptor substrates containing a terminal Gal β (1-3)GlcNAc unit (Weinstein *et al.* 1982, Wlasichuk *et al.* 1993). The copious presence of Gal β (1,3) terminal residues in virulent *Leishmania* parasites (McConville *et al.* 1990), probably results in the higher expression of α 2,3-linked Sia in the virulent promastigotes. By confocal microscopy, we could show the presence of the α 2,3-sialyltransferase and α 2,6-sialyltransferase enzymes mainly in the Golgi of the parasites (Figure 7). All eukaryotic sialyltransferases have in common the presence in their catalytic domain of four conserved peptide regions [sialylmotifs L (large), S (small), motif III, and VS (very small)] that are hallmarks for sialyltransferase identification (Jeanneau *et al.* 2004, Harduin-Lepers *et al.* 2005). Since, no antibody against any parasite derived sialyltransferase is available, and we had previously observed that the human β 1,4-galactosyltransferase could recognize *L.donovani* β 1,4-

galactosyltransferase, we used antibodies against the human enzymes to stain the parasite sialyltransferase enzymes.

Conclusion

Taken together, our data suggests an association between the virulence of *L.donovani* promastigotes and the expression of sialoglycans, which precludes the activation of signal transduction pathways leading to effective host cell invasion.

Data Analysis

Data were expressed as mean \pm SD and evaluated by the student's t test. *P* values of <0.05 were assumed to be statistically significant.

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Disclosure

There is no financial/commercial conflict of interests.

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