The *Trypanosoma cruzi* membrane mucin AgC10 inhibits T cell activation and IL-2 transcription through L-selectin

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Keywords: cell surface molecules, cytokines, T lymphocytes, parasitic protozoan

Abstract

*Trypanosoma cruzi* infection is associated with a severe T cell unresponsiveness to antigens and mitogens characterized by a decreased IL-2 synthesis and by nitric oxide (NO) production. Although spleen cell unresponsiveness to ConA was less severe in infected IFN-γR−/− or inducible nitric oxide synthase (iNOS)−/− mice than in control littermates, IL-2 inhibition was as severely impaired. Ag C10, a *T. cruzi* mucin, inhibited T cell proliferation as well as IL-2 secretion and IL-2 mRNA induction in response to mitogens and to anti-CD3. This effect took place at the transcriptional level since Ag C10 was able to inhibit IL-2 promoter-driven transcription. Moreover, the transcription of reporter genes controlled by CD28RE, NFAT or AP-1, but not by NF-κB sites, were inhibited by AgC10 to different degrees, although the greatest effect was observed for NFAT. In agreement with that, overexpression of NFAT significantly reverted Ag C10 inhibition of IL-2 transcription. AgC10 also inhibited early steps of T cell activation as tyrosine phosphorylation of the tyrosine kinase ZAP-70 and the adapter protein SLP-76. AgC10 binds to T cell surface through CD62L, and antibodies to CD62L inhibited T cell proliferation and IL-2 secretion and transcription as efficiently as AgC10. Indeed, AgC10 did not inhibit activation by T cells from CD62L-deficient mice (Sell−/−). Our results suggest that Ag C10, through binding to L-selectin, was able to inhibit different activation pathways that lead to inhibition of IL-2 secretion and T cell proliferation. This was independent of NO and IFN-γ.

Introduction

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas’ disease, which affects several million people in South and Central America (1,2). This parasite exists in at least three morphologically distinct forms: infective (metacyclic or blood trypomastigotes), insect borne (epimastigotes) which replicate in the vector, and intracellular replicative (amastigotes) which grow and replicate intracellularly in a variety of mammalian cells (3). From a clinical point of view, *T. cruzi* infections proceed in two phases. In the acute phase, circulating blood trypomastigotes and local inflammation at the sites of infection are observed. During the chronic phase, circulating parasites cannot be observed by inspection of blood but progressive tissue damage occurs involving the oesophagus, colon and heart (1,2).

Several alterations of the immune response have been described in this disease. Among them, *T. cruzi* acute infection is associated with a severe immunosuppression, measured as the loss of cellular proliferation to mitogens and antigens, which is thought to facilitate dissemination and establishment of the parasite. Different mechanisms mediated by various cell types have been ascribed to immunosuppression. Thus, some reports have pointed to T cells, including γ/δ T cells (4) as well as to adherent cells (5) or immature myeloid cells (6) as suppressor cells. Besides, inhibition of IL-2 synthesis (7), reduced cell surface expression of IL-2R by spleen cell cultures obtained from infected mice (8), as well as human peripheral blood cells (9) have been observed. Apoptosis of CD4+ T cells has been also previously described in acute *T. cruzi* infection (4), and T cells obtained from infected mice have an enhanced TCR activation-induced cell apoptosis that may explain this unresponsiveness (10). Increased NO secretion (11) has been accounted as one mechanism responsible for explaining immunosuppression and recently, CD11b+Gr1+ myeloid cells have been identified by our group as responsible for this NO-mediated immunosuppression (6). Other soluble substances, including suppressive cytokines...
and TGF-β, IL-4 of IL-10 and prostaglandins, released upon contact with parasite-derived antigens, have also been involved in the mechanism of T. cruzi immunosuppression (12–14). Finally, elevated levels of IFN-γ and TNF-α are produced by spleen cell cultures from infected mice (15), which in turn induce high levels of NO (6).

Mucins comprise a heterogeneous group of O-glycosylated glycoproteins that display relevant roles in adhesion, which seem to be required for the interaction between unicellular parasites with host cells during invasion (16). Trypanosoma cruzi mucins participate in parasite interaction with host cells (17), including invasion and/or survival (18), and are able to alter macrophage functions (19,20). Previous evidence suggested the existence of at least two sets of mucin-like molecules in T. cruzi (21). One of them, present in the insect forms, migrates in the 35–55 kDa range (20,22), in contrast with mucins derived from tissue-derived trypomastigote forms that migrate as a broad band of 60–200 kDa (23). The protein portions of these mucins are encoded by a heterogeneous gene family (24), which appears to be differentially expressed in the different stages (21). The lipid nature of their GPI anchors also differs from epimastigote/metacyclic forms and trypomastigotes (25).

In this paper we have addressed the immunosuppressive mechanism that takes place during acute T. cruzi infection, especially related to suppression of IL-2 production and T cell activation. We found that acutely infected iNOS−/− mice have less marked immunosuppression than control littermates but identical decrease of IL-2 production. AgC10, a T. cruzi mucin, present in insect forms and amastigotes, was able to block IL-2 with IL-2 transcription. Moreover, this effect seems to involve factors NFAT and AP1 and IL-2 promoter activation, followed by transduction proteins. This led to inhibition of the transcription factors NFAT and AP1 and IL-2 promoter activation, followed by a decrease in IL-2 transcription. Moreover, this effect seems to be dependent on AgC10 interaction with L-selectin.

Methods

Mice and parasites

Trypanosoma cruzi Tulahuen (T) strain of T. cruzi was a gift from Dr John David (Harvard, USA). Specific pathogen-free mice of the following control strains, C57Bl/6 and 129/SvEv (Sv129) and the strains with the same background but with disrupted iNOS (iNOS−/−), IFN-γ receptor (IFN-γR−/−) or L-selectin genes (Sell−/−), respectively, were obtained from Jackson Laboratories, Bar Harbor, USA, and maintained in the Centro de Biología Molecular animal facilities at the Universidad Autónoma. Groups of mice (8–12 weeks old) were infected with T. cruzi Tulahuen strain by intraperitoneal injection of 10⁷ blood trypomastigotes obtained from mice previously infected with frozen trypomastigotes. Parasitemia was measured as previously described (26). The animal research described in this paper complied with national and European Union legislation and with related codes of practice.

Purification of AgC10

In vitro stationary phase T. cruzi cultures, which contain a mixture of epimastigotes and metacyclic forms (27), were extracted with organic solvents as described (20). Briefly, parasites (10⁸), washed with PBS three times, were extracted twice with 10 volumes of chloroform/methanol/water (1:2:0.8, v/v) at room temperature and the insoluble material removed by centrifugation at 10 000 g for 15 min. The supernatants were dried under a stream of nitrogen and portioned between 1-butanol and H₂O (2:1, v/v). Under these conditions, AgC10 partitioned into the aqueous phase. The upper butanol-rich phase was removed and the lower aqueous phase was re-extracted with H₂O-saturated butanol (1:2, v/v). Aqueous phases were pooled, lyophilized and resuspended in Buffer A (0.1 M ammonium acetate buffer containing 5% 1-propanol). This sample was loaded onto a column of octyl-Sepharose CL4B (10 × 1 cm) (Sigma, St Louis, MO) equilibrated in buffer A at a flow rate of 4 ml/h at 4°C. The column was washed with 20 volumes of buffer A followed by 5 volumes of a discontinuous gradient with 15, 20, 25, 30, 35, 40, 60 and 100% of 1-propanol in water at a flow rate of 15 ml/h. The AgC10-containing fractions (tested by ELISA or western blot with mAb C10) were pooled, partitioned in 1-butanol/H₂O (2:1, v/v) and rechromatographed on octyl-Sepharose. The immunoreactive material from the column was pooled, lyophilized and dissolved in PBS. This procedure leads to >98% pure AgC10 (20).

Spleen cell cultures

Spleen cell (SC) suspensions were prepared from infected mice at 14 days post infection or from control uninfected mice as indicated. SC were depleted of erythrocytes by hypotonic lysis with distilled water and resuspended in RPMI-1640 complete medium containing 5% FCS, 2 mM l-glutamine, penicillin (100 U/ml) and streptomycin (100 ng/ml) (Gibco Laboratories, Grand Island, NY). Once SC were obtained from Sv129 mice, CD4 and CD8 cells were purified using MACS CD4* and CD8* isolation kit (Miltenyi Biotec, Germany), following the manufacturer’s instructions. Where indicated, 5 µg/ml Con A (Sigma Chemical Co.), 2 mM l-NMMA (N⁵-monomethyl-L-arginine, Calbiochem-Behring Corp., La Jolla, CA), 2 µg/ml anti-CD3 and growing concentrations of AgC10 (1, 5, 10, 15 or 20 µg/ml) or 10 µg/ml of anti-CD62L (Mel14) (Biosource) were added at time 0 without changing the medium. The cultures were dried under a stream of nitrogen and portioned between 1-butanol and H₂O (2:1, v/v) and rechromatographed on octyl-Sepharose. The immunoreactive material from the column was pooled, lyophilized and dissolved in PBS. This procedure leads to >98% pure AgC10 (20).

Lymphoproliferation assays

SC were cultured in triplicate in flat-bottomed 96-well plates at 2 × 10⁵ cells/well (200 µl/well). After incubation at 37°C and 5% CO₂ for 48 h, each well received 1 µCi [³H]thymidine (Amersham Ltd, Buckinghamshire, UK). The cultures were terminated 18 h later by automated harvesting and were then processed for measurement of incorporated radioactivity in a liquid scintillation counter.

Measurement of IL-2 and nitric oxide production

SC were cultured in 24-well flat-bottomed plates at 1.2 × 10⁶ cells/well with the correspondent stimulus at 600 µl/well. Cultures were incubated at 37°C, 5% CO₂ for 24 h, and the supernatants were harvested. IL-2 was detected by a two-site
sandwich ELISA (Endogen, Woburn, MA). NO was measured as nitrite accumulated in the supernatants by using the Griess reaction (28).

RNA isolation and analysis
SC (3 \times 10^6) were seeded in 12-well plates and stimulated with 5 \mu g/ml of Con A in the presence of growing concentrations of AgC10 and RNA was isolated by using TRIzol reagent (Life Technologies) following manufacturer’s instructions. Total RNA (20 \mu g/ml) was separated by agarose-formaldehyde and transferred to a nylon membrane as previously described. A 0.9 kb genomic probe containing the mIL2 gene was used to detect IL-2 mRNA, and a 0.9 kb mouse Pst-\beta-actin fragment was used as a quantitative control.

Cell transfection assays
Jurkat human lymphoblastoid cells, clone E6-1, were maintained in RPMI-1640 complete medium containing 5% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 ng/ml streptomycin (Gibco Laboratories). Cells were seeded at a density of 5 \times 10^5 cells/ml and transfected using LipofectAMINEPLUS\textsuperscript{TM} reagent (Invitrogen) following the manufacturer’s instructions. Briefly, 2 \times 10^6 cells were incubated at 37°C and 5% CO\textsubscript{2} with 4 \mu l of lipofectamine, 6 \mu l of PLUS reagent and 0.5 of the following plasmids: pLL2luc, pNFATluc (29); pNF3Tkluc (30); pCD28RE/AP1luc (31); or AP-1(p-73col)-luc (32). These were transfected alone or in combination with 0.5 \mu g of the expression plasmids for NFAT1 (33), the members of NF-\kappaB, c-rel and p65 (34) or the component of AP1, c-jun (35). The transfection process was carried out in 1 ml of OPTIMEM\textsuperscript{TM} (Invitrogen) for 4 h. After this time, cells were centrifuged and cultured in RPMI-1640 complete medium containing 2% FCS and stimulated for 16 h with 1 \mu M of PHA or 15 ng/ml of anti-CD62L (Leu-8) (Biosource, Camarillo, CA) where indicated, centrifuged and lysed with 50 \mu l of lysis buffer from Luciferase Assay System Kit (Promega) and the luciferase activity was measured in 20 \mu l of supernatant obtained from the lysed extracts, following the manufacturer’s instructions (35).

Immunoprecipitation and western blot assays
Jurkat cells (5 \times 10^6) were cultured in 6-well flat-bottomed plates with the corresponding stimuli (15 ng/ml of TPA plus 1 \mu M of calcium ionophore, 1 \mu M of PHA or 2 \mu g/ml of anti-CD3) for 5 min in the presence or absence of AgC10. After stimulation, cells were centrifuged, washed with PBS and lysed for 30 min with 50 \mu l of NP40 buffer (10 mM NaF enriched with the protease inhibitors aprotinin and leupeptin at 2 \mu g/ml, pepstatin 1 \mu M and PMSF 1 mM and 100 \mu M of the phosphatase inhibitor Na\textsubscript{2}VO\textsubscript{4}). Cells were centrifuged and the supernatants were incubated with 25 \mu l of protein A-Sepharose (Sigma) for 1 h. After centrifugation, supernatants were collected and 1.5 mg of the obtained protein concentration were incubated at 4°C in agitation with 1 \mu l 4G10 (antibody against phosphotyrosine) for 4 h. After this the immune complexes were captured with 40 \mu l of protein A-Sepharose for 16 h at 4°C in agitation. After several washes in NP40 buffer, the extracts were separated by SDS–PAGE (10% polyacrylamide or 8%) and later subjected to western blotting with the appropriate antibodies (anti-phospho SLP-76 and anti-phospho ZAP-70).

Flow cytometric analyses
SC obtained from Sv129 mice were cultured or not, with 15 \mu g/ml of AgC10 for different periods of times. After AgC10 treatment, SC were incubated at 4°C for 20 min with 10 \mu g/ml of anti-CD62L–FITC antibody (Mel 14– from Biosource) in 50 \mu l of staining buffer (PBS 2% FCS), and then fixed with PBS 1% paraformaldehyde before analyzing them by flow cytometry.

Statistical analysis
Experimental differences over the controls were analyzed by the Student’s t-test. Probability values P > 0.05 were considered non-significant. All the experiments described were performed at least twice in order to warrant the reproducibility of the results.

Results

NO- and INF-\gamma-independent immunosuppression in the acute phase of T. cruzi infection
Previous studies have extensively documented a severe immunosuppression in the acute phase of T. cruzi infection. In agreement with this, we have found that splenocytes (SC) taken from T. cruzi-infected Sv129 or C57Bl/6 mice, at 14 days post infection, have a greatly reduced proliferative response to mitogens as Con A (Fig. 1A). NO-dependent mechanisms have been accounted as responsible for this suppression (11). However, SC from IFN-\gammaR\textsuperscript{−/−} and iNOS\textsuperscript{−/−} infected mice, which are unable to produce NO in response to Con A, still presented a significant unresponsiveness, although lower (ranging between 50–70% of control responses) than SC from infected wild-type littermates, thus indicating the existence of NO- and INF-\gamma-independent suppressive mechanisms (Fig. 1). In those mice immunosuppression was related to parasitemia (Fig. 1B). Moreover, this unresponsiveness cannot be overcome by l-NMMA treatment, discarding residual NO production as responsible for this effect (data not shown).

On the other hand, immunosuppression has been also associated with decreased IL-2 expression (7). In agreement with those results, IL-2 production by activated SC from T. cruzi-infected Sv 129 or C57Bl/6 mice was greatly reduced (5–10% of control responses) as compared with SC obtained from uninfected mice, despite the great differences in absolute parasitemia suggested that a parasite component was
involved. First, we tested whether parasite extracts inhibit T cell proliferation. *Trypanosoma cruzi* extracts inhibited SC proliferation by 50–70% in response to Con A (data not shown), as previously reported. We have previously shown that Ag C10, a mucin expressed in metacyclic trypomastigotes, epimastigotes and amastigotes, was able to downregulate IL-2 production in human PBMC (36). Thus, we tested whether Ag C10 was able to inhibit the proliferative response of SC from uninfected C57Bl/6 mice to T cell mitogens as Con A (Fig. 2A), or to anti-CD3/CD28 stimulation (data not shown), in a dose-dependent manner. A decrease in the production of IL-2 in the same cultures induced by AgC10 was observed, IL-2 production being somewhat more sensitive to AgC10 inhibition (Fig. 2A). Moreover, a monoclonal antibody to Ag C10 was able to prevent the inhibition observed with Ag C10, confirming that AgC10 and not a minor contaminant of AgC10 preparation was responsible for this effect (Fig. 2B). In addition, Ag C10 was able to inhibit proliferation and IL-2 production by SC from iNOS/C255/C255 mice activated with Con A as efficiently as the inhibition observed in SC obtained from control C57Bl/6 mice (Fig. 2C). The AgC10 immunosuppressive effect on SC was also observed with purified CD4+ or CD8+ cells upon stimulation with either Con A or anti-CD3, suggesting that this effect was taking place mainly through activation pathways on both types of T cells (Fig. 2D). Moreover, AgC10 did not cause cell death or any toxicity to mitogen or anti-CD3-activated cells from any mouse strain. Thus, cell death was <2% either when cells were treated only with AgC10 or in combination with the stimuli.

**Fig. 1.** NO- and IFN-γ-independent immunosuppression in *T. cruzi*-infected mice. iNOS/C255/C255 and IFN-γR/C255/C255 mice as well as their littermate controls, C57BL6 and Sv129, were intraperitoneally infected, or not, with *T. cruzi* parasites as described in the Methods. At the peak of parasitemia (14 days post infection), SC were obtained and cultured with ConA. (A) Proliferation was measured as [3H]thymidine incorporation after 72 h of stimulation, and NO production was measured by the Griess reaction. (B) Immunosuppression in iNOS/C255/C255 mice is related to parasitemia. Uninfected and 14-days-infected iNOS/C255/C255 mice were sacrificed and SC proliferation was measured in response to Con A. Parasitemia levels were measured as the number of parasites/ml in blood. (C) IL-2 production by SC from infected mice. After 24 h stimulation of SC, supernatants were collected and IL-2 concentration was determined by ELISA. Results are the means ± SD of three different experiments, with three mice per group in each one. All values in presence of AgC10 were significantly different from untreated controls (P < 0.001).
AgC10 inhibits IL-2 transcription

IL-2 production in T cells is mostly regulated at the transcriptional level. After TCR stimulation, different intracellular pathways are activated and IL-2 synthesis takes place (37). Once the inhibitory effect of AgC10 on IL-2 production was observed, we tested whether AgC10 was affecting IL-2 mRNA. This mucin inhibited IL-2 mRNA induction by Con A-stimulated SC in a dose–response manner, almost completely suppressing it (95% inhibition) at 20 μg/ml (Fig. 3A). Next, we tested whether the effect of AgC10 on IL-2 production was taking place at the transcriptional level. For this, we transfected a human T cell line, Jurkat, with a reporter plasmid expressing luciferase under the control of the human IL-2 promoter. AgC10 was able to inhibit IL-2 promoter activation after TPA plus calcium ionophore, or PHA stimulation by an average of 70% in the four experiments performed (Fig. 3B). AgC10 had no effect on basal IL-2 promoter activity (data not shown). IL-2 transcription after T cell activation requires the cooperative activity of several transcription factors that bind to IL-2 promoter regulatory sequences (37). Among those sites,
CD28RE (where c-rel and AP-1 bind), NFAT/AP-1 and NF-jB play a prominent role. Thus, we tested the effect of AgC10 on transcription driven by promoters containing exclusively any of those sites. AgC10 was able to inhibit CD28RE, NFAT and AP-1, but not NF-jB-driven promoters in various degrees although to a significantly lesser extent than the full IL-2 promoter. The NFAT-luc and CD28 RE-luc reporter genes were the most sensitive to AgC10 inhibition (Fig. 3C).

As an alternative approach to test if the inhibition of the activation of a particular transcription factor by AgC10 was involved in IL-2 inhibition, those factors were overexpressed in Jurkat cells by transient transfection, and we tested whether they could block or reduce AgC10 inhibitory activity. For this, Jurkat cells were transfected with plasmids expressing NF-jB family members (c-rel which binds to CD28RE site or p65/relA which binds to the kB site), a member of AP-1 complex (c-jun) or NFAT1. By using this approach, AgC10 inhibition of IL-2 promoter could not be completely reverted by any of these transcription factors alone. Again, NFAT seems to be the most sensitive factor to AgC10 inhibition since its overexpression greatly reverted AgC10 inhibition of IL-2 promoter (Fig. 3D). Those differences were not dependent on variations in the expression levels of the corresponding proteins, since all of them were expressed in transfected cells in apparently quite similar amounts as detected by western blotting (data not shown).

**Ag C10 inhibits an early step of T cell activation**

Right after T cell activation, once antigen is presented to the TCR-CD3 complex, protein kinases that belong to src family, such as the src family kinases, become activated. These kinases phosphorylate a variety of substrates, including transcription factors. AgC10 inhibits IL-2 transcription

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**Fig. 3.** AgC10 inhibits IL-2 transcription. (A) C57BL6 SC were stimulated for 48 h with 5 μg/ml of Con A in the presence or absence of growing concentrations of AgC10 (1, 5, 10 and 20 μg/ml), except the unstimulated controls (C). After stimulation, RNA was isolated and 20 μg were electrophoresed in agarose gel, transferred to a nylon membrane and hybridized with IL-2 and β-actin probes. Numeric values indicate the relative absorbance of the specific RNA bands. A representative experiment of the three performed is shown. (B and C) Jurkat cells were transiently transfected with 0.5 μg of IL-2 promter or multimerized CD28RE/AP1, NF-kB, AP1 or NFAT promoters coupled to luciferase reporter gene. Once transfected, cells were stimulated with 15 ng/ml of TPA plus 1 μM of calcium ionophore (B and C) or with PHA 2 mM (B), and treated with 15 μg/ml of AgC10 where indicated for 16 h. Cells were lysed and luciferase activity was quantified and normalized by measuring protein concentration in each sample. Data is represented as the mean ±SD of three different experiments. The P-values were: pIL-2 (P < 0.001); CD28RE (P < 0.01); NFAT (P < 0.001); NF-jB (n.s.); AP1 (P < 0.01). (D) Jurkat cells were co-transfected with 0.5 μg of pIL-2luc and 0.5 μg of the expression plasmids for NFAT, c-rel, p65, c-jun or its correspondent empty plasmids as controls. After stimulation with 15 ng/ml of TPA plus calcium ionophore 1 μM, in the presence or absence of 15 μg/ml of AgC10, luciferase activity was quantified and normalized as previously mentioned and represented as % of activation by TPA/Io. Only the reversion for NFAT was statistically significant (P < 0.001).
like Fyn and Lck, become activated and are able to phosphorylate ITAM sequences that are present in the TCR and CD3 intracytoplasmic domains. This process is followed by the coupling of other tyrosine kinases, such as ZAP70, and adapter proteins, such as SLP76 (38). The fact that many of the transcription factors that become activated after T cell stimulation and bind to IL-2 promoter to produce its maximum activation are affected in various degrees by AgC10 suggested that the effect of AgC10 could take place at an earlier event after the T cell triggering common to those pathways. To test this, we studied whether AgC10 affected the phosphorylation of tyrosine kinases and adapter proteins coupled to the TCR–CD3 complex after T cell activation. As shown in Fig. 4, AgC10 strongly inhibited SLP76 (~80%) and less efficiently ZAP70 (~40%) phosphorylation in tyrosine residues after TPA/lo or PHA activation of human jurkat T cells.

**AgC10 acts through L-selectin**

We have previously shown that AgC10 binds to L-selectin (CD62L) in human macrophages (20). Moreover, there are recent evidences showing that L-selectin acts as a signaling transducing receptor in T cells, affecting early events after T cell triggering (39). To study whether Ag C10 was acting on T cells through its binding to L-selectin, we first tested if Ag C10 was able to bind to L-selectin in T cells. As shown in Fig. 5, Ag C10 prevented the binding to mouse SC of a monoclonal antibody against CD62L.

Next, we tested if specific antibodies to L-selectin could mimic Ag C10 immunosuppressive activity. Mel-14, a mAb specific for mouse L-selectin, inhibits mouse T cell proliferation as well as IL-2 secretion induced by anti-CD3 in a similar way that Ag C10 does (Fig. 6A). This effect also takes place at the transcriptional level, since addition of a mAb specific for human L-selectin (Leu-8) to human Jurkat T cells also blocks induction of IL-2 transcription, measured as induction of IL-2 promoter-driven transcription after stimulation with TPA plus ionophore. This mAb to L-selectin did not have any affect on the basal activity of IL-2 promoter (Fig. 6B). The expression of L-selectin in jurkat T lymphocytes was on average 6 × 10^4 at the basal level (data not shown). AgC10 appeared to be acting directly via L-selectin, and not indirectly by mediating L-selectin shedding, since its inhibitory effect on cellular proliferation was also observed in the presence of TAPI-2, a specific inhibitor of L-selectin shedding (Fig. 6C).

To corroborate that the AgC10 inhibitory effect was taking place through L-selectin, we studied the effect of AgC10 on activation of SC obtained from Sell^-/- mice. As shown in Fig. 6(D), the marked inhibitory effect of AgC10 on proliferation to Con A when SC were obtained from control littermates (Sv129) was not observable on SC obtained from Sell^-/- mice, clearly confirming that AgC10 requires L-selectin to exert its inhibitory activity.

**Discussion**

Immunosuppression of T cell response during *Trypanosoma cruzi* infection in humans as well as in mice has been extensively
documented (4, 7, 8). This suppression is characterized by a lack of proliferative response to mitogens and antigens, and by strongly diminished IL-2 secretion. However, the cellular and molecular mechanisms underlying this inhibition, as well as the connection between those two phenomena are not yet fully understood. Diverse mechanisms regarding the responsible suppressor cell as well as the responsible mechanism, decreased IL-2/IL-2R and increased NO or PGs, were described (6, 7, 11–14, 40).

By taking advantage of iNOS−/− mice, we have evidenced the co-existence of at least two different mechanisms of immunosuppression. Furthermore, inhibition of IL-2Rα expression, which is controlled by IL-2 levels (42), is thought to be dependent on parasitemia levels in vivo and of parasite concentration in vitro. Thus, it is likely that the inhibition of IL-2/IL-Rα pathway is mostly mediated by parasite-derived molecules. Our data fit with this since unresponsiveness in iNOS−/− mice increases with parasitemia.

Our results, together with those of references (6) and (11) suggest a model to explain the underlying immunosuppression

Fig. 6. Involvement of L-selectin on AgC10 inhibition of T cell activation. (A) Sv129 mice SC were stimulated with 5 μg/ml of ConA for 72 h, in the presence or absence of 20 μg/ml of AgC10 or 10 μg/ml of anti-CD62L (Mel 14). Proliferation was measured as [3H]thymidine incorporation. After 24 h stimulation of SC, supernatants were collected and IL-2 concentration was determined by ELISA. Results are the means ±SD of triplicate cultures from three different experiments. (B) Jurkat cells were transiently transfected with 0.5 μg of IL-2 promoter coupled to luciferase (pIL-2Luc). Once transfected, cells were stimulated with 15 ng/ml of TPA plus 1 μM of calcium ionophore, and treated with 15 μg/ml of AgC10 or 10 μg/ml of anti-human CD62L mAb Leu8 where indicated, for 16 h. AgC10 and Leu8 were added to the culture 1 h before the stimuli. Cells were then lysed and luciferase activity was quantified and normalized by measuring protein concentration in each sample. Data are represented as the mean ±SD of three different experiments. All values in presence of AgC10 (P < 0.001) or anti-CD62L antibodies (P < 0.005) were significantly different. (C) Sv129 SC were stimulated as in (A), in the presence or absence of AgC10 (20 μg/ml), alone or combined with l-selectin shedding inhibitor, TAPI-2 (50 μM). Proliferation was measured as in (A). G1 and G2 represent two different groups of three mice each. All values in presence of AgC10 were significantly different from controls (P < 0.001). Moreover, no statistically significant differences were observed in presence or absence of TAPI-2. (D) l-selectin-deficient mice (Sell−/−) or littermate control (wt) SC, were stimulated with 5 μg/ml of ConA for 72 h, in the presence or absence of 20 μg/ml of AgC10, and proliferation was measured as previously mentioned. Results are the mean of two different experiments with three mice in each group in each experiment.
in acute *T. cruzi* infections. Thus, infection induces the colonization of the spleen of a suppressive immature Gr1^+^CD11b^+^ myeloid population. At the same time, parasite-derived molecules activate T cells to produce large amounts of IFN-γ and TNF-α that in turn activate those myeloid cells to secrete large amounts of NO. This NO can affect the proliferation of T cells either directly or by inducing apoptosis. Simultaneously, parasite-derived molecules can suppress directly the activation of both CD4 and CD8 T cells by blocking IL-2 and IL-2Rα synthesis. The predominance of one or other mechanism may depend on environmental and genetic factors as well as on the parasitemia levels. Moreover, the co-existence of both mechanisms ensures that T cell activation is effectively blocked during *T. cruzi* acute infection, to facilitate the dissemination and establishment of this parasite in the infected host. For *T. cruzi*, which needs to survive in chronically infected hosts in the presence of a specific immune response, it would not be surprising that more than one mechanism be concomitantly active.

The inhibition of host cytokine IL-2 synthesis by parasites appears to play a very important role in the pathology of the infections caused by these organisms (43). Nevertheless, little is known about the molecules responsible for these effects. In *T. cruzi* infection, the parasite component that induces this IL-2-mediated immunosuppression is not fully characterized. Previous reports have identified *T. cruzi* glycoinositolphospholipids (GIPL) (44,45) as potent suppressors of T cell proliferation to mitogens associated with low IL-2 and IL-2R expression. However, the cellular and molecular mechanisms underlying this inhibition were not addressed. Besides, not all GSL from all strains of *T. cruzi* are immunosuppressive, suggesting that they are not the most important molecules involved. Mucins are other possible candidates. In general, mucins from *T. cruzi* blood trypomastigotes are stimulatory rather than inhibitory (46). On the other hand, AgC10 is an abundantly expressed mucin present in metacyclic trypomastigotes, epimastigotes and amastigotes but not in blood trypomastigotes (20,27), as well as in the serum of infected mice (de Diego J. L., unpublished results). We have shown that AgC10 mimics some of the effects previously associated with *T. cruzi* infection as IL-2 and IL-2R downregulation (36). Mucins expressed by colon cancer cells also have immuno-suppressive properties, by strongly inhibiting IL-2 production (47–49). Thus, *T. cruzi* AgC10 mucin is a good candidate for the IL-2 suppressive activities both in CD4 and CD8 T cells. Recently, a single *T. cruzi* mucin gene expressed in eukaryotic cells has been described to induce T cell anergy, characterized by IL-2 decrease (50). The *T. cruzi* mucin family contains about 500 members divided into two main subfamilies (21). Thus, it is likely that more than one mucin could contribute to the immunosuppression in acute *T. cruzi* infection.

Moreover, it has been proposed that inhibition of IL-2 gene is the critical factor in immunosuppression in acute murine *T. cruzi* infection (51). IL-2 is regulated primarily at the transcriptional level. The IL-2 promoter has been extensively studied in humans and mice (37). From those studies, it is becoming clear that IL-2 transcription is the paradigm of cooperativity, requiring the coordinate activity of various transcription factors such as NFAT, p65 and c-rel and AP-1, which bind in a single or coordinate form to various promoter regions, including combined sites NFAT/AP-1 and CD28RE, where c-rel/AP-1 binds. In fact, the coordinate action of all of them is required for a full activation, and deletion or mutation of one of those sites significantly depresses IL-2 transcription. Ag C10 was able to induce a complete downregulation of IL-2 transcription. However, its effects on the response of the individual sites to mitogenic stimulation was much less pronounced, suggesting that the combined effect on several of them, most likely CD28RE/AP-1 and NFAT, can lead to the complete inhibition of the full gene. Despite this, NFAT activation seems to be the most sensitive target of AgC10 inhibition, since a reporter plasmid under the control of those sites was the most efficiently inhibited by AgC10 and moreover overexpression of NFAT, strongly although not completely, reverted AgC10 inhibition.

*Trypanosoma cruzi* infection in *vivo* is characterized by inhibition of binding of transcription factors to the enhancers of the IL-2 promoter (51), especially AP-1 (a component of CD28RE) and NFAT, whereas the effect was not so prominent with NF-κB. This fits extremely well with the observed activity of AgC10 in *vivo*, and points out to an important pathophysiological role of AgC10 in *T. cruzi* infection.

Besides, we have found that AgC10 inhibits such early steps of T cell activation as tyrosine phosphorylation in the TCR-complex adapter protein SLP-76 and in the TCR recruited ZAP-70 tyrosine kinase. SLP-76 is required for NFAT and AP-1 activation (52,53), as well as for CD28 responses (54) and IL-2 induction. Similarly, ZAP-70 is required for both NFAT, CD28RE and AP-1 activation and IL-2 induction (54–56). This fits with the observed effects of AgC10 on T cell activation and may explain why AgC10, by inhibiting SLP-76 and ZAP-70, is affecting NFAT, AP-1 and CD28RE-dependent pathways simultaneously, leading to almost complete suppression of IL-2 transcription.

We have also shown that AgC10 binds to L-selectin in T cells. This confirms our previous results in human macrophages (20). The effects of AgC10 on T cells seem to be mediated via its interaction with L-selectin since it did not suppress Sell–/– SC proliferation to mitogens. AgC10 did not seem to indirectly affect L-selectin by inducing its shedding from the membrane, since no changes in AgC10 immunosuppressive activity were observed when cells were treated with AgC10 in the presence of the L-selectin inhibitor TAPI-2. Since AgC10 did not have any suppressive effects on Sell–/– mouse cellular proliferation or IL-2 production, it is unlikely that earlier activation that affects IL-2 promoter transcriptional activation, such as ZAP 70 and SLP 76 phosphorylation, should be affected in those mice by AgC10. Although due to the mucin-like nature of AgC10 and its sticky characteristics as mucin, we cannot discard that AgC10 is binding to other cell surface molecules. Moreover, mAb specific for L-selectin inhibited T cell proliferation, IL-2 synthesis and IL-2 promoter-driven transcription as AgC10 did in both human and mouse T cells.

Selectins are adhesion molecules mediating the transient interaction of leukocytes with endothelial cells, which has been described as rolling. L-selectin is constitutively expressed on almost all leukocytes, including T cells (57). Moreover, recent evidence indicates that L-selectins also function as signal transducing receptors which induce the activation of several intracellular signaling cascades (58,59). Moreover, GlyCAM-1,
a soluble L-selectin ligand, also activates T cells (60). Thus, in T lymphocytes, L-selectin triggering results in a src-tyrosine kinase dependent activation of the MAP-kinase JNK (58), which is most important in the control AP-1 activity (61). Besides, L-selectin stimulation leads to activation of p56lck, calcium release and subsequent activation of calcineurin, which dephosphorylates NFAT (39). AgC10 inhibits some of the pp56lck-mediated effects as Tyr-phosphorylation of both SLP-76 and ZAP-70 and NFAT and AP-1 activation. Thus, it is very likely that Ag C10 may interfere with GlyCAM-1 or L-selectin src-mediated co-stimulatory signaling leading to NFAT and AP-1 activation and resulting in the observed immunosuppression.

In summary, our results have shed some light on the molecular mechanism by which immunosuppression takes place on the acute phase of Chagas’ disease. AgC10 may be released to the serum and, through an L-selectin-dependent mechanism, blocks phosphorylation of the TCR/associated signal transduction proteins ZAP-76 and SLP-76. This leads to inhibition of the transcription factors NFAT and AP1, followed by a decrease in IL-2 transcription. This represents a previously undescribed mechanism used by parasites to avoid potentially damaging immune responses, that serves them to evade the host’s immune response. Finally, AgC10 has the potential to be used as reagent to explore the regulatory mechanism governing lymphocyte activation.

Acknowledgements

We thank the personnel from the animal facilities of the CBMSO, as well as Carmen Punzón and Gloria Escríbano for their excellent technical assistance. This work was supported by grants from the Ministerio de Educacion y Cultura, Fondo de Investigaciones Sanitarias, Comunidad Autónoma de Madrid and the Fundación Ramón Areces.

Abbreviations

AP1 activated protein 1
Con A concavalin A
GPI glycosphosphatidylinositol
iNOS inducible nitric oxide synthase
io calcium ionophore
L-NMMA N(G)-monomethyl-L-arginine
NFAT nuclear factor activated T cells
NF-κB nuclear factor κB
NO nitric oxide
SC spleen cells

References
