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**“GLYCOPATHOLOGY OF CHAGAS DISEASE.
ROLE OF GALECTINS IN THE OUTCOME OF
THE IMMUNE RESPONSE DURING THE
EXPERIMENTAL *TRYPANOSOMA CRUZI*
INFECTION”**

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ABSTRACT

Galectins are an evolutionary conserved family of galactoside-binding proteins. It has been shown that these proteins play important roles as regulators of homeostasis, because they control inflammatory response, cell adhesion, cell growth, apoptosis and immune response. They are differentially expressed by various immune cells and their expression levels depend on cell activation and differentiation, as well as on pathological conditions. Galectins not only bind host ligands, but they can bind to several glycans on pathogen surface, as it has been shown for viruses, bacteria, nematodes or parasites. The interaction of galectins with pathogens leads to activation of the immune system and can mediate cell-pathogen interactions.

Regarding *T. cruzi* infection, it has been reported that galectin-3 binds to the parasite surface promoting adhesion to host cells *in vitro*, and it is known as well that galectin-1 and -3 modulate B cell response and macrophage activity during the course of infection.

In this work, we first investigated whether recombinant human galectins are able to bind *T. cruzi in vitro*. Our results confirm the reported observation regarding the ability of galectin-3 to bind *T. cruzi* as well. In addition, we observed that other members of the galectin family can bind *T. cruzi* surface, like galectin-1, 4, 7 and 8 in a specific and differential way regarding binding intensity and specificity. Moreover, galectins presented a higher avidity for infective forms of *T. cruzi* present in the host, amastigotes and trypomastigotes, than the non-infective forms, reflecting the changes that occur in the parasite during the metacyclogenesis process in order to become infective and face up the new challenge that the host means for the survival of the parasite. *T. cruzi* has developed during the evolution mechanisms to module and escape from the host immune response. Here we show that *T. cruzi* is able to proteolitically process galectin-3, -4 and -8, resulting in truncated galectins that preserve the Carbohydrate Recognition Domain, but lack the multivalency associated to many of the galectin biological functions. The processing of galectins by *T. cruzi* is shown to be dependent of Zn-metalloproteases and collagenases. When these proteases were inhibited, recombinant galectins induced parasite death, suggesting that galectins could act as direct microbicide agents, meanwhile this ability is counteracted by specific proteases in *T. cruzi* implicated in galectin cleavage.

The results obtained *in vitro* suggest an important role of galectins during *T. cruzi* infection, and this was confirmed *in vivo* after experimental murine infection. In general, galectins were highly regulated during the course of infection, in different

spleen cell subsets and in the heart, both in cardiac cells and infiltrating immune cells. The role of galectin-3 during the outcome of the infection was studied using genetically deficient mice. The lack of galectin-3 led to a higher parasitemis during *T. cruzi* infection. Galectin-3 deficient mice mounted an altered humoral response, in the sense that they generated antibodies against the glycan epitope Galb1-3GlcNAcb1-3Galb1-X, structure not immunogenic in wild type mice. In addition, they presented a diminished inflammatory and immune response, in concordance with the reported pro-inflammatory functions reported for galectin-3. Galectin-3 knock-out mice showed a misbalanced cytokine serum levels during infection, down-regulation of inflammatory genes associated to resistance to *T. cruzi* infection, IFN- γ , iNOS, IL-12 and COX-2, and up-regulated expression of the Toll Like Receptors, an important family of pathogen recognition receptors implicated in *T. cruzi* recognition. Parasite burden in the heart of infected galectin-3 deficient mice was not significantly different from wild type mice, but in contrast, showed a dramatically reduction in the number of infiltrating immune cells, suggesting a pivotal role of galectin-3 in cell recruitment to the site of infection.

In an attempt to elucidate the immunological mechanisms that led to these abnormalities, we differentiated galectin-3 deficient dendritic cells *in vitro*, which showed a reduced TNF- α and IL-1 production after *T. cruzi* infection. This deficiency was correlated to a deficient TLR2-TLR1 heterodimer surface formation, due to the lack of TLR-1 surface expression. The defects in dendritic cells responses due to the lack of galectin-3, could explain the systemic alterations observed in the experimental infection of deficient mice.

Taken all the data together, galectins might play key roles in innate and adaptive responses during *T. cruzi* infection by modulating parasite recognition, interactions with host cells, parasite clearance and initiation and progression of the outcome of the immune response mounted against *T. cruzi*.

RESUMEN

Las galectinas son una familia de proteínas de unión a carbohidrato altamente conservadas a lo largo de la evolución. Se ha demostrado que estas proteínas juegan un papel importante como reguladores de la homeostasis, debido a su capacidad de modular la respuesta inflamatoria, adhesión celular, crecimiento celular, apoptosis y respuesta inmune. Son expresadas diferencialmente por diferentes células del sistema inmune, y sus niveles de expresión dependen del estado de activación y diferenciación celular, así como de procesos patológicos. Las galectinas no solo unen ligandos propios del hospedador, si no que pueden unirse también a ligandos presentes en la superficie de organismos patógenos como virus, bacterias, nematodos o parásitos. La interacción de las galectinas con organismos patogénicos conduce a la activación del sistema inmune y puede mediar interacciones célula-patógeno.

Con respecto a la infección por *T. cruzi*, ha sido descrito que galectina-3 se une a la superficie del parásito potenciando la adhesión a células del hospedador, y se sabe que galectina-1 y -3 modulan la respuesta de células B y macrófagos durante el desarrollo de la infección.

En el presente trabajo, investigamos primero si galectinas humanas recombinantes eran capaces de unirse *in vitro* a *T. cruzi*. Nuestros datos confirmaron que galectina-3 es capaz de unirse a *T. cruzi*. Además, observamos que otros miembros de la familia de galectinas pueden unirse también a *T. cruzi*, como galectina-1, -4, -7 y -8 de una manera específica y diferencial en lo relativo a la intensidad de la unión y a su especificidad. Además, galectinas recombinantes mostraron una afinidad mayor por las formas infectivas de *T. cruzi* que están presentes en el hospedador, amastigotes y trypomastigotes, que las formas no infectivas, reflejando los profundos cambios que ocurren en el parásito durante el proceso de metaciclologénesis con el objetivo de ganar infectividad y afrontar el nuevo reto que el hospedador supone para el parásito y su supervivencia. *T. cruzi* ha desarrollado durante la evolución mecanismos para modular y escapar de la respuesta inmune del hospedador. En este trabajo mostramos que *T. cruzi* es capaz de procesar proteolíticamente la galectina-3, -4 y -8, conduciendo a la aparición de galectinas truncadas que preservan intacto el dominio de reconocimiento a carbohidrato, pero pierden la multivalencia asociada a muchas de sus funciones biológicas. El procesamiento de las galectinas por *T. cruzi* resulta ser dependiente de Zn-metaloproteasas y colagenasas. Cuando estas proteasas son inhibidas, las galectinas recombinantes inducen muerte en el parásito, sugiriendo que las galectinas podrían actuar como agentes microbicidas *per se*, mientras que esta capacidad es contrarrestada

por *T. cruzi* mediante proteasas específicas para producir cortes en la secuencia de las galectinas.

Los resultados obtenidos *in vitro* inducen a pensar que las galectinas juegan un papel prioritario durante la infección por *T. cruzi*, como fue confirmado *in vivo* después de la infección experimental murina. En general, las galectinas fueron altamente reguladas durante el curso de la infección, en distintos tipos celulares procedentes de bazo de animales infectados, y en el corazón, tanto en células cardíacas y en células inmunes infiltradas. El papel de la galectina-3 durante el desarrollo de la respuesta inmune fue analizado usando ratones genéticamente deficientes. Estos ratones mostraron una mayor parasitemia, y generaron anticuerpos contra el epítopo glicano Galb1-3GlcNAcb1-3Galb1-X, estructura no inmunogénica para ratones salvajes. Además, mostraron una respuesta inflamatoria disminuida, en concordancia con trabajos previos señalando el papel pro-inflamatorio de la galectina-3. Ratones deficientes en galectina-3 mostraron un desajuste en los niveles de citoquinas en suero durante la infección, regulación a la baja de genes asociados a la resistencia a la infección como IFN- γ , iNOS, IL-12 and COX-2, y regulación a la alza de la familia de receptores Toll Like, importantes en el reconocimiento de *T. cruzi*. La carga parasitaria en el corazón de ratones deficientes en galectina-3 no fue significativamente diferente de los ratones salvajes control, pero mostraron una reducción drástica en el número de células inmunes infiltradas, indicando un papel central de la galectina-3 en el proceso de reclutamiento de células inmunes a los sitios de infección.

Intentando profundizar en los mecanismos inmunológicos que condujeron a estas anomalías, se diferenciaron *in vitro* células dendríticas deficientes en galectina-3, las cuales mostraron una producción muy reducida de TNF- α y IL-1 después de infectarlas con *T. cruzi*. Esta deficiencia se correlaciona con una deficiente presentación en la superficie celular del complejo TLR1-TLR2, debido a una ausencia de TLR-1 en la membrana celular. Este defecto, producto de la falta de galectina-3, en la respuesta inmune de células dendríticas podría explicar las alteraciones sistémicas observadas en los ratones deficientes durante la infección experimental.

Considerando todos los datos, podemos concluir que las galectinas deben jugar un papel clave durante el establecimiento de la respuesta inmune innata y adaptativa, debido a sus implicaciones en el reconocimiento de *T. cruzi*, interacciones del parásito con células hospedadoras, eliminación del parásito e iniciación y progreso de la respuesta inmune montada contra *T. cruzi*.

INTRODUCTION

1. Chagas disease, history, etiological agent, distribution and clinical manifestations.

Chagas disease is a chronic illness caused by the protozoan *Trypanosoma cruzi*. It was first described by Carlos Chagas in 1909 (Chagas 1909; WHO 2006), but the disease was not seen as a major public health problem until the 1960s. He discovered that the intestines of Triatomidae harboured an unknown specie of the genus *Trypanosoma*, a flagellate protozoan. He named the parasite that causes the disease *Trypanosoma cruzi*, recalling the Brazilian physician and epidemiologist Oswaldo Cruz. Carlos Chagas identified the causative agent, vector, host, clinical manifestations and epidemiology of the disease.

Chagas disease currently affects 16–18 million people, with some 100 million (25% of the whole Latin American population) at risk of acquiring the disease, killing around 50,000 people annually. It is estimated that the disease is responsible of 649,000 DALYs every year (Disability Adjusted Life Years, the number of healthy years of work lost due to premature death and disability) (WHO 2006). The disease is distributed in the Americas, from Southern Argentina to the Southern United States, covering 18 countries distributed in 2 different ecological zones, the Southern Cone and the Northern South America, Central America and Mexico. Nowadays, there is an increasing risk of infection in the developed countries, like the European Union and Spain, due to migration movements.



Illustration 1: geographical distribution of Chagas disease. Endemic areas of infection in humans are highlighted in red.

T. cruzi is transmitted by hemiptera from the family *Reduviidae*, such as *Triatoma*, *Rhodnius* or *Panstrongylus*, via the vector faeces after a bug bite. *T. cruzi* life cycle is really complex. An infected triatomine insect vector feeds on blood and releases trypomastigotes in its feces near the site of the bite wound. Those affected, by scratching the site of the bite, causes trypomastigotes to enter the host through the wound, or through intact mucosal membranes, such as the conjunctiva. Then, inside the host, the trypomastigotes invade cells, where they differentiate into intracellular amastigotes. The amastigotes multiply by binary fission and differentiate into trypomastigotes, then are released into the circulation as bloodstream trypomastigotes. These trypomastigotes infect cells from a variety of biological tissues and transform into intracellular amastigotes in new infection sites. The bloodstream trypomastigotes do not replicate. Replication resumes only when the parasites enter another cell or are ingested by another vector. The reduviid bug, also known as 'kissing' bug, becomes infected by feeding on human or animal blood that contains circulating parasites. The ingested trypomastigotes transform into epimastigotes in the vector's midgut. The parasites multiply and differentiate in the midgut and differentiate into infective metacyclic trypomastigotes in the hindgut, completing the life cycle.

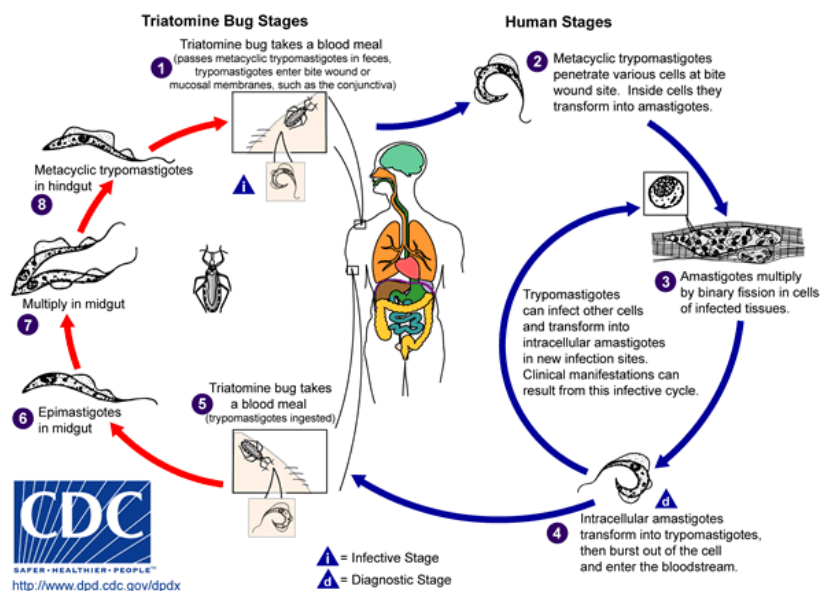


Illustration 2: Schematic representation of the life cycle of *T. cruzi*. *T. cruzi* is naturally transmitted by hemiptera from the family *Reduviidae*. During blood sucking, the insect defecates on the skin (1), carrying the infective metacyclic trypomastigotes that enter the mammalian host and infect local cells (2). Parasites inside the cells turn into amastigotes which multiply intracellularly (3). Some amastigotes transform again into trypomastigotes that disrupt the cell (4) infecting new cells and enter the bloodstream. Bugs are infected when they feed on blood containing parasites (5). Once inside the insect, *T. cruzi* transform into epimastigote form (6), multiplies by binary fission (7) and differentiate into infective metacyclic trypomastigotes (8), thus completing the life cycle.

Two different transmission cycles exist for *T. cruzi*: one related to wild, non-human vertebrate hosts (over 100 mammalian species like opossums and armadillos), called the 'sylvatic cycle'; and another one, dependent on home-dwelling hemiptera and involving humans and household animals. This is called 'domestic cycle'. Humans are considered to be a very new host for *T. cruzi*. In fact, it is estimated that the first contact with humans occurred in the late Pleistocene, 30000-15000 years ago, meanwhile the parasite emerged as a specie over 150 million years (Briones, Souto et al. 1999).

However, *Trypanosoma cruzi* can also be transmitted through blood transfusions, organ transplantation, transplacentally, breast milk, oral ingestion (Yoshida 2008) and in laboratory accidents. Children can also acquire Chagas' disease while still in the womb. According to the World Health Organization, the infection rate in Latin American blood banks varies between 3% and 53%, a figure higher than of HIV infection and hepatitis B and C (WHO 2006).

This high blood prevalence has made that the original distribution of the disease is being modified nowadays due to population movements and immigration, and new cases have been reported in other regions like United States and Europe, turning into a new public health problem in countries where the disease was unknown, mainly due to blood transfusions.

T. cruzi infection progress has been divided into successive acute and chronic stages, according to our current pathogenic knowledge of Chagas disease. Following the parasite infection, there is a short acute phase showing an abundant parasitemia, but frequently with very mild and unspecific symptoms and the infection can be unnoticed. A small sore can develop where the parasite enters the body. When the inoculation site is the conjunctival mucous membranes, the patient may develop unilateral periorbital edema, conjunctivitis, and preauricular lymphadenitis. This constellation of symptoms is referred as Romana's sign. Most patients results to be asymptomatic in the acute phase, making difficult the diagnosis. In addition to those asymptomatic patients, others develop symptoms that include fever, enlarged and painful lymph nodes and fatigue. This can last up to four weeks, and then these symptoms disappear. Death in acute Chagas disease can be related to heart failure and/or meningitis in immune-compromised patients but more commonly, patients enter a symptomless phase known as indeterminate phase that can last for 10-20 years. During this phase the parasitemia is virtually undetectable, and the parasites are invading most organs of the body. Only one third of the individuals with indeterminate infections will develop clinical

manifestations. The disease has a very variable clinical presentation, affecting nervous system, digestive system or heart. Thus, chronic infections result in various neurological disorders, including dementia, damage to the heart muscle, and sometimes dilation of the digestive tract (megacolon and megaesophagus) (Lopes, Rocha et al. 1989; Adad, Andrade et al. 1991), as well as weight loss. Swallowing difficulties may be the first symptom of digestive disturbances and may lead to malnutrition. However, considering the epidemiology data, the most devastating manifestation of the disease is the Chagas cardiomyopathy. After several years of an asymptomatic period, 27% of those infected develop cardiac damage, 6% develop digestive damage, and 3% present peripheral nervous involvement. Several cardiac symptoms are associated to the disease, including premature contractions, ECG abnormalities, enlarged heart, altered heart rate or rhythm, heart failure and cardiac arrest (Tanowitz, Kirchhoff et al. 1992). Only the chemotherapeutic agent Benznidazole is used for the treatment of chagasic patients, despite is highly toxic and has limited efficacy, especially in the chronic phase of the disease.

Two primary hypotheses are proposed to account for pathogenesis in chronic *Trypanosoma cruzi* infections. One claims that the pathology is directly linked to the parasite persistence in infected tissues (Zhang and Tarleton 1999; Tarleton 2001), resulting in chronic inflammatory reactivity. The other one postulates an autoimmune basis for the pathology (Kierszenbaum 1986; Levin, Kaplan et al. 1993; Soares, Pontes-De-Carvalho et al. 2001; Girones, Cuervo et al. 2005; Girones, Carrasco-Marin et al. 2007). According to this one, the parasite presence is not crucial for the Chagas pathology, since there are not a big number of parasites in some affected organs during the chronic phase, but it claims that *T. cruzi* infection induces immune responses which are targeted at self tissues. However, both events are not mutually exclusive, and they could occur simultaneously during Chagas disease (Girones and Fresno 2003; Kierszenbaum 2005).

It has been proposed that the diversity in the clinical manifestations of Chagas disease may depend on the genetic host background (Andrade, Machado et al. 2002), and also on the genetic diversity of the parasite. Different studies have demonstrated that *T. cruzi* is a very heterogeneous taxon (Macedo and Pena 1998; Devera, Fernandes et al. 2003; Macedo, Machado et al. 2004). DNA markers like rDNA or miniexon gene have generated a consensus about the existence of two major phylogenetic lineages

within the taxon (Zingales, Souto et al. 1998), that has been designated as T cruzi I and T cruzi II.

The relevance of this genetic variability can be a determinant of differential tissue tropism or distribution, and consequently of the clinical forms of the disease (Montamat, De Luca D'Oro et al. 1996; Andrade, Machado et al. 1999) Thus, it is natural to think that not all *T. cruzi* strains are able to infect humans, and that different strains can show different clinical manifestations (Vago, Andrade et al. 2000). The genetic structure of *T. cruzi* populations is even more complex, because there are different subgroups described in every single lineage *T. cruzi* I or *T. cruzi* II (Montamat, De Luca D'Oro et al. 1996; Brisse, Dujardin et al. 2000). Recently, it has been identified five lower phylogenetic subdivisions in the TcII group, proposing the existence of six discrete lineages in total (Brisse, Dujardin et al. 2000; Brisse, Verhoef et al. 2001; Sturm, Vargas et al. 2003). Tc I and Tc II are associated to the 'sylvatic cycle' and 'domestic cycle' respectively. Cases of human infection by *T. cruzi* I strains are rare and usually asymptomatic. However, a correlation between genetic variability and clinical symptoms of the disease has not yet been established, because the available diversified methods used to type or group *T. cruzi* strains may face drawbacks that interfere with the results. They are, for instance, the isolation of *T. cruzi* populations from hosts that harbor more than a single strain or the selection of populations during the maintenance of strains in laboratory conditions or during their amplification in experimental animals or in cultures (Devera, Fernandes et al. 2003).

2. Immune response

2.1 Innate and immune response against *T. cruzi*

T. cruzi triggers a complex immune response. Soon after the parasite overcomes the skin barrier, it is recognized by the immune system in order to activate a proper immune response. Complement and acute phase proteins bind to *T. cruzi*, and mediates the first interaction between parasite and phagocytic cells. Subsequently, other host receptors interact with the parasite. Toll Like Receptors, TLRs, are the best characterized receptors that identify parasite components, but others can collaborate, like the mannose receptor or the dectin family. TLRs are pathogen recognition receptors (PRRs) associated to the innate immune system. The family consists of several

members. TLR-2, TLR-4 and TLR-9 are known to recognize *T. cruzi* (Bafica, Santiago et al. 2006; Tarleton 2007). Moreover, mice deficient in the adaptor molecule MyD88 were unresponsive to *T. cruzi* (Campos, Closel et al. 2004), in a very similar fashion shown by TLR-2 and TLR-9 deficient mice. TLR-4 works as a homodimer on the cell surface, meanwhile TLR-2 needs to form heterodimers either with TLR-1 or TLR-6 in order to trigger intracellular signals (Sandor, Latz et al. 2003). PRRs recognize pathogen associated molecular patterns (PAMPs), molecular structures found only in pathogens but not in the host. Thus PRRs in general and TLRs particularly, can distinguish self from nonself components. Glycoconjugates presents on *T. cruzi* are recognized by TLRs in DCs, leading to cytokine and chemokine production: IL-8, IL-12, macrophage inflammatory protein-1- α and monocyte chemoattractant protein-1 (MCP-1/CCL2) (Ouaissi, Guilvard et al. 2002). This recruits macrophages to the infection site, cells that are infected by the parasite. Macrophages and DCs secrete high amounts of pro-inflammatory cytokines, like IL-12 and IL-1, which activates natural killer cells to produce interferon- γ (IFN- γ) (Aliberti, Cardoso et al. 1996). This IFN- γ acts over macrophages and DCs to induce TNF- α production, that synergistically with IL-12 and TNF- α produce nitric reactive species (NO) through up-regulation of the enzyme iNOS, which in turn, leads to parasite killing (Munoz-Fernandez, Fernandez et al. 1992; Abrahamsohn and Coffman 1996). The parasite avoids the early immune response and spreads through the body invading tissues. During the acute phase, *T. cruzi* can infect almost every nucleated cell. Immune response against *T. cruzi* also involves activation of the humoral and cellular components of the adaptive response. Immune response induces a polyclonal expansion of T (CD4⁺ and CD8⁺) and B cells. Despite this polyclonal activation, there is a high humoral and cellular immunosuppression. The mechanisms of such immunosuppression range from downregulation of IL-2 receptor in activated T cells (Majumder and Kierszenbaum 1996), to immature myeloid suppressor cells (Goni, Alcaide et al. 2002) or to the enhanced cell death of lymphocytes (Lopes and DosReis 1996; Zuniga, Motran et al. 2000). This functional deficiency has been implicated in the ability of the parasite to evade the immune response and persist in the host. Although the balance Th1/Th2 (which appears to result from mutual functional antagonism exerted by IFN and IL-10) is important for the outcome of the immune response in other parasite diseases like

Leishmaniosis, there is a co-existence of both types of response during Chagas disease(DosReis 1997). Th1 T cells produce IFN- γ , increasing TNF- α and NO by

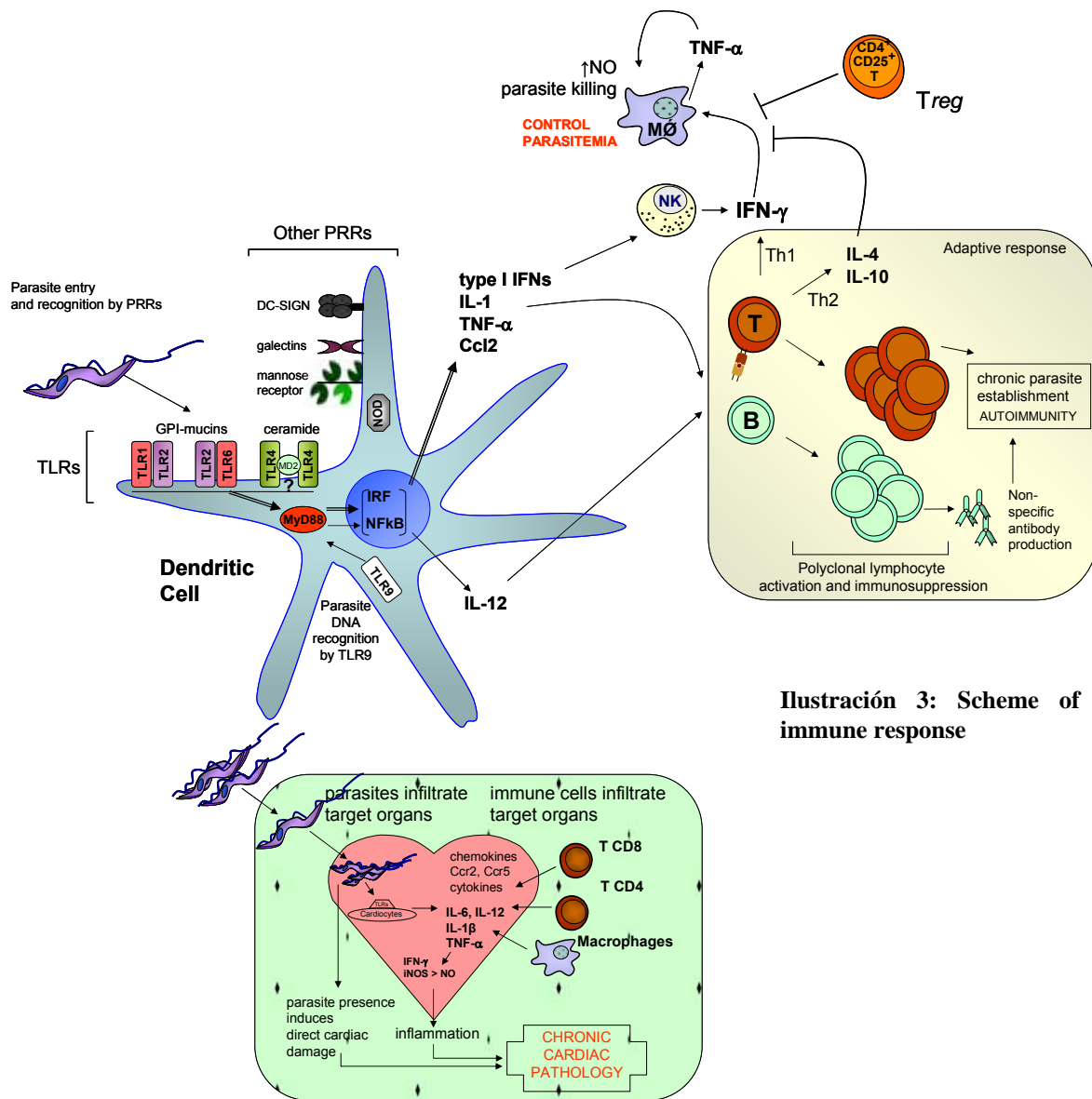


Ilustración 3: Scheme of immune response

macrophages, in a classical LPS-dependent macrophage activation. The polyclonal expansion affects also to the B cell repertoire, accompanied by a huge increase in immunoglobulin production. However, most of these antibodies are not effective, because they are non-specific for *T. cruzi*, and this could favor the appearance of autoimmune processes. When parasites disappear from the blood stream, the strong Th1 response is controlled by Th2-like cytokines (IL-4, IL-10) (Hunter, Ellis-Neyes et al. 1997; Abrahamsohn, da Silva et al. 2000) and regulatory T cells. Once the acute phase

is over, parasites are virtually undetectable in the blood of patients, and they restrict their presence to target tissues, commonly the heart. Parasite burden in the heart is elevated during the acute phase, and heart invasion by *T. cruzi* leads to chemokine production by cardiomyocytes (Ccl5/RANTES, CXCL3/MIP-2 β), cytokines (IL-6, IL-1 β , TNF- α) and NO (Zhang and Tarleton 1996; Chandrasekar, Melby et al. 1998; Machado, Martins et al. 2000). The only presence of this cytokines induce parasite killing by NO-dependent mechanism in macrophages (Villalta, Zhang et al. 1998), and besides this creates an inflammatory scenario and recruits infiltrating CD4⁺ T cells, CD8⁺ T cells and macrophages. IFN- γ produced by T cells induces NO production by macrophages increasing parasite killing. After the acute phase, the parasite burden in heart is gradually reduced, but a significant number of infiltrating cells still remain in the heart, supporting the hypothesis of autoimmunity processes as a cause of Chagas cardiomyopathy.

2.2 The Danger model, a new link between innate and adaptive response

For over 50 years immunologists have based their work on the idea that the immune systems functions by distinguish between self and nonself. Nowadays, a new theory is emerging in the field, attempting to complete the inherent problems of the self versus nonself classical theory, and it has been named 'the Danger Model'. In this Model, innate immunity is more concerned with damage inducing by invading pathogens than with the 'foreignness' of invading pathogen. Then, immunity is called into action by alarm signal coming from the injured cells. For instance, the presence of certain intracellular molecules out of the usual location serves to activate the subsequent innate immune events that will lead to the acquired immunity. Thus, danger signals initiate the immune response, although the original pathogen is the responsible of the activation of such danger signals. In homeostatic condition, although there is antigen presentation, the lack of these danger signals keeps the immune system in a non-reacting status. (Brown and Lillicrap 2002; Matzinger 2002), The model is an effort to understand the initiation of immune response, and how self and non-self is distinguish by the immune system, or in other words, how pathogens generate an immune response, meanwhile self components do not.

3. Glycoconjugates present in *Trypanosoma cruzi*.

Some protozoa present a simple life cycle, like *Trichomonas*, but others like *Leishmania* sp. or *T. cruzi* present a really complex life cycle as exposed previously. *T. cruzi* colonizes different habitats during its life cycle, from the intestinal tract of an insect to the cytoplasm of a mammalian cell, with a brief passage by the blood of the vertebrate host. An adaptation of the parasite to these different environments should occur and, consequently, differences on the composition of the plasma membrane should happen, because the surface of parasitic protozoa plays a primary role in the interaction with the environment. A thick coat of glycoconjugates covers the surface of *Trypanosoma cruzi*, and the nature and composition of these glycoconjugates change on the different parasite biological stages. It is known that glycans are of great importance in parasite protection, attachment to insect guts, interaction with host cells and host immunomodulation, since most of the PRRs described so far in *T. cruzi* are glycosylated.

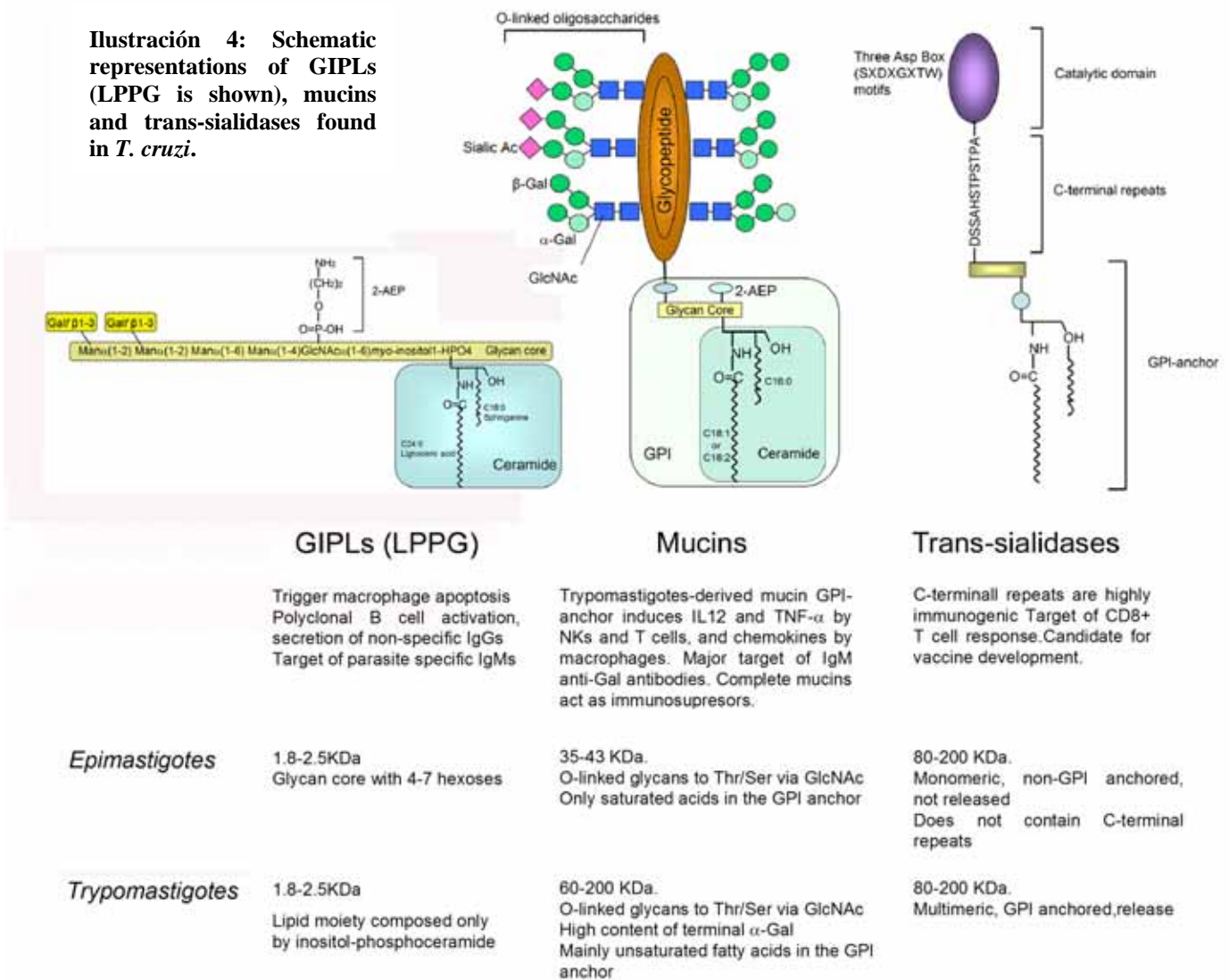
Glycosylphosphatidylinositol (GPI)-anchored molecules are the most abundant molecules present on the parasite surface, and are indeed essential to control activation of the innate immune system as well as initiation of the acquired host immune response in the vertebrate host. (GPI)-anchored molecules are basically GIPLs, mucins and trans-sialidases.

3.1 Structure and function of GIPLs.

GIPLs are small molecules composed by a conserved GPI glycan core, containing the conserved motif $\text{Man}\alpha 1-4\text{GlcNac}\alpha 1-6\text{phosphatidylinositol(PI)}$ and a ceramide moiety. Additional differences may be found in the lipid tails and carbohydrate branches linked to the conserved glycan core. Based on this, GIPLs can be grouped in three different types. Type I GIPLs ($\text{Man}\alpha 1-6\text{Man}\alpha 1-4\text{GlcNac}\alpha 1-6\text{PI}$) are the main plasma components of epimastigotes and metacyclic trypomastigotes (Previato, Gorin et al. 1990; Carreira, Jones et al. 1996). GIPLs are the dominant glycolipids present on all the developmental stages of *T. cruzi*, but are much less abundant in trypomastigotes than in epimastigotes (Golgher, Colli et al. 1993). The most common glycoproteins of trypanosomatids are anchored by

glycoinositolphospholipids (GIPLs) to their plasma membrane. In addition, free GIPLs have been described, for instance the lipopeptidophosphoglycan (LPPG) which is a major component of the surface of *T. cruzi* epimastigotes. It has been demonstrated the presence of an immunogenic epitope in LPPG containing β -galactofuranose (Gal β) (Schnaidman, Yoshida et al. 1986). The ability of GIPLs to modulate the immune response has been clearly demonstrated. They have a wide range of non-specific activities in macrophages (Freire-de-Lima, Nunes et al. 1998), NK cells (De Arruda Hinds, Previato et al. 1999), T (Gomes, Previato et al. 1996; Bellio, Liveira et al. 1999; Oliveira, Peixoto et al. 2004) and B lymphocytes (Bento, Melo et al. 1996; Bilate, Previato et al. 2000).

Ilustración 4: Schematic representations of GIPLs (LPPG is shown), mucins and trans-sialidases found in *T. cruzi*.



3.2 Structure and function of the mucin-like glycoprotein family.

The major protein components of the *T. cruzi* surface are mucin-like proteins. Mucins are GPI-anchored peptides enriched in hydrophilic amino acids (threonine, serine, lysine and glycine) that serve as scaffold for the extensive addition of O-glycans (Schenkman, Ferguson et al. 1993; Previato, Jones et al. 1994; Previato, Jones et al. 1995; Serrano, Schenkman et al. 1995). The oligosaccharides of *T. cruzi* mucins represent approximately 60% of the total mass of the glycoprotein, are highly branched and contain Gal β , Gal α and sialic acids units besides O-linked GlcNAc. The mucins expressed by different stages of *T. cruzi* are highly polymorphic. Those from epimastigotes and metacyclic trypomastigotes migrate in SDS-polyacrilamide gels as double or triple bands of 35-43 and 35-50 KDa (Di Noia, Sanchez et al. 1995). In contrast, in tissue cultured trypomastigotes mucins are much bigger, in the range of 60-220 KDa (Freitas-Junior, Briones et al. 1998; Previato, Sola-Penna et al. 1998). Mucins are responsible of many of the immunomodulatory properties of *T. cruzi*. α -galactosyl epitopes are targets of the abundant lytic anti-Galactose antibodies from acute and chronic chagasic patients (Freitas-Junior, Briones et al. 1998; Previato, Sola-Penna et al. 1998), and the trypomastigote mucin GPI-anchor can alone induce the synthesis of proinflammatory cytokines and nitric oxide by macrophages (Camargo, Almeida et al. 1997), but the same fraction obtained from epimastigotes did not show such properties. In contrast, a purified complete mucin, AgC10, inhibits pro-inflammatory cytokine production in macrophages, (de Diego, Punzon et al. 1997; Alcaide and Fresno 2004), showing the complexity of the immunomodulation that these molecules are able to.

3.3 Structure and function of members of the trans-sialidase family.

Several surface antigens of *T. cruzi* have been grouped in a superfamily defined by the presence of a consensus amino acids sequences homologous to bacterial sialidases (de Diego, Punzon et al. 1997; Alcaide and Fresno 2004). They are a highly polymorphic family of glycoproteins, but the glycan moiety is not so important for this family as it is for the mucins. Active trans-sialidases catalyze the transfer of terminal sialic acid from the host glycoconjugates to parasites mucins.

Although the name of family is trans-sialidase, not all the subgroups present trans-sialidase (TS) activity. The family can be subdivided based on their sequences into four different subgroups: Type I includes proteins with catalytic activity like TCNA (“trypomastigote cultured neuraminidase”), SAPA (“shed acute-phase antigen”) and TS clone 154 (Pereira, Mejia et al. 1991; Pollevick, Affranchino et al. 1991; Uemura, Schenkman et al. 1992; Pollevick, Sanchez et al. 1993). The rest of the subgroups do not present activity. Family II comprises a number of trypomastigote surface antigens designated generically as Tc-85 KDa (Peterson, Wrightsman et al. 1986; Kahn, Colbert et al. 1991). Family III is represented by the 160 KDa antigens located on the flagellar region of trypomastigotes (Norris, Schrimpf et al. 1997). The unique members described for the family IV are the antigens Tc-13.1 and Tc-13.5. They contain amino acids repetitions not related to the other groups.

The importance of trans-sialidases for *T. cruzi* survival remains unclear, but might be relevance due to the existence of multiple copies of genes encoding each of these proteins. Many of the Tc-85 KDa proteins have been implicated in a number of processes important for parasite like adhesion to host cells and extracellular matrix proteins (Giordano, Chammas et al. 1994; Giordano, Chammas et al. 1994; Alves 1996; Giordano, Fouts et al. 1999), although this subgroup lacks trans-sialidase activity. Type I trans-sialidases catalyze the transference of sialic acid to β -galactosyl acceptor molecules. As *T. cruzi* is not able to synthesize sialic acid, such transglycosidase activity allows the parasite to acquire sialic acid from the host. The host sialic acid is added to the galactosyl terminal residues present on the abundant mucin-like proteins on the parasite surface. The importance of this acquisition of sialic acid is not yet well understood, but it seems that the sialic acid can help the parasite to adhere and penetrate non-phagocytic cells (Ming, Chuenkova et al. 1993; Schenkman, Vandekerckhove et al. 1993).

However, the abundance of sialic acid on the parasite surface might indicate that sialylation have a primary protective role, protecting the parasite from the environment like a physical barrier and a strong negative charge, and changing terminal glycans in host cells and parasite. Galactosyl residues are exposed now in the host cells, meanwhile the parasite hides its own galactosyl terminal residues, abundantly expressed in the surface mucins. Interestingly, mucin sialylation increases the resistant of *T. cruzi* to lysis mediated by chagasic anti-galactosyl antibodies (Pereira-Chioccola, Acosta-Serrano et al. 2000).

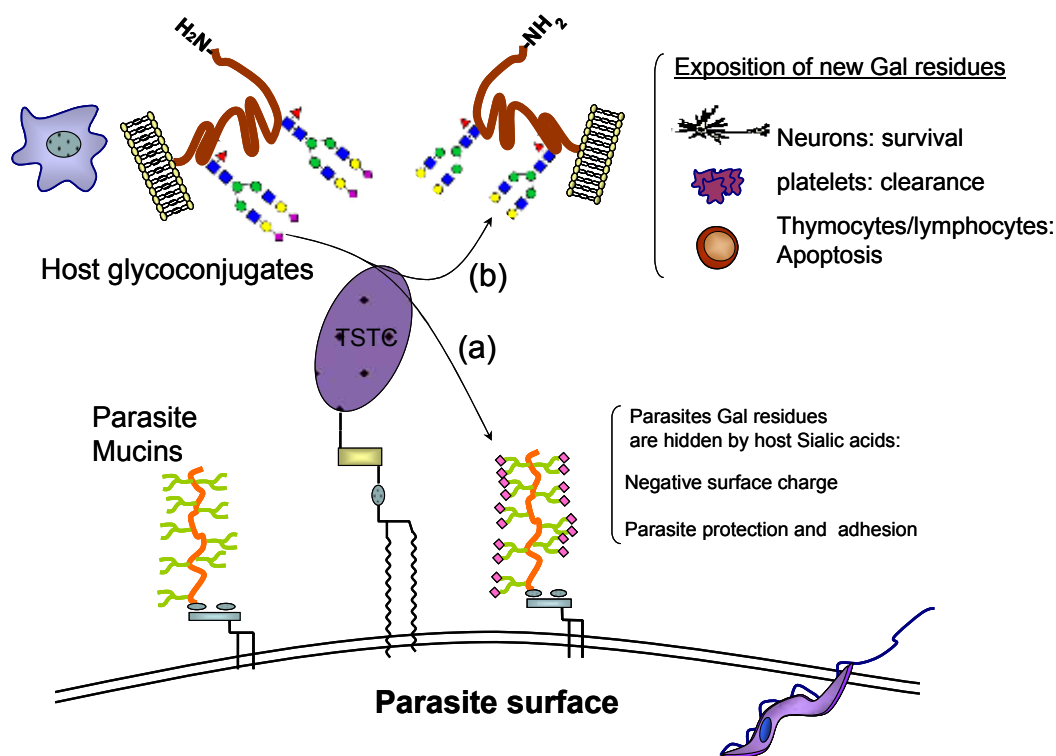


Illustration 5: *T. cruzi* Trans-sialidase (TSTC) transfers sialic acids from host glycoconjugates to parasite mucins. TSTC modifies terminal glycans, both parasite mucins (a) and host glycoconjugates (b).

4. Glycoconjugates in the mammalian host.

The glycoconjugates are important for the biology of *T. cruzi*, but they play also an outstanding role in the vertebrate host. Indeed, the glycobiology is emerging as a new relevant field for a lot of biological processes. Glycans cover the surface of all mammalian cells. They are added to protein and lipid backbones, thanks to a process called glycosilation. This take place during glycoprotein biosynthesis from the endoplasmic reticulum to the golgi and membrane. During this journey to the cell, membrane glycans are important for a proper protein folding being a quality control in the endoplasmic reticulum, for intracellular transport and targeting, sorting in the endoplasmic reticulum, golgi and secretion (Helenius and Aebi 2001; Helenius and Aebi 2004; Molinari 2007). Glycosilation is mediated by a complex set of enzymes, termed glycosiltransferases and glycosidases. Glycosiltransferases catalyze the transfer of a sugar to a substrate, and glycosidases are enzymes that catalyze the hydrolysis of glycosidic bonds in glycan structures. Glycosilation is a very dynamic process.

Expression of glycosyltransferases and glycosidases glycan profile change between cell types, and these enzymes are highly regulated according to cell metabolism, activation or pathological conditions. However, some types of glycosylation occur outside the secretory pathway. In mammals, N-acetylglucosamine has been found linked to serine or threonine residues in many cytoplasmic and nuclear proteins (Hart, Kreppel et al. 1996). For instance, it has been reported the importance of this O-glycosidation in the oncogenic protein p53 affecting phosphorylation sites, and regulating by the balance of both events activity and stability of the protein (Yang, Kim et al. 2006).

The importance of glycosylation is shown by the fact that >1% of the total genome encodes genes for the glycosylation machinery, 50% of the proteins are glycosylated in mammals (Apweiler, Hermjakob et al. 1999), 80% of membrane proteins, and is by far the most common post-translational modification, highly evolutionary conserved. The classes of glycoconjugates found in eukaryotic cells are divided according to the nature of the linkage of the sugar moiety to the aglycone (the non sugar part of the molecule, a protein or a lipid). Within the mammalian glycosylation repertoire, N-glycosylation and O-glycosylation have been identified, and this leads to three different types of glycoconjugates, N-glycans, O-glycans and glycosaminoglycans. An N-glycan is a sugar chain covalently linked to an asparagine residue within the consensus peptide sequence Asn-X-Ser/Thr. O-glycans are typically linked to the peptide chain via N-Acetyl-galactosamine to a serine or threonine residue, although other types of O-glycans do exist (Schachter 2000; Yan and Lennarz 2005). Glycosaminoglycans (often termed proteoglycans) are also linked to serine or threonine, but they are linear, produced by different biosynthetic pathways and are often highly sulphated (Esko and Selleck 2002).

Historically, the importance of glycans has been attributed to metabolism and structural properties, but glycans influence the structure and function of the mature glycoproteins that are attached at, thus controlling molecular recognition, cell interactions and signal triggering for cellular functions.

The way that glycans can modify protein functions is associated to the high variability that can be found in these molecules. Meanwhile nucleotides and amino acids form linear polymers, monosaccharides form branched structures, which means a huge number of three-dimensional different structures. For example, using 20 amino

acids, around 64 millions of different combinations can be achieved. However, using only 6 hexopyranoses as much as 10^{12} different combinations could be possible. From a cellular point of view, structure is synonymous of information. Besides, the same peptide sequence can hold different glycans, leading to the possibility to have different glycoforms with identical protein backbone, expanding even more the diversity linked to glycans; this fact joins glycoconjugates to a primordial role in the context of molecular recognition. Nevertheless, there is no communication without the proper tools to interpret the code, and the biological information that glycans encode should be decoded by specific receptors, able to recognize it. In order to recognize these glycans, there are various classes of glycan binding proteins (CBPs), or lectins. CBPs are proteins of non-immune origin able of recognize specific glycan structures without metabolic sugar modification, turning the structural information into biological information. The term “lectin” tends to refer nowadays to CBPs present in plants, meanwhile CBPs is used for animal lectins.

CBPs are implicated in molecular recognition phenomena, and this is so in part due to the surprisingly fine specificity that CBPs show to ligands presented by distinct glycoconjugates. There are several families of CBPs, some recognize not only mammalian glycans, but they can also recognize glycans present on the surface of several pathogens. This reflects their dual function in host-pathogen recognition and biological cell responses. On the other hand, some pathogens may also express specific glycan epitopes not present in vertebrate host, recognized also by other mammalian CBPs. Four CBPs families are predominantly intracellular and four generally function outside the cell. The intracellular CBPs are the calnexin family, M-type, L-type and P-type, and are located in luminal compartments of the secretory pathway and function in the trafficking, sorting and targeting of maturing glycoproteins. The extracellular CBPs enclose the C-type (collectins, selectins, mannose receptor, and others), R-type, I-type (siglecs) and galectins. They are either secreted into the extracellular matrix or body fluids, or localized to the plasma membrane, and mediate a range of functions including cell adhesion, cell signalling, glycoprotein clearance and pathogen recognition. These eight families are the best known, but recent findings point to the existence of additional new groups of animal lectins - F-box lectins, ficolins, chitinase-like lectins, F-type lectins and intelectins - some of which have roles complementary to those of the well-established lectin families. More information about animal CBPs can be found at

Glycan recognition through CBPs participate in multiple mechanism of cellular regulation, from protein folding to processes involved in cell-cell communication, adhesion, cellular homeostasis receptor activation and signal transduction (Ohtsubo and Marth 2006). Several CBPs play an outstanding role within the immune system, like C-type lectins (van Kooyk and Geijtenbeek 2003), siglecs (Crocker, Paulson et al. 2007; Varki 2007) and galectins. C-type lectins and Siglecs are antigen-uptake receptors that facilitate antigen presentation to T cells by APCs. Galectins are more versatile molecules able to modulate the physiology of different immune cells by acting as adhesion proteins, cytokine regulators or cell activators. The features of the galectin family will be exposed in detail.

5. Galectins.

5.1 General Concepts

Galectins are a quite new family of animal glycan binding proteins highly conserved throughout animal evolution. Although the first galectin was discovered in 1979, it was only in the late 1990s that a growing body of experimental evidences made the scientific community look at these proteins with interest. Galectins are defined by a conserved carbohydrate recognition domain (CRD) of about 130 amino acids with affinity for β -galactosides, as it was defined in 1994 (Barondes, Castronovo et al. 1994). Galactose is a necessary but insufficient requirement for high-affinity binding. Most mammalian galectins bind preferentially to glycoconjugates containing the ubiquitous disaccharide N-Acetyllactosamine ($\text{Gal}\beta 1\text{-3GlcNAc}$ or $\text{Gal}\beta 1\text{-4GlcNAc}$). Binding to individual lactosamine units is low, K_d around $1\mu\text{M}$, but avidity increases when they bind to lactosamine disaccharide in repeating units (polylactosamines). However, monosaccharides flanking the basic lactosamine unit modify the affinity of each galectin for the different glycoconjugates, due to the subtle differences in the CRD structure of individual members.

To date, 15 mammalian galectins have been identified in a wide variety of tissues from several species are more likely to be discovered. Galectins are subdivided into three different groups according to their structure: monomeric, tandem-

repeat and quimeric galectins (Hirabayashi and Kasai 1993). Monomeric galectins have only one CRD. Galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15 are the member of this group. Although they are monomers, monomeric galectins can dimerize depending on the concentration. Tandem repeat galectins are composed by two CRDs, which can have different sugar specify in same galectins. The two CRDs present in the protein are joined covalently by a peptide linker of variable length, and sometimes the length is the different between isotypes of the same galectin member, like galectin-8 (Hirabayashi and Kasai 1993). Members of this group are galectin-4, -6, -8, and -9. The last group is formed only for one protein, galectin-3. Gal-3 is composed by a CRD fused to an N-terminal domain. The N-terminal domain shows a high degree of homology with collagen, composed by tandem repeats aminoacids sequences. This collagen-like domain is responsible for the protein oligomerization that can be observed in soluble galectin-3. Galectin-11, also known as GRIFIN (galectin-related inter-fiber protein) is special within the family, in the sense that this protein lacks galactoside binding activity

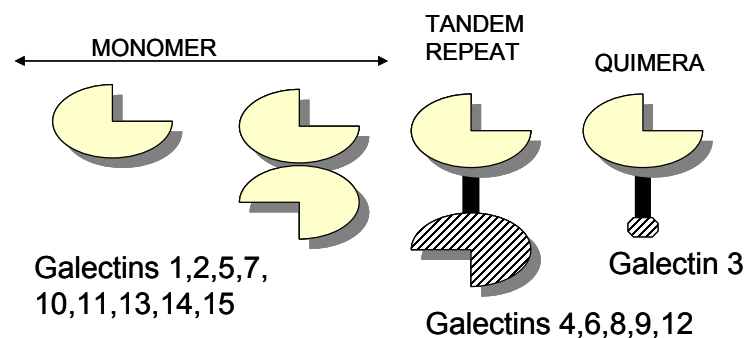


Ilustración 6: The galectin family

(Ogden, Nunes et al. 1998). It is expressed only in the lens, and it was included in the galectin family due to sequence homology, but its CRD lacks seven essential amino acids required for sugar binding, at least in mammals. For this reason its inclusion in the gatin family has been debated for many authors. However, a homologous GRIFIN has been found in zebrafish in lens, showing in this case carbohydrate binding activity (Ahmed and Vasta 2008). Many galectins are either mono-, bi- or multivalent with regards to their carbohydrate binding activities. Monomeric galectins can form non covalent dimmers, tandem-repeat galectins have two CRDs in their sequence and galectin-3 forms olligomers and lattices when it binds to complex glycoconjugates on

the cell surfaces. This multivalency is a critical feature for their biological function, because this allows galectins to cross-link different receptors on the cell surface, triggering so intracellular signals that will address cell biology (Fred Brewer 2002). Biological functions of galectins are not exclusively depending on receptors cross linking. It has been described for Gal-3 the formation of lattices on the cell surface. This lattice is formed because Gal-3 is able to form oligomers, and the effect is a modification of surface receptors motility and clustering, altering the receptor threshold to trigger signals inside the cells. It is well described this effect on the TCR motility on T cells. Remarkably, the responsiveness of cells to individual members of galectin can fluctuate depending on the glycoconjugates expressed on the cell surface, and glycan composition is the result of selective glycosyltransferases activity, enzymes that are regulated according to the differentiation and activation of the cells.

It has been shown that galectins are present in all the cellular locations, nucleus, cytoplasm and cell surface. Galectins are synthesized in the cytoplasm, but they can be either traslocated to the nucleus or can be secreted, despite they do not contain signal peptides to direct them through the classical endoplasmic reticulum-Golgi apparatus secretion system (Mehul and Hughes 1997; Hughes 1999). This unorthodox mechanism has been described for Gal-1 and -3 secretion. They are secreted to non-classical secretory pathways, and once outside the cell, they can bind to, and cross link multiple glycoconjugates present on the cell surface, in the extracellular matrix or even on the surface of pathogens present during an infectious process. Besides, this unusual route might prevent galectins to bind to nascent glycoproteins.

Some galectins are distributed in a wide variety of tissue and cell types, meanwhile others are restricted to more specific locations. For example, galectin-1 is expressed ubiquitously, and galectin-4 is expressed only in the digestive tract under normal physiological conditions (Huflejt and Leffler 2004). Galectins are commonly expressed in immune cells (Rubinstein, Ilarregui et al. 2004). They are found in activated macrophages (Sato and Hughes 1994; Rabinovich, Iglesias et al. 1998; Rabinovich, Riera et al. 1999; Correa, Sotomayor et al. 2003; Sano, Hsu et al. 2003), activated B cells (Zuniga, Rabinovich et al. 2001; Acosta-Rodriguez, Montes et al. 2004), activated T cells (Blaser, Kaufmann et al. 1998; Joo, Goedegebuure et al. 2001), dendritic cells (Vray, Camby et al. 2004) and NK cells (Hanna, Bechtel et al. 2004). Recently it has been also shown elevated expression of galectins in CD4⁺CD25⁺ regulatory T cells (Ocklenburg, Moharregh-Khiabani et al. 2006; Garin, Chu et al.




2007; Kubach, Lutter et al. 2007). Expression of galectins during activation, differentiation and infectious processes is highly regulated in immune cells, pointing out the important role that galectins play during the immune response. Galectins have been implicated in cell-cell adhesion, cell-ECM interaction, immunomodulation, inflammation, cell growth or chemotaxis (Rabinovich, Baum et al. 2002; Liu 2005; Rabinovich, Toscano et al. 2007), functions that will be exposed below. All of these functions are of great importance during the development of the immune response and inflammatory processes, and together with the fact that galectins are markedly up-regulated and secreted by several cell types in response to pathogen invasion, have prompted to several research groups to postulate that galectins are “danger signals” (Sato 2002; Sato and Nieminen 2004).

The wide diversity that has been reported in galectins functions is in concordance with the broad distribution of individual galectins in different tissue, as well as in the subcellular locations. Most of the functions attributed to galectins have been shown to be extracellularly, interacting with glycosylated surface molecules to regulate intracellular signalling. However, some intracellular functions has also been postulated, like regulation of pre-mRNA splicing (Dagher, Wang et al. 1995; Vyakarnam, Dagher et al. 1997), protection (Yu, Finley et al. 2002) and induction of apoptosis (Kuwabara, Kuwabara et al. 2002) controlling the release of cytochrome c from the mitochondria.

So, galectins operate at different levels of innate and adaptive response, but in addition, and to make the scenario even more complex, β -galactosides-related carbohydrates are commonly present in pathogen-associated glycoconjugates, that are potential ligands for galectins. This can affect almost every immune event triggered by galectins: i) pathogen recognition that in turn will mount the proper response against specific pathogens, ii) adhesion promotion of pathogen to host cells and extracellular components, iii) galectins-mediated response in the host, including for example regulation of apoptosis, cytokine balance and cell adhesion and migration. There is in the literature a growing number of pathogens that can bind galectins. The consequences of galectin-pathogen interaction are being revealed by the use of galectin deficient mice models, mainly Gal-3^{-/-} mice, as it will be showed below in detail. As several examples of pathogens recognized by galectins, it can be mentioned the following. Gal-1 acts as a soluble factor that enhances adhesion of HIV to host cells (Ouellet, Mercier et al. 2005;

Mercier, St-Pierre et al. 2008). Gal-3 and Gal-9 bind *Leishmania* sp. (Pelletier and Sato 2002; Pelletier, Hashidate et al. 2003), Gal-3 binds mycolic acids of *Mycobacterium tuberculosis* (Barboni, Coade et al. 2005), and also *Klebsiella pneumoniae* (Mey, Leffler et al. 1996), the helminth *Schistosoma mansoni* (van den Berg, Honing et al. 2004) and *Candida* sp., (Kohatsu, Hsu et al. 2006) showing antifungal activity.

Nevertheless, most of the galectin functions reported to date have been demonstrated using recombinant proteins added exogenously to different cell types. Whether endogenous galectins exert all these proposed activities remains to be clarified. It is worthy to mention this point, because when in vitro experiments are done, several important features about galectin way of action are not been considering. i) Concentration of galectins in solution affects their glycan affinity and multivalency, thus their ability to cross link several receptors. ii) subcellular location is essential to address galectin function. For example, Gal-3 is an anti-apoptotic factor in T cells when it is intracellular overexpressed, but induces apoptosis of the same cells when is added in vitro extracellularly. iii) glycans that are the natural ligands of galectins can change their sugar composition depending on the physiopatological status. In the last years, new approaches have been made, by knocking out with siRNA specific galectin genes and using knock out animal models. This will be extremely useful to address the real functions of galectins. Despite that, it is available now a lot of information about galectin functionality that will be exposed now, focusing on their immunomodulatory and inflammatory abilities.

	T cell activation	T cell apoptosis	Cytokine production	Cell adhesion and migration	Regulatory T cells
 Galectin-1	-	+ Extracellularly	- IL-2 (T cells) - TNF α (T cells) - IFN γ (T cells) + IL-5 (T cells) + IL-10 (T cells) + TGF β (T cells)	- Adhesion and transendothelial migration of T cells	+
 Galectin-3	-	+ Extracellularly - Intracellularly	- IL-5 (Eosinophils and T cells) - IL-12 (DCs) + IL-1 (Monocytes) + IL-2 (T cells) + IL-8 (Neutrophils)	+ Adhesion to ECM + Chemoattraction of macrophages	?
 Galectin-9	?	+ Extracellularly	- IFN γ (Th1 cells) + IL-12 (DCs)	+ Chemoattraction of eosinophils - CD44-mediated T cell adhesion	?

Anderson et al., 2007

6. Galectin-1

6.1 General Aspects

Galectin-1 was the first protein discovered in the family. It has been named previously L-14, galaptin and BHL between other names. It is encoded by the LSGALS1 gene, located on chromosome 22q12. Galectin-1 occurs as a monomer of around 14KDa as well as a non-covalent homodimer consisting of subunits of one CRD. The integrity of the dimer is maintained through interaction in the hydrophobic core, region separated from the CRD (Lopez-Lucendo, Solis et al. 2004). This gives to the dimer a high stability in molecular terms. Nevertheless, one of the main properties of the protein is that it spontaneously dissociates at low concentration (K_d 7 μ M) into monomeric form (Cho and Cummings 1995), that it is still able to bind to glycans but with lower affinity and different specificity (Leppanen, Stowell et al. 2005). Interestingly, each form is associated with different biological activities. The arrangement of lactosamine disaccharides in multiantennary repeating chains (up to 3 branches) increases the binding avidity for dimeric gal-1 (dGal-1), but in contrast there is no increase when the recognition lactosamine unit is repeated in a string glycan (Schwarz, Ahmed et al. 1998; Ahmad, Gabius et al. 2004). Although terminal galactose residues are important for dGal-1 binding, dGal-1 binds well to α 3-sialylated and α 2-fucosylated terminal N-acetyllactosamine, but not to α 6-sialylated or α 3-fucosylated terminal N-acetyllactosamine (Amano, Galvan et al. 2003; Leppanen, Stowell et al. 2005). The importance of this structural differences has been proved in the case of thymocyte selection, because activity of the ST6Gal I sialyltransferase leads to express α 6-sialylated N-glycans containing. This avoids gal-1 binding and the subsequent induction of galectin mediated apoptosis, and therefore promotes cell survival (Amano, Galvan et al. 2003). This example shows the very close relation between glycan structure consequence of selectively glycosyltransferases expression and cell response mediated by galectins.

Gal-1 is present both inside and outside the cells. It has been described in the cytosol and cell nuclei. It is secreted to the extracellular medium, in a way independently of the classical endoplasmic reticulum-Golgi pathway, and outside the cells can bind glycoconjugates on the cell surface or on the extracellular matrix. The

secreted pathway of Gal-1 is still unclear. It seems that Gal-1 is secreted in a manner similar to the pro-angiogenic factor FGF-2 or interleukin-1 β , requiring some unidentified integral membrane proteins. It has been proposed that the gal-1 export pathway might imply sugar recognition and the Na⁺/K⁺ -ATPase pump, because a selective inhibitor of the sodium pump, ouabain, inhibits these export processes (Nickel 2005). Extracellular functions of gal-1 are associated to the sugar binding activity, meanwhile intracellular functions are commonly CRD independent.

Gal-1 is expressed broadly in the organism. It has been located in skeletal, smooth and cardiac muscle, neurons, thymus, kidney, spleen, placenta, etc (Chiariotti, Salvatore et al. 2004). Regarding immune cells, gal-1 is expressed by a variety of cells in central and peripheral immune compartments, including thymic epithelial cells (Baum, Pang et al. 1995), activated T cells (Blaser, Kaufmann et al. 1998; Gonzalez, Rubinstein et al. 2005), macrophages (Rabinovich, Iglesias et al. 1998) and activated B cells after *T. cruzi* infection (Zuniga, Rabinovich et al. 2001).

6.2 Galectin-1 in inflammation and immune response

Modulation of cell adhesion and migration.

Several reports describe the role of Gal-1 during cell migration. Cell migration is the net result of adhesion and invasion. It has been published several reports pointing out the role of Gal-1 in the two processes, but most of the work has been done in relation to tumour cells. Gal-1 increases the adhesion of various normal and cancer cells to ECM via the cross linking of glycosylated integrins. Gal-1 transiently increases availability of β 1 integrins, modulating adhesion of smooth muscle cells (Moiseeva, Williams et al. 2003). Gal-1 can also mediate aggregation of human melanoma cells, and induce adhesion between cancer and endothelial cells, process that favours the dispersion of the tumour cells (Glinsky, Huflejt et al. 2000). Gal-1 has been described as a marker of high cell invasiveness in mammary carcinoma cell lines, and binds to a number of ECM components like laminin, fibronectin and vitronectin. Regarding T cells, it has been shown a negative regulation of T-cell recruitment to the endothelium mediated by Gal-1, under both physiological and pathophysiological conditions, and Gal-1 inhibited interleukin-1 β -induced polymorphonuclear leukocytes recruitment into

the mouse peritoneal cavity under experimental inflammation(La, Cao et al. 2003; Norling, Sampaio et al. 2008).

Regulation of cell growth.

Effects of gal-1 in the regulation of cell growth are positive or negative depending on the cell types involved, cell activation status and might be influenced by factors related to the Gal-1 itself, like the balance of monomeric versus dimeric forms, and intracellular versus extracellular location (Scott and Weinberg 2004). Gal-1 is mitogenic for various types of murine and human cells, including spleen cells, lymphocytes (Symons, Cooper et al. 2000) and various vascular cells (Sanford and Harris-Hooker 1990; Moiseeva, Javed et al. 2000). On the other hand Gal-1 inhibits proliferation of other cell types like neuroblastoma (Kopitz, von Reitzenstein et al. 2001) and stromal bone marrow cells (Andersen, Jensen et al. 2003). In addition, Gal-1 inhibits antigen-induced proliferation of CD8⁺ T cells (Blaser, Kaufmann et al. 1998).

T cell homeostasis and survival.

Gal-1 has emerged as a new regulator of T cell homeostasis. Indeed, this is the most studied and best understood function of Gal-1. It has already been said, that Gal-1 inhibits proliferation of activated CD8 T cells, but regulation of T cell response is much more regulated by Gal-1. Gal-1 controls T cell central and peripheral tolerance (Sotomayor and Rabinovich 2000). During T cell development in Thymus, negative selection of potentially auto reactive T cell clones may occur, in order to avoid autoimmune disease. The mechanisms underlying thymocyte selection are not well understood, but it has been shown that Gal-1 expressed on thymic epithelial cells induces selective apoptosis of immature thymocytes (Perillo, Uittenbogaart et al. 1997), suggesting a potential role for this protein in the processes of negative/positive selection within the thymic microenvironment. Moreover, once T cells are activated in the periphery, to maintain cellular homeostasis and to protect activated mature T cells from the continued secretion of potentially harmful amounts of cytokines, these activated cells must be removed by apoptotic mechanisms. This apoptosis used to be mediated by Fas-Fas interaction, but other molecules seem to be involved in the induced apoptosis. Gal-1 induced apoptosis of activated T cells in the periphery but not of resting T cells (Perillo, Pace et al. 1995), and the cell death seems to be dependent on the expression of the core 2 β -1,6 N-Acetylglucosaminyltransferase (C2-GnT I) (Galvan, Tsuboi et al.

2000; Nguyen, Evans et al. 2001). This enzyme is responsible of the core branched glycan structure present on O-glycans of the surface proteins that lead to Gal-1 dependent apoptosis. Thus, cell lines lacking this glycosyltransferase are resistant to cell death induced by Gal-1. Interestingly, differential glycan expression has been shown in different T cell subsets, and this leads to a different susceptibility to Gal-1 induced death. Meanwhile Th1 and Th17 T cells has been shown to undergo Gal-1 mediated apoptosis, Th2 were resistant, due to a differential sialylation of cell surface glycoproteins (Toscano, Bianco et al. 2007). CD45 was the first glycoprotein demonstrated to be a galectin-1 receptor in activated T cells (Perillo, Pace et al. 1995; Walzel, Schulz et al. 1999). Subsequently, other counter-receptors were demonstrated to bind Gal-1, like CD43, CD2, CD3 and CD7. Gal-1 binding to activated T cells results in a dramatic redistribution of these glycoproteins into segregated membrane microdomains. However, although deletion of CD45 confirmed its importance in gal-1 mediated cell death, gal-1 is still able to induce apoptosis in CD45-deficient T cells (Fajka-Boja, Szemes et al. 2002). The signal transduction events that gal-1 triggers into the cell to promote apoptosis involve several intracellular mediators like the activation of Lck/ZAP-70/MAPK signalling pathway (Ion, Fajka-Boja et al. 2006) , Bcl-2 function modulation, cytochrome c release from the mitochondria, caspase-8 (Matarrese, Tinari et al. 2005) and induction of specific transcription factors like NFAT, AP-1 (Rabinovich, Alonso et al. 2000) and NF-kappaB (Koh, Lee et al. 2008). Gal-1 also suppresses the production of the pro-inflammatory cytokine IL-2 (Vespa, Lewis et al. 1999), and favors the anti-inflammatory IL-10 (van der Leij, van den Berg et al. 2004), effect that was enhanced when a recombinant covalently dimeric galectin-1 was used (van der Leij, van den Berg et al. 2007). Moreover, Gal-1 decreased TNF- α and IFN- γ production by IL-2 activated T cells (Rabinovich, Ariel et al. 1999). Taken all the data together, Gal-1 seems to be a key regulator of T cell homeostasis, first acting during the central tolerance, and later controlling the activated T cells. Several facts support this hypothesis: Gal-1 is ubiquitously expressed, and is overexpressed in organs of immune privilege, such as retina, testis or placenta. Here, gal-1 might ensure the rapid elimination of inflammatory T-cells, protecting so the integrity and function of the tissue. And finally, under inflammatory conditions, high amounts of Gal-1 are secreted by macrophages, antigen-induced T cells and B cells. This lead to the killing of effector T cells after the completion of the immune response.

In addition to this pro-apoptotic activity, Gal-1 has been shown to modulate the cytokine balance under pathological conditions. Gal-1 has efficiently skews the cytokine profile from Th1 to Th2. This is showing promising new therapeutic approaches for the treatment of inflammation-related diseases including graft versus host disease (Baum, Blackall et al. 2003), arthritis (Rabinovich, Daly et al. 1999), autoimmune retinal disease (Toscano, Commodaro et al. 2006) colitis and nephritis (Tsuchiyama, Wada et al. 2000), and could be useful in some neurodegenerative diseases and in cancer (Rabinovich 2005).

Galectin-1 functions in other immune cell types.

Compelling evidence has been accumulated regarding the effects of galectin on T cell fate, but limited information is available on how galectin-1 may impact other immune cell types. In concordance with the anti-inflammatory properties of this protein, Gal-1 has been shown to inhibit IL-12 and nitric oxide production by macrophages in vitro, and Gal-1 inhibited the classical pathway of NO production and iNOS expression after LPS macrophage activation, increased arginase activity, and favored the alternative route of L-arginine metabolism (Correa, Sotomayor et al. 2003). It has been reported, that Gal-1 reduces IFN- γ -induced MHC II expression and antigen presentation (Barrionuevo, Beigier-Bompadre et al. 2007). The effects of Gal-1 over dendritic cells (DCs) have also been studying. Gal-1 maturated DCs showed an activation status similar to the one after LPS treatment, in the means of up-regulation of activation markers (CD80, CD86, CD83, HLA-DR), IL-6 and TNF- α production. In contrast, Gal-1 did not induce IL-12 production by DCs, as LPS does (Fulcher, Hashimi et al. 2006). This suggests a possible role of Gal-1 during initiation of innate immune response. It has been attempted to take advantage of this Gal-1 ability over DCs to treat type I diabetes in mice. Engineered DCs overexpressing Gal-1 have been shown useful to increase the apoptosis of T cells and to reduce the number of IFN-gamma-secreting CD4+ T cells in pancreatic lymph nodes (Perone, Bertera et al. 2006)

Galectin-1 in infectious diseases and during Chagas disease.

Some of the Gal-1 functions have been deeply described, like regulation of T cell homeostasis, and many others need still to be clarified. Nevertheless, as immune and inflammatory modulator, it is clear to think that Gal-1 might play an outstanding

role during the mounting and resolution of the immune response. However, there are not many works about the role of galectin-1 during infectious diseases. It has been shown the implications of gal-1 during HIV infection, because gal-1 acts as an adhesion molecule that promotes virus attachment to host cells (Ouellet, Mercier et al. 2005; Mercier, St-Pierre et al. 2008), and because HIV infection changes the glycan composition of infected T cells, enhancing their susceptibility to Gal-1 mediated cell death (Perone, Bertera et al. 2006).

In regard to our experimental model, the *Trypanosoma cruzi* infection, it has been published an up regulation of gal-1 in macrophages and B cells after parasite infection in the murine model by the same group in 2001 (Zuniga, Gruppi et al. 2001; Zuniga, Rabinovich et al. 2001). They showed that Gal-1 expression is up-regulated in macrophages, both in vivo and in vitro, after *T. cruzi* infection, and that Gal-1 is secreted to the extracellular medium in response to the parasite infection. Interestingly, they showed that Gal-1 is able to control macrophage activity and survival. Low concentration of Gal-1 was able to increase *T. cruzi* intracellular replication in vitro and reduced the inflammatory macrophage activity, because IL-12 secretion was diminished, as well as nitric oxide levels. This led to increase parasite replication. However, a higher concentration of Gal-1 showed a very different activity in the modulation of macrophage activity, because higher Gal-1 concentrations were found to trigger macrophage apoptosis inhibiting parasite replication. Gal-1 was also up-regulated in B cells during *T. cruzi* infection, mainly in the cytosol, although it was also found that activated B cells secreted Gal-1. This secreted Gal-1 did not induce apoptosis in B cell, but it did in activated T cells. Moreover, secreted Gal-1 by B cells inhibits INF- γ by T cells during infection. It is likely that activated B cells regulate T cell activity through the expression of Gal-1, but this results needs still to be confirmed. Another point is that Gal-1 could be implicated in autoimmunity processes during Chagas disease, because it has been shown the occurrence of anti-Gal-1 auto antibodies in the sera from infected patients, both in acute and in chronic stages. Because Gal-1 expression was highly up-regulated in the hearts of these patients, the authors speculate with a possible link between Gal-1 expression and Chagas cardiomyopathy, by means of autoimmunity processes (Giordanengo, Gea et al. 2001), but whether these antibodies have some clinical relevance or not, is still under controversy.

7. Galectin-3

7.1 General aspects

Galectin-3 is the sole member of the chimera-type galectins. It is encoded by the LSGALS3 gene, located on chromosome 14q21-22. Other names attributed to this protein were Mac-2 antigen, CBP35 or IgEBP. The monomer in solution is a protein of 35 KDa. Gal-3 is composed by two different domains. One is the C-terminal domain, composed by 130-135 amino acid residues. This is the carbohydrate recognition domain, and this is what defines the molecule as a galectin. Like other galectins, this domain is arranged in 12 β strands and it has affinity for lactose (Lac) and N-acetyllactosamine (LacNAc). The affinity of Gal-3 is increased for complex branched ligands (Hirabayashi, Hashidate et al. 2002). Gal-3 acts as a receptor for ligands containing poly-N-Acetyllactosamine sequences which consist of many disaccharide units: Gal β 1,4 GlcNAc bond to each other by β 1,3 linkage. Extension at the non-reducing end of the disaccharide units with NeuNAc α 2,3 or with GalNAc α 1,3 and Fuc α 1,2 substituents greatly enhances affinity for galectin-3 (Hughes 2001). The molecular interaction of human galectin-3 with the LacNAc moiety is well characterized thanks to structural and mutagenic studies. The arginine in position 144 is known to interact with the GlcNAc residues linked to the O-3 of the terminal galactose (Seetharaman, Kanigsberg et al. 1998).

Galectin-3 is so far unique in the family in having an extra, long and flexible N-terminal domain. This domain is composed by around 100 amino acid residues, and it is made up of repetitive sequences rich in proline, glycine, tyrosine and glutamine, and lacking hydrophobic residues (Krzeslak and Lipinska 2004; Rubinstein, Ilarregui et al. 2004). It has been reported that the N-terminal domain has 33.5% of homology with collagen α 1 (II) chain of bovine cartilage (Raz, Pazerini et al. 1989), so it is also designated as collagen-like domain. The N-terminal domain contains sites for phosphorylation, (Ser 6, Ser 12), that seems to be implicated in the regulation of its intracellular location and in the regulation of its activities, like control of apoptosis (Mazurek, Conklin et al. 2000; Yoshii, Fukumori et al. 2002). Although this domain lacks sugar binding activity, it has been recently shown by molecular modelling and mutagenic analysis that it contributes together with the CRD in oligosaccharide binding

through Tyr¹⁰² and adjacent residues for some fucosylated sugar structures (Barboni, Bawumia et al. 2000). However, the N-terminal domain is essential to regulate Gal-3 function because it is also responsible for the oligomerization of the protein (Massa, Cooper et al. 1993), and oligomers are critical for cross-linking of surface receptors and signal transduction. Gal-3 can form oligomers composed up to 5 units, and is able to form lattices on the cell surface. These lattices are important because they control protein motility. In this way, Gal-3 modulates receptor clustering, segregation and activation threshold on the cell surface (Rabinovich, Toscano et al. 2007). One example is the interaction of galectin-3 with the T-cell receptor (TCR). The interaction is promoted by modifications on glycan composition of the TCR, mainly by the activity of the N-Acetylglucosaminyltransferase, MGat5, and results in a lattice formation that restricts TCR motility and down-regulation of the T cell response (Demetriou, Granovsky et al. 2001).

Gal-3 has been found in the cytosol, nucleus and attached to the cell surface. As it has been mentioned before, Gal-3 can be actively secreted to the extracellular milieu via a reticulum-golgi independent pathway, in response to inflammatory stimulus like LPS or infections. In the case of Gal-3, it is likely that the N-terminal domain contains signals that address the protein to this non-conventional secretion pathway. It has been shown that a hamster CRD Gal-3 fragment lacking the N-terminus, when expressed in transfected Cos cells, is not secreted (Menon and Hughes 1999). Moreover, the addition of the Gal-3 N-terminus to a non-related cytosolic protein makes the protein to be secreted outside the cell (Mehul and Hughes 1997). Immunohistochemical studies have indicated that the first step for Gal-3 secretion is the accumulation of Gal-3 at the cytoplasmic side of the plasma membrane, and then vesicles containing Gal-3 are pinched off. The transfer of the protein to the plasma membrane domains seems to be dependent on heat shock proteins and other molecular chaperones. The capture of Gal-3 to the membrane domains is mediated by the Src protein family of tyrosine kinases (Zlatkine, Mehul et al. 1997).

The wide distribution of Gal-3 in the different cell compartments reflects its multivalent functionality, as it will be exposed below. There have been described several glycosylated surface ligands for Gal-3, like extracellular matrix components and membrane integrins. The extracellular recognition of these ligands is usually glycan-dependent via CRD, and is related to cell-cell interaction, adhesion and signal transduction. There have been described some intracellular proteins as Gal-3 ligands,

and interactions have been described to be CRD-dependent or independent, depending on the protein. Intracellular ligands are cytokeratins (Goletz, Hanisch et al. 1997), Gemin4 (Park, Voss et al. 2001) and Bcl-2 (Yang, Hsu et al. 1996). The presence of Gal-3 in the cellular nucleus appears to be associated with alternative pre-RNA splicing (Patterson, Wang et al. 2004), and regulation of gene transcription (Lin, Pestell et al. 2002). Gal-3 stabilized transcription binding factor in the cyclin D₁ promoter region and induced trans-activation of CREB and SP1 transcription factors to activate promoter activity. Since CREB and SP1 elements are present in many promoter regions of many genes, it is reasonable to speculate that Gal-3 could be regulating other gene expression. It has been shown two possible mechanisms to explain nuclear import of Gal-3 to the nucleus, one a passive diffusion and another one an active transport (Nakahara, Oka et al. 2006). An increasing effort is being done regarding this event, because the import of Gal-3 to the nucleus is, in part, the malignant phenotype of various cancers.

Although it was found in many normal tissues, Gal-3 expression in adults, similarly to its expression during embryogenesis, is mainly related to the epithelial cells and myeloid cells. However, Gal-3 is expressed in other tissues under pathological conditions, like infections or many tumours: human pancreas, colon and thyroid carcinomas. This is not surprising, taking into account the many biological functions that Gal-3 is modulating: cell-cell adhesion, cell-extracellular matrix interactions, immunoregulation, inflammation, chemotaxis and pathogen recognition.

7.4 Galectin-3 in inflammation and immune response

A large body of work has demonstrated the role of galectin-3 in the regulation of the function of immune cells. Gal-3 expression has been detected in activated macrophages, eosinophils, neutrophils, activated B cells, mast cells, epithelium of the gastrointestinal and respiratory tracts (Frigeri and Liu 1992; Truong, Gruart et al. 1993; Truong, Gruart et al. 1993; Liu, Hsu et al. 1995; Acosta-Rodriguez, Montes et al. 2004). Many studies have been performed using exogenously added recombinant galectins, but in the last years the use of Gal-3 deficient mice has provided additional evidence to consider this protein an important mediator during inflammatory and immune processes. Thus, Gal-3 is able to recognize many pathogens, being a new candidate for a new type of pathogen recognition receptor (PRR). In general, Gal-3 is a powerful pro-

inflammatory signal and specific Gal-3 functions related to inflammation and immunology will be shown.

Regulation of apoptosis and cell growth

Galectin-3 has been shown to be both a pro- and anti-apoptotic molecule. In general, intracellular Gal-3 acts as an inhibitor of apoptosis, meanwhile extracellular Gal-3 is a pro-apoptotic factor. Apoptosis in lymphocytes exemplifies this fact. Gal-3 transfected Jurkat T cells (Yang, Hsu et al. 1996) and Gal-3 transfected Burkitt lymphoma cells (Hoyer, Pang et al. 2004) were more resistant to anti-Fas mediated death. In contrast, when Gal-3 was added exogenously to lymphocytes or T cell lines, it induced apoptosis by clustering of the surface glycoprotein CD71 (Stillman, Hsu et al. 2006). Studies in cancer lines that have been transfected with Gal-3 cDNA showed contradictory results too. Meanwhile overexpression of Gal-3 in human breast carcinoma cell line BT594 induced apoptosis (Lee, Song et al. 2003), it protected from apoptosis in human bladder carcinoma cell line J8 (Lee, Song et al. 2003). As neither of these cell lines express endogenous Gal-3, differences can be attributed to the expression of different Gal-3-associated proteins in each cell line. Gal-3 was found to be up-regulated in keratinocytes during UV-B-induced apoptosis, acting as an anti-apoptotic mediator (Lee, Song et al. 2003), and induced mast cell apoptosis by oxidative stress and mitochondrial permeability (Suzuki, Inoue et al. 2008).

The molecular mechanism that Gal-3 triggers when acts intracellularly to prevent apoptosis are not yet completely understood. It seems that Gal-3 prevents mitochondrial damage and therefore inhibits cytochrome c release, down-regulating caspase activation. In addition, it has been established that Gal-3 binds Bcl-2 (Yang, Hsu et al. 1996). There is 48% of similarity between Gal-3 and Bcl-2, and it can be found within the CRD of Gal-3 the NWGR motif (residues 180-183 of the human Gal-3). This motif is essential for the anti-apoptotic activity of Gal-3, since the activity disappears when the glycine present in this motif is substituted for an alanine (Akahani, Nangia-Makker et al. 1997). Gal-3 it is the only galectin found to have this motif. Interaction with the death receptor family CD95 (APO-1/Fas) could be another way used by Gal-3 for its anti-apoptotic function (Fukumori, Takenaka et al. 2004).

Gal-3 was also shown to modulate cell growth. Proliferation and differentiation of certain cell types is accompanied by increased or suppressed expression of Gal-3, depending on the cell type. Moreover, exogenous Gal-3 can induce or inhibit cell growth. Gal-3 was able to induce outgrowth of neuritis from dorsal root ganglia explants (Pesheva, Kuklinski et al. 1998), rat mesangial cells (Sasaki, Bao et al. 1999) and human lung fibroblast (Inohara, Akahani et al. 1998). On the other hand, Gal-3 inhibits cell proliferation of granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced rat bone marrow cells (Krugluger, Frigeri et al. 1997) and MDCK cells (Bao and Hughes 1999). It seems that Gal-3 is able to modulate specific signal pathways related to cell growth and proliferation. Gal-3 has been shown to interact with the oncogenic K-Ras protein in its activated status (Elad-Sfadia, Haklai et al. 2004), enhancing the duration and strength of the K-Ras signal. In addition, Gal-3 interacts with N-Ras and H-Ras proteins, loading them into active complexes (Shalom-Feuerstein, Levy et al. 2008).

Modulation of cell adhesion

Gal-3 is able to modulate cell-cell and cell-extra cellular matrix interactions in account of its multivalency, acting as a bridge between glycosylated components of the extracellular medium. In a sugar-dependent way, Gal-3 binds extracellular matrix components like laminin (Woo, Shaw et al. 1990; Sato and Hughes 1992; Ochieng and Warfield 1995), fibronectin (Sato and Hughes 1992), tenascin (Probstmeier, Montag et al. 1995), and membrane integrins present on the surface of the macrophages, the main membranes molecules implicated in cell adhesion: CD11b/18, $\alpha1/\beta1$, N-CAM, LAMP-1,2 (Dong and Hughes 1997; Ochieng, Leite-Browning et al. 1998). Gal-3 also binds the heavy chain of CD98, induces CD98 dimerization, showing that this is not only a passive event, but it promotes integrin activation (Hughes 2001). So, Gal-3 is able to mediate cell adhesion of different cell types on extra cellular matrix, in a positive or a negative way. It promotes adhesion of human neutrophils to laminin (Kuwabara and Liu 1996) and to endothelial cells (Friedrichs, Torkko et al. 2007), supports eosinophil rolling and adhesion in a process mediated by the integrin VCAM-1 (Rao, Wang et al. 2007) and mediates binding of L-selectin-triggered lymphocytes to dendritic cells (Swarte, Mebius et al. 1998). In contrast, Gal-3 can inhibit adhesion, as observed in thymus, where Gal-3 disrupts thymocyte interactions with thymic epithelial cells.

Although not deeply discussed in this thesis, it is worth mentioning that Gal-3 is an important factor for many processes related to cancer and cell adhesion, like metastasis and cancer progression, and Gal-3 it is being considering nowadays as a possible therapeutic target for cancer treatment (Hasan, Ashraf et al. 2007).

Chemotaxis and Cell activation in vivo and in vitro models.

Extracellular Gal-3 was first found to be a potent chemoattractant for monocytes and macrophages (Sano, Hsu et al. 2000), as observed both in vitro and in vivo. The recruitment of these phagocytic cells to the sites of infection is in concordance with the pro-inflammatory role attributed to this protein. High concentrations of Gal-3 are needed for its chemotactic activity, and both protein domains are necessary for macrophages chemotaxis. Recently it has been found that Gal-3 can recruit other cell types, like neutrophils during *Streptococcus pneumoniae* infection (Sato, Ouellet et al. 2002; Nieminen, St-Pierre et al. 2008). Moreover, Gal-3 is a potent immune cells activator. Gal-3 in monocytes and macrophages induces superoxide anion production, increases the pro-inflammatory cytokine IL-1 and potentiates the phagocytosis (Sano, Hsu et al. 2003), what defines the classical macrophage activation. However, endogenous Gal-3 has been implicated in the regulation of alternative macrophage activation (associated with exposure to IL-4/IL-13 and distinct from those induced by IFN- γ), through a Gal-3 feedback loop stimulated by IL-4, via activation of CD98 and PI3K activation (MacKinnon, Farnworth et al. 2008). These inflammatory activities have been also observed in other cell types. Therefore, in neutrophils, Gal-3 also activates NADPH-oxidase, and enhances the respiratory burst in exudated and LPS-activated neutrophils. Gal-3 mediates the degranulation of eosinophils and mast cells, possibly through the cross-linking of Fc ϵ RI and/or Fc ϵ RI-IgE complex. The exposure of Jurkat human T cells activates the cells, as evidenced by IL-2 production. Besides these positive effects, suppressive actions of Gal-3 on myeloid cells have also been observed. Exposure of human eosinophils, peripheral blood mononuclear cells (PBMC) and an antigen-specific T cell line to Gal-3 leads to inhibition of IL-5 production.

The effects of Gal-3 on cell activation shown till now have been done mainly by use of exogenous protein. The role of Gal-3 in the inflammatory response has been confirmed in the last years in Gal-3 deficient mice. These animals do not exhibit any developmental or morphological abnormalities, and young adult mice have not shown

so far apparent phenotypes under standard laboratory conditions. However, targeted disruption of gal-3 gene in mice results in a diminished inflammatory response during many infections and diseases. In an inflammatory scenario, it has been shown that Gal-3 deficient mice showed reduced number of granulocytes compared to wild-type animals after thioglycolate-induced peritonitis (Colnot, Ripoche et al. 1998). In agreement with this result, two years later another research group showed that Gal-3^{-/-} mice developed fewer inflammatory cell infiltrations in the peritoneal cavities than the wild-type mice in response to thioglycollate broth treatment, mainly by a reduced number of infiltrated macrophages (Hsu, Yang et al. 2000). Besides, Gal-3^{-/-} macrophages were more susceptible to inducible apoptosis, pointing out that Gal-3 could favor not only the initiation of the immune response, but the prolongation over time of such response.

The lack of Gal-3 protected mice against induced asthma reactions, developing the mice significantly less airway hyper-reactivity (Zuberi, Hsu et al. 2004). This protection was attributed to the capacity of Gal-3 to skew the cytokine balance to a lower Th2 response.

These facts prompted several groups to study the role of Gal-3 in the development of infections defined by a strong Th2 response, like helminth infections. In this regard, the results obtained from *Schistosoma mansoni* infection of Gal-3^{-/-} mice have given contradictory data. On one hand, Bickle et al (Bickle and Helmbj 2007) showed that Gal-3 is not affecting neither the humoral nor the cellular response against *S. mansoni*, and therefore they showed that there were no changes in the Th2 response and no differences in hepatic granulome formation. On the other hand, Breuilh et al (Breuilh, Vanhoutte et al. 2007), and Oliveria et al (Oliveira, Frazao et al. 2007) independently showed that Gal-3^{-/-} developed reducer liver granulomas and mount a biased cellular and humoral Th1 response, implicating Gal-3 in the T cell priming by dendritic cells. Moreover, it was previously described the ability of Gal-3 to recognize specific glycan structures present on helminths. Gal-3 bound the epitope GalNAc1-4GlcNAc (LDN), abundantly present on the surface of *S. mansoni*, and they co-localized in the schistosome egg-induced granulomas in the liver, supporting the primary role of Gal-3 in the immune response against this parasite (van den Berg, Honing et al. 2004; Van de Vijver, Deelder et al. 2006). A higher Th1 immune response was mounted in Gal-3^{-/-} mice when they were infected with the intracellular parasite *Toxoplasma gondii*, probably due to a higher IL-12 production by dendritic cells (Bernardes, Silva et al. 2006). This was corroborated by the fact that spleen cells

from *S. mansoni* infected Gal-3^{-/-} mice produced more IFN- γ compared to the control animals, something similar to the data showed previously by Breuilh and Oliveira. It is noteworthy to mention that Gal-3^{-/-} mice infected with *T. gondii* showed a reducing number of macrophages and CD8⁺ cells in all the organs analyzed except for the lungs, showing that the Gal-3 functions depends on the specific tissue as well as the infectious agent. Gal-3 has been shown to play a role during *Streptococcus pneumoniae* infection. First, it was observed that Gal-3 was expressed in lungs of infected mice, promoting adhesion of neutrophils to endothelial cells, and Gal-3 resulted to enhance neutrophil phagocytosis, acting like a bacteriostatic agent *in vitro*. Results after infection of Gal-3^{-/-} mice showed that knock-out mice developed a more severe pneumonia, and presented a lower number of recruited leukocytes, mainly neutrophils, in infected lungs (Sato, Ouellet et al. 2002; Farnworth, Henderson et al. 2008; Nieminen, St-Pierre et al. 2008). In contrast to helminths infections, where Gal-3^{-/-} dendritic cells showed no defect in TNF- α production after infection, Gal-3^{-/-} mice infected with the yeast *Candida albicans* showed a reduced expression of the Th-1 promoting cytokine TNF- α compared to control animals (Jawhara, Thuru et al. 2008). The absence of this model showed a reduced inflammation and abolished the response of TLR-2 to *C. albicans*, through diminished TNF- α production. Interesting enough, this reflects that Gal-3 can trigger different immune response depending on the infective agent.

Gal-3 seems to play an important role on the outcome of the immune response during *T. cruzi* infection, although there are still many points that remain unclear. Today it has been demonstrated *in vitro* that Gal-3 can promote adhesion of *T. cruzi* to host cells, as it was shown using human coronary artery smooth muscle cells (Kleshchenko, Moody et al. 2004), and to coated laminin surfaces (Moody, Ochieng et al. 2000). However, the possible implications of Gal-3 during *T. cruzi* adhesion have not been demonstrated *in vivo*. From *in vivo* experiments it was shown that Gal-3 addressed B cell fate during infection, dendritic cell migration and thymocytes apoptosis. In regard to B cells, Gal-3 was up-regulated after infection. Up-regulated Gal-3 skewed plasma immunoglobulin producing B cell to a memory B cell phenotype, in a process that implies IL-4 signalling through the transcription factor Blimp-4 (Acosta-Rodriguez, Montes et al. 2004). It has been shown an increase of Gal-3 expression in dendritic cells during *T. cruzi* infection, and a subsequent altered migration (Vray, Camby et al. 2004), and a role of Gal-3 during the thymus atrophy, regulating death of cortical immature

thymocytes, since gal-3^{-/-} mice did not show the thymus atrophy observed during Chagas disease (Silva-Monteiro, Reis Lorenzato et al. 2007).

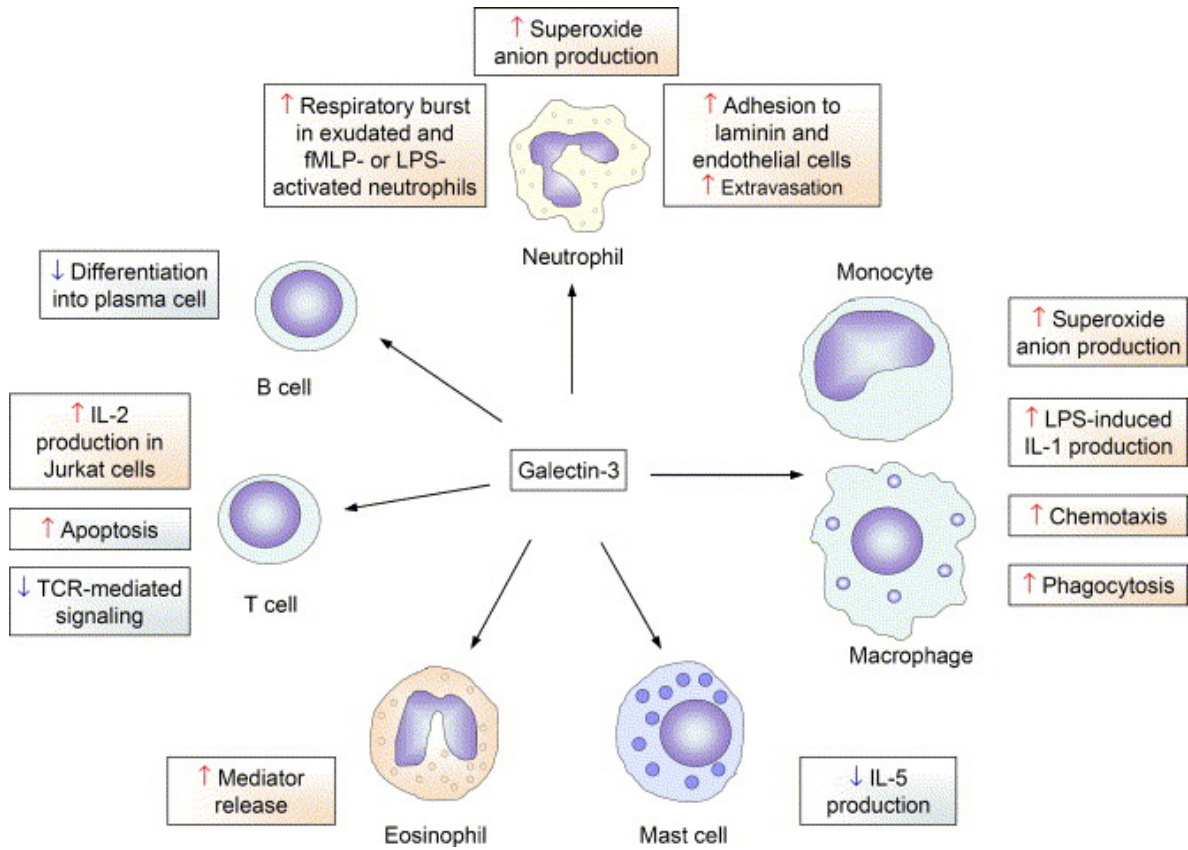


Ilustración 7: The effects of galectin-3 on immune cells. Red upwards arrows indicate positive effects and blue downward arrows negative effects. Pro-inflammatory effects of galectin-3 are indicated in red shaded boxes, anti-inflammatory effects in blue shaded boxes. fMLP:formyl-methionyl-leucyl-phenylalanine (J. Dumic et al. 2006)

8. Other galectins

The best studied galectins are Gal-1 and Gal-3. However, they are only two of the fifteen members described so far. In the recent years there have been described new functions for these “unknown” galectins of great relevance for the outcome of inflammatory and immune processes.

8.1 Galectin-9

Gal-9 was first cloned from mouse embryonic kidney (Wada and Kanwar 1997), and soon it was found in tumor tissues of Hodgkin’s disease that is frequently characterized by blood and tissue eosinophilia (Tureci, Schmitt et al. 1997). It is found in liver, small intestine, thymus, kidney, spleen and cardiac and skeletal muscle. In immune cells it is expressed by T cells and eosinophils. It is not well understood its role under pathogenic conditions, but it is known that Gal-9 expression is up-regulated by different cell types after incubation with pro-inflammatory cytokines. It is up-regulated and released in astrocytes (Yoshida, Imaizumi et al. 2001) and endothelial cells (Imaizumi, Kumagai et al. 2002) after IL-1 β and IFN- γ treatment respectively, and Jurkat T cells increase Gal-9 expression and release after PMA treatment (Chabot, Kashio et al. 2002).

Gal-9 is a tandem-repeat galectin, thus composed by two CRDs. Several isoforms have been described (Spitzenberger, Graessler et al. 2001), according to their different length of the peptide that join the two homologous CRDs. It has been also named ecalectin, because one of the first findings about this protein was that it possesses one of the most powerful eosinophil-specific chemoattractant activity (ECA), *in vitro* and *in vivo* (Hirashima 1999; Hirashima 2000). Carbohydrate binding of Gal-9 is needed for its ECA activity, since mutations of the CRDs completely abolished this activity, and it seems that the protein needs the two CRDs to exert it, because the recombinant CRDs separately did not show any chemoattractant activity (Matsushita, Nishi et al. 2000). Gal-9 is not only a potent eosinophil chemoattractant, but it is also an eosinophil activator as revealed by the fact that Gal-9 is able to induce superoxide production in eosinophils (Matsumoto, Hirashima et al. 2002).

Gal-9, like Gal-1, plays a role in immunomodulation of the T cell response. Both galectins induce apoptosis of activated T cells and thymocytes, although they seem to bind different glycoconjugates receptors and trigger different pathways (Bi, Earl et al.

2008). Indeed, the pathways involved in Gal-9-induced apoptosis might vary in different T cells and status. Gal-9 induces apoptosis of activated CD4⁺ and CD8⁺ T lymphocytes via the Calcium calpain caspase 1 pathway (Kashio, Nakamura et al. 2003), but in Jurkat T cells has been shown that Gal-9 induces apoptosis through an independent caspase mechanism (Lu, Nakagawa et al. 2007). Recently, it has been reported several works pointing out that the role of Gal-9 in the modulation of T cell response is much more specific, controlling specially the Th1 response. Gal-9 interacts with Tim-3, a member of the TIM family of proteins (T-cell immunoglobulin mucin) specific for Th1 cells. Binding of Gal-9 to Tim-3 leads Th1 cells to death and inhibition of IFN- γ production (Zhu, Anderson et al. 2005; van de Weyer, Muehlfeit et al. 2006). Tim-3 has been found in alloreactive CD8⁺ cytotoxic T cells, and that makes these CD8 T cells susceptible of Gal-9 mediated apoptosis (Wang, He et al. 2007).

The role of Gal-9 in the control of the immune response is confirmed by the fact that Gal-9 is able to suppress collagen-induced arthritis in the mouse model by suppressing the generation of Th17 cells and promoting the induction of regulatory T cells (Seki, Oomizu et al. 2008). In addition, LPS-induced production of TNF- α , IFN- γ and IL-12 does not occur in Gal-9 transgenic mice, meanwhile Gal-9 deficient mice became susceptible to the LPS-induced anaphylactic shock (Tsuboi, Abe et al. 2007).

Regarding other immune cell types, it has been shown that Gal-9 induces maturation of dendritic cells, inducing IL-12 and Th1 cytokine production, in a carbohydrate independent way (Dai, Nakagawa et al. 2005).

8.2 Galectin-4

Gal-4 has a restricted expression to the digestive tract (Gitt, Colnot et al. 1998), and it was first involved in crosslinking the lateral cell membranes of the surface-lining epithelial cells (Wasano and Hirakawa 1999). There are two works that link Gal-4 to T cell response in gastrointestinal tract. One claims the Gal-4 is able to induce T cell activity, increasing IL-6 expression, and exacerbating chronic colitis (Hokama, Mizoguchi et al. 2004). On the contrary, the other one proposes that Gal-4 is ameliorating the T cell response, inducing apoptosis of activated T cells, and reducing TNF-, IL-6, IL-8, IL-10 production (Paclik, Danese et al. 2008).

8.3 Galectin-8

Galectin-8 was cloned in 1995 (Hadari, Paz et al. 1995), and it has been shown to be widely expressed in tumoral tissues (Bidon-Wagner and Le Pennec 2004). The protein was described as a tandem-repeat galectin since it was composed by two different CRDs linked by a peptide linker. However, it has been reported that the gene LGALS8 could encode for six isoforms of Gal-8 by alternative splicing, three of them a tandem-repeat like protein, and three related to the monomeric galectin structure (Bidon, Brichory et al. 2001). Like other galectins, is a secreted protein, and as a soluble protein interacts with extracellular matrix components, modulating cell adhesion phenomena. Gal-8 is related to Gal-4, but unlike this last one that it is confined to the intestine and stomach, Gal-8 is expressed in liver, kidney, cardiac muscle, lung and brain. In addition to its role in cancer progression, it has been demonstrated other functions for Gal-8. First, it was shown that Gal-8 could modulate cell adhesion by means of interaction with specific integrins, as it was shown for human cancer cells, neutrophils and Jurkat T cells (Hadari, Arbel-Goren et al. 2000; Nishi, Shoji et al. 2003; Yamamoto, Nishi et al. 2008). In the recent years new articles have been published, suggesting that Gal-8 functions are closer to other galectins regarding immunomodulatory properties than it was expected. For example, Gal-8 is able to modulate neutrophil functions, inducing their adhesion, but inducing super oxide production too (Nishi, Shoji et al. 2003), an essential event in bactericidal function of neutrophils. It was shown that only the N-terminal CRD is needed to induce super oxide production, although other capacities of Gal-8 need the implication of both CRDs. Gal-8 was recently found in the thymus, showing a pro-apoptotic activity of the thymocytes subset CD4(high) CD8(high) (Tribulatti, Mucci et al. 2007). It is likely that Gal-8, as well as Gal-1 and -3, participate in thymocyte selection during T cell development. Related to apoptosis, it has been published that Gal-8 is able to expose phosphatidylserine in the promyelocytic HL-60 cell line, but surprisingly, independently of apoptosis (Stowell, Arthur et al. 2008). Regulation of Gal-8 functions could be more complicated, since this work shows that Gal-8 could form dimers through the N-terminal domain. Further work needs to be done to elucidate the role of Gal-8 in the immune system, but the current data suggest a strong implication of this galectin in regard to immunomodulation.

OBJECTIVES

- 1- Study the ability of recombinant galectins belonging to the three different subgroups within the protein family to bind to infective and non-infective forms of the parasite *T. cruzi* and define the functional consequences of such interaction.
- 2- Define the expression profile of galectins during the course of murine experimental infection with *T. cruzi* in immune cells and in the heart tissue.
- 3- Establish the role of galectins in the outcome of the immune response mounted against *T. cruzi* by means of experimental infection of genetically galectin deficient mice.

MATERIAL AND METHODS

1. Purification and either fluorescein isothiocyanate or biotin labelling of recombinant proteins.

Expression plasmid pQE9 containing the human galectin-1 sequence was kindly provided by Dr. Elena Moisevaa (Leicester Warwick Medical School, UK). The protein was purified as described previously (Andersen, Jensen et al. 2003). Expression plasmids for human galectin-3 and galectin-4 were provided by Dr. Hakon Leffler (Institute of Laboratory Medicine, Sweden)(Patnaik, Potvin et al. 2006) . Expression plasmid pGEX containing the human galectin-7 sequence were provided by Dr. Thierry Magnaldo (Institut Gustave Roussy, France) (Magnaldo, Bernerd et al. 1995). Four expression plasmids pGEX4T2 containing the human galectin-8 sequence and the mutant galectin-8 R69H, galectin-8 R233H, and galectin-8 R69H/R233H sequences were provided by Dr. Nozomu Nishi (Kagawa University, Japan) (Nishi, Shoji et al. 2003). All recombinant human galectins were overexpressed and purified using a clone of *Escherichia coli* BL21 containing each expression plasmid. Briefly, bacteria cultures were incubated with 1mM isopropyl-1-thiogalactopyranoside for 3h at 37°C to induce recombinant protein production. Bacteria were pelleted, resuspended in phosphate-buffered saline supplemented with 4mM EDTA, 2mM β -mercaptoethanol and 1mM lactose (PBS-MELac) together with a protease inhibitor cocktail. After sonication, bacteria were centrifuged to obtain the soluble fraction, where the recombinant proteins were present. They were purified by affinity chromatography, by Ni-NTA chromatography in the case of hrGalectin-1, hrGalectin-7, hrGalectin-8, hrGalectin-8 mutants and GST were purified by glutation-Sepharose (Sigma) affinity chromatography. hrGalectin-3 and hrGalectin-4 were purified using α -lactose-agarose (Sigma). All recombinant galectins were proved to have affinity for α -lactose-agarose, except the galectin-8 R69/R233, which lacks lactose binding activity. Proteins were stored in PBS-MELac at 4 °C. The purity of recombinant galectins was determined by coomassie-blue staining after SDS-PAGE, and their sugar binding capacity was periodically tested.

Biotin-labelled galectins were prepared using the EZ-Link[®] Sulfo-NHS-Biotin purchased from Pierce, according to manufacturer's instructions, but 5 mM lactose was added into the coupling buffer, to avoid chemical modification of the active site in the carbohydrate recognition domain. Recombinant galectins were incubated in PBS 5mM lactose with fluorescein isothiocyanate isomer I (Sigma) to label fluorescently

recombinant galectins. Biotin or FITC-labelled proteins were dialysed against PBS-MELac to remove the excess of unbound reagent.

2. Galectins binding to *T. cruzi* and cleavage

Flow cytometry was used to evaluate galectin binding to the surface of different biological stages of *T. cruzi*. Isolated epimastigotes, amastigotes and trypomastigotes of *T. cruzi* Y and Dm28c strains were washed three times with cold PBS, and then they were incubated with FITC-labelled recombinant galectins (2- to 20 μ M) at 4°C for 20 minutes in the presence or absence of lactose (50 mM). After incubation, parasites were washed three times with cold PBS to eliminate unbound galectins, and they were fixed in 1% paraformaldehyde in PBS for 30 minutes. After washing again with cold PBS, relative fluorescence intensity was measured on a FACScalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo analysis software.

To study the binding and cleavage of purified galectins by *T. cruzi*, the parasites (1×10^7) were incubated with recombinant galectins (2 μ M) in 250 μ l of serum-free RPMI containing 25 mM Hepes:PBS (1:1 ratio) at 37 °C with agitation (800 rpm/min) for the indicated time for the cleavage assay. After the incubation, parasite-free supernatants were obtained by spinning at 3000 rpm for 5 min. The supernatants were fractionated by SDS-PAGE, and proteins in the gels were stained by CBB staining. Alternatively, fractionated proteins on the SDS-PAGE gels were transferred to the nitrocellulose filters and the galectins-related fragments were detected by specific anti-galectin antibodies: mouse anti-human galectin-1 (Novo Castra, clone 25C1) μ g/mL, mouse anti-human galectin-3 (Novo Castra, clone 9C4) μ g/mL, mouse anti-human galectin-4 (R&D Systems, clone198616) 2 μ g/mL, mouse anti-human galectin-7 (R&D Systems, clone198614) 2 μ g/mL and mouse anti-human galectin-8 (R&D Systems, clone 210608) 2 μ g/mL. Membranes were incubated with horseradish peroxidase (HRP) goat anti-mouse antibody (1:1000; Pierce) as secondary antibody. Proteins were detected using Supersignal reagent (Pierce). To inhibit cleavage, various protease inhibitors were added at the concentration ranging from that recommended by the manufacture to 10 times higher. The protease inhibitors used were: pepstatin, (1-10 μ M), leupeptin (1-10 μ M), aprotinin (0.3-3 μ M), EDTA (1-10mM), phenylmethanesulphonylfluoride (0.1-1mM), orto-phenantroline (0.5-5mM), bestatin (13-130 μ M), antipain (50-500 μ g/ μ L), HgCl₂ (2-10 μ M), TLCK (2-50 μ M), TPCK (2-50 μ M), E-64 (2-50 μ M).

3. N-terminal Peptide Sequencing

Galectins fragments that were generated by incubating recombinant galectins with *T. cruzi* were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Galectin fragments were located by staining with 0.5% Ponceau S solution. N-terminal protein sequencing of galectin-3 fragments was performed on a protein sequencer (Perkin Elmer, Procise 494) in Centro de Investigaciones Biológicas (Madrid)

4. Adhesion assays of *T. cruzi* to host cells

To study the parasite adhesion to non-adherent cells like the monocyte cell line THP-1 or murine dendritic cells, *T. cruzi* trypomastigotes were labelled with the fluorescent dye CFSE (Molecular Probes). Parasites were incubated 10 minutes at 4°C with 5 mM CFSE in cold PBS solution and then they were extensively washed to remove the excess of CFSE. Mammalian cells (10^5 cells/assay) cells were resuspended in RPMI medium and CFSE-labelled parasites were added to the cells (cell:parasite ratio 1:3), they were incubated 15 min at 4 °C with or without recombinant galectins at indicated concentrations. 50 mM lactose was added during the incubation time to inhibit galectin carbohydrate mediated effects. Afterwards, the cells were fixed in 1% paraformaldehyde in PBS. Fluorescence attached to the cells was measured on a FACScalibur flow cytometer (BD Biosciences), gating the analysis in the cell population, more complex and bigger than the free parasites, allowing us to distinguish between free and cell attached parasites.

To study adhesion of *T. cruzi* to LLcMK2 cell line, cells were cultured in microtiter plate wells and trypomastigotes were added to each cell in a 1:10 cell: parasite ratio, in the presence or absence of recombinant galectins (0.2-2 μ M) at 4°C for 15 minutes. After PBS washing, the number of parasites attached to the cells was counted directly by optical microscopy.

5. Preparation of Galectin-3 Affinity Columns and identification of galectin ligands on *T. cruzi*

To prepare galectin immobilized columns, 5-10 mg of recombinant galectin-3 were covalently coupled to HiTrap NHS-activated columns according to manufacturer's instructions (Amersham Biosciences), including 5mM lactose in the coupling buffer. Parasites were lysated in buffer B, composed by 0,2 M NaCl, 20 mM tris-HCl, 0,1% CHAPS and protease inhibitors cocktail. Parasites were sonicated, and soluble fraction was separated by centrifugation at 14.000 rpm for 15 min. The soluble fraction was applied to the previously set up galectin column. The column was extensively washed, and eluted with buffer B 150 mM lactose. Eluate was someted to SDS-PAGE cromatography, and transferred to nitrocellulose filters. The *T. cruzi* AgC10 mucin was identified between the galectin-3 ligands by using a specific monoclonal antibody previously prepared in the laboratory (Alcaide and Fresno 2004). rhGalectin-7-GST and rhGalectin-8-GST were coupled in a non-covalent way to glutation-Sepharose columns, and soluble fraction of parasite lysate was loaded onto the columns. After washing, lactose was used to elute the column, and as describe before, AgC10 parasite mucin was found to be present in the eluate.

6. Cell lines and parasites

Vero, LLcMK2 and THP-1 cells were grown in RPMI complete medium containing 5% FCS, 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100 ng/ml) (Gibco, Grand Island, NY) at 37°C in an atmosphere containing 5% CO₂. Six different strains of *T. cruzi* were maintained: Y, CL-Brener and P2 strains, as Tc2 lineage, and Dm28c, CT and CQ strains , as Tc1 lineage, according to the genetic dimorphism seen in *T. cruzi*(Souto, Fernandes et al. 1996). Epimastigotes forms of *T. cruzi* were grown in LIT (liver infusion triptose) medium (Castellani, Ribeiro et al. 1967) containing 10% FCS at 28°C. Intracellular amastigotes were obtained from infected Vero cells after lysis. Pure culture trypomastigotes were collected from the extracellular medium of Vero cells 3 to 4 days after infection, centrifuged at 2000 x g for 10 min, and recovered from the supernatant 3 h later, when parasites left the bottom due to their motility.

7. Dendritic Cells culture

Femurs from healthy mice were removed, and dendritic cells (DCs) were derived by culture of bone marrow cells in complete RPMI medium (2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, 10% FCS) supplemented with 10% granulocyte–macrophage colony-stimulating factor (GM-CSF)-transfected X63 myeloma cell line-conditioned medium and 50 μ M 2-mercaptoethanol for 7 days under a humidified atmosphere of 5% CO₂ at 37°C. X63 myeloma cell line was kindly provided by Dr. Margaret Harnett (University of Glasgow, Scotland). Half of the medium was replaced with fresh medium every two days during one week, and then the cells became to exhibit morphology typical of immature DCs. They expressed CD11c, MHCII surface markers, and low expression of co-stimulatory molecules CD80 and CD86, as assessed by flow cytometry assay. These phenotype corresponds to immature DCs, and these DCs were either exposed overnight to 1 μ g/mL LPS (*Escherichia coli* serotype O26:B6 [Sigma-Aldrich]), or infected overnight with 10 trypomastigotes (Y strain) per cell. Cells were then collected and washed to analyze surface marker expression by flow cytometry, and supernatants were collected for further cytokine measurement.

8. Mice, experimental infection and analysis of the parasitological parameters

Six to 8-weeks-old male and female mice were used for experimental infection. BALB/c and C57BL/6 mice were purchased from Harlan Laboratories, and galectin-1^{-/-}, galectin-3^{-/-}, galectin-9^{-/-} mice were supplied by The Consortium for Functional Glycomics (La Jolla, USA). Mice were maintained and bred under pathogen-free conditions in compliance with European norms (Convention 1986) in the animal facilities (Universidad Autónoma Madrid). Mice were infected with 2x10³ Y strain Trypomastigotes by means of intraperitoneal injection. All in vivo experiments were done in groups of 5 mice, and animals were sacrificed at days 14 and 28 after infection, keeping a group of non-infected animals as control. Parasitemia levels were determined every 2 days by directly visualizing and counting parasites in tail vein blood. To study the humoral response, mice were bled at 7, 14, 21, 28 and 42 days after infection, and non-infected mice were bled as controls. Soluble *T. cruzi* antigens were used to coat the microtiter plate wells, and an Enzyme-linked Immunosorbent Assay

(ELISA) was carried out to quantify the total amount of IgG1 and IgG2a present in mouse serum able to recognize *T. cruzi* antigens. Specific HRP-anti mouse IgG1 and IgG2a (Nordic Immunology) were used to detect the primary antibodies after addition of specific HRP substrate.

9. Protein expression and Western blot

Heart protein extracts were prepared using a PT 1300 D homogenizer (Polytron). For Western blot analyses, 20 µg of tissue extract was fractionated on SDS–10% polyacrylamide gel and transferred to a nitrocellulose membrane. Primary antibodies were used as follows: anti-mouse galectin-1 (R&DSYSTEMS, clone 201066) 5 µg/mL, anti-mouse galectin-3 (R&DSYSTEMS) 1 µg/mL. Membranes were incubated with horseradish peroxidase (HRP) rabbit anti–goat antibody (1:10,000; Sigma-Aldrich) or goat anti–mouse antibody (1:1000; Pierce) as secondary antibody. Proteins were detected using Supersignal reagent (Pierce).

10. Flow cytometry

For detection of surface markers, cells were washed with cold PBS and were incubated with 10% FCS in PBS solution to block unspecific immunoglobuline interactions. Cells were incubated for 20 minutes at 4°C with specific antibodies, and they were extensively washed with cold PBS to eliminate unbound antibodies. Spleen cells were stained for anti-mouse CD3, anti-mouse CD11c, anti-mouse F4/80 and anti-mouse CD45R/B220 in conjunction with anti galectins antibodies to detect galectin surface presence on the surface of different cell types within the spleen cells subpopulations. Dendritic Cells were first detached from the petri dish, as they are adherent cells, stained with antibodies specific for CD11c in conjunction with antibodies specific for major histocompatibility complex (MHC) class II, CD80 and CD86 along with the relevant isotype controls (BD Pharmingen). Flow cytometry was carried out using a FACScalibur Immunocytometry System (BD Pharmingen) and data were analyzed using Flow Jo software. Primary antibodies were used as follows: anti-mouse galectin-1 (R&DSYSTEMS, clone 201066) 50 µg/mL, anti-mouse galectin-3 (R&DSYSTEMS) 5 µg/mL, anti galectin-4 (Santa Cruz Biotechnologies) 2 µg/mL, anti

galectin-8 (Santa Cruz Biotechnologies) 2 µg/mL, anti galectin-9 (Santa Cruz Biotechnologies) 2 µg/mL, phycoeritrin conjugate anti-mouse CD45R/B220 (BD Pharmingen, clone RA3-6B2) 10 µg/mL, phycoeritrin conjugate anti-mouse CD4 (BD Pharmingen, clone RM4-5) 4 µg/mL, phycoeritrin conjugate anti-mouse CD8 (BD Pharmingen, clone 53-6.7) 4 µg/mL, phycoeritrin-Cy5 conjugate anti-mouse CD3e (BD Pharmingen, clone 145-2C11) 10 µg/mL, phycoeritrin conjugate anti-mouse CD11c (BD Pharmingen, clone HL3) 10 µg/mL, fluorescein-5-isothiocyanate conjugate anti-mouse CD11c (BD Pharmingen, clone HL3) 10 µg/mL, phycoeritrin conjugate anti-mouse F4/80 (eBioscience, clone BM8) 4 µg/mL, along with the isotype controls phycoeritrin conjugate rat IgG2a, phycoeritrin conjugate rat IgG2b, fluorescein-5-isothiocyanate conjugate rat IgG2a, phycoeritrin conjugate armenian hamster IgG1. Alexa-Fluor[®] 488 rabbit anti-goat IgG (Molecular Probes) 0.75 µg/mL and Alexa-Fluor[®] 488 rabbit anti-rat IgG (Molecular Probes) 3 µg/mL were used as secondary antibodies when it was necessary.

11. Quantitative real-time polymerase chain reaction (PCR) and reverse-transcription (RT) PCR.

The heart and spleen from non-infected and infected mice were removed. Heart DNA was isolated using the High Pure PCR Template Preparation Kit (Roche). 100 ng of genomic DNA were used for parasite detection, using quantitative PCR as reported before (Piron, Fisa et al. 2007). Total parasite DNA was quantified using pure parasite DNA as calibration curve.

RNA was extracted from heart and spleen by use of Trizol[®] reagent (Invitrogen) according to manufacturer's instructions, and the obtained RNA was subsequently cleaned up by use of RNeasy Mini Kit (Qiagen). Traces of genomic DNA contamination were removed using DNase I (Qiagen) on-column during RNA purification. Total isolated RNA concentration and purity was checked by UV spectrophotometry. RNA was first reverse transcribed into cDNA using a reverse transcriptase (Super Array Bioscience Corporation) and the resulting cDNAs were used as template for subsequent PCR amplification using primers specific for selected genes. Gene expression of individual genes was performed using the Real Time[®] SYBR Green PCR Master Mix (Super Array Bioscience Corporation). Quantification of gene expression was calculated using the comparative threshold cycle (C_T) method,

normalized to both the actin and GAPDH housekeeping genes and efficiency of the RT reaction (relative quantity, $2^{-\Delta\Delta CT}$). Results are shown as fold increase gene expression, comparing relative quantity expression of one sample versus another control one that was set as control, as indicated in each case. The genes analyzed are the followings: NM_021297 Toll-like receptor 4, NM_011604 Toll-like receptor 6, NM_030682 Toll-like receptor 1, NM_011905 Toll-like receptor 2, NM_031178 Toll-like receptor 9, NM_008495.2 galectin 1, NM_025622.1 galectin 2, NM_010705.1 galectin 3, NM_010706.1 galectin 4, NM_010707.1 galectin 6, NM_008496.4 galectin 7, NM_018886.2 galectin 8, NM_010708.1 galectin 9, NM_008401 Integrin alfa M, NM_010578 Integrin beta 1, NM_010493 Icam1, NM_010104 Endothelin 1, NM_011345 E-Selectin, NM_011346 L-Selectin, NM_011347 P-selectin, NM_011333 Ccl-2, NM_009915 Ccr-2, NM_009917 Ccr-5, NM_007482 Arginase I, NM_009705.1 Arginase II, NM_010927 iNOs-2, NM_013693 TNF- α , NM_00837 IFN- γ , NM_011198 COX-2, NM_009283 Stat1, NM_021704 CXCL12, NM_010177 FasL, NM_054039 FOXP3, NM_009969 Csf2, NM_009424 Traf6, NM_007639 Cd1d1, NM_008689 Nfkb1, NM_011487 Stat4, NM_146145 Jak1, NM_010851 MyD88, NM_008352 IL12p40, NM_010558 IL5, NM_007393 Actin beta, NM_008084 GAPDH

<i>GeneBank</i>	<i>Description</i>	<i>GeneBank</i>	<i>Description</i>	<i>GeneBank</i>	<i>Description</i>
NM_021297	Toll-like receptor 4	NM_010493	Icam1	NM_021704	CXCL12
NM_011604	Toll-like receptor 6	NM_010104	Endothelin 1	NM_010177	FasL
NM_030682	Toll-like receptor 1	NM_011345	E-Selectin	NM_054039	FOXP3
NM_011905	Toll-like receptor 2	NM_011346	L-Selectin	NM_009969	Csf2
NM_031178	Toll-like receptor 9	NM_011347	P-selectin	NM_009424	Traf6
NM_008495.2	galectin 1	NM_011333	Ccl-2	NM_007639	Cd1d1
NM_025622.1	galectin 2	NM_009915	Ccr-2	NM_008689	Nfkb1
NM_010705.1	galectin 3	NM_009917	Ccr-5	NM_011487	Stat4
NM_010706.1	galectin 4	NM_007482	Arginase I	NM_146145	Jak1
NM_010707.1	galectin 6	NM_009705.1	Arginase II	NM_010851	MyD88
NM_008496.4	galectin 7	NM_010927	iNOs-2	NM_008352	IL12p40
NM_018886.2	galectin 8	NM_013693	TNF- α	NM_010558	IL5
NM_010708.1	galectin 9	NM_00837	IFN- γ	NM_007393	Actin, beta,
NM_008401	Integrin alfa M	NM_011198	COX-2	NM_008084	GAPDH
NM_010578	Integrin beta 1	NM_009283	Stat1		

12. Confocal immunofluorescence

Parasites were adhered to pre-treated Biobond[®] cover slips, and then they were fixed and permeabilized with acetone. Fixed parasites were incubated with recombinant FITC labelled galectins at 4 °C at 0.2-2 μ M galectin concentration. Unbound galectins were removed by extensive washes with PBS.

Hearts were fixed in 4% paraformaldehyde in PBS solution, incubated in 30% sucrose solution, embedded in Tissue-Tek[®] O.C.T. compound (Sakura) and frozen. 10- to 15 μ m thick sections were fixed in paraformaldehyde in PBS solution. Sections were washed, 10% FCS and 10% mouse serum were used to block unspecific binding, and were incubated with the following antibodies: 50 μ g/mL rat anti-mouse galectin-1 (RDSsystems), 10 μ g/mL goat anti-mouse galectin-3 (RDSsystems), 2 μ g/mL rat anti-mouse CD68 (Serotec), μ g/mL rat anti-mouse CD4 (eBioscience), μ g/mL rat anti-mouse CD8 (ebioscience). Secondary antibodies were used for detection as follows: 4 μ g/mL alexa fluor[®] 488 conjugated rabbit anti-goat (Molecular Probes), 4 μ g/mL alexa fluor[®] 555 conjugated goat anti-rat (Invitrogen). To-Pro-3 (Invitrogen) was used for nucleic acid staining, and IgG (Jackson ImmunoResearch) and rat IgG (BD Transduction) were used for negative isotypes controls. Images were obtained using an LSM510 meta confocal laser coupled to an Axiovert 200 (Zeiss) microscope. ImageJ software was used for later image analysis, in order to quantify the number of CD68+, CD4+ and CD8+ cells stained in each field.

13. Cytokine quantification

FASTQuant[®] Microspot Assays for Cytokine Quantification (Whatman) was used to detect cytokines in mice serum samples. Serums from 5 individual mice, either infected (14 days after infection) or non-infected mice were pooled and the presence of IL-1 β , IL-5, IL-13, IL-2, IL-6, TNF α , IL-4, IL-10 and IFN- γ was measured. The protocol was done according to manufacturer's instructions. Briefly, protein array slides were blocked and incubated with either serum samples or with cytokine standards overnight. After washing, slides were incubated first with biotinylated anti-cytokines antibodies, and second with secondary Cy5-labelled antibody. A Microarray Scanner (Agilent) was used for image acquisition, and data analysis was done by XXXXX .

Analysis of cytokines from the supernatant of infected (trypomastigotes:cell ratio 10:1), LPS activated (1 μ g/mL) and RPMI control dendritic cells was conducted using a mouse Th1/Th2 cytometric bead array kit (Bender MedSystems). It was analyzed on a FACSCalibur flow cytometer. In this method, fluorescent polystyrol beads are coupled with antibodies specific to the analytes to be detected, a biotin conjugated antibody mix is added, which binds to the analytes bound to the capture antibodies. Streptavidin-Phycoerythrin (PE) is added the last, which binds to the biotin conjugates. Beads are differentiated by their sizes and distinct spectral signature by flow cytometry. Standard curves were determined for each cytokine from a range of 27–20000 pg/ml. The lower limit of detection for the method, according to the manufacturer is 0.7–12.7 pg/ml, depending on the analyte. The following cytokines were measured: GM-CSF, IFN- γ , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF- α . IL-12p70 was measured too in dendritic cells supernatants, in this case performing a quantitative enzyme linked immunoabsorbent assay (ELISA), the Mouse IL-12 p70 ELISA Ready Set-Go!, purchased from eBioscience.

14. Statistical analysis

Arithmetic means and standard deviation of the means were calculated. The Student's *t*-test was used to determine the statistical significance of the differences observed. Differences were considered statistically significant when $p < 0.05$.

RESULTS

1. INTERACTION GALECTINS-T. cruzi IN VITRO

1.1 Galectins bind specifically T. cruzi, and the affinity of galectins is higher for infective stages of the parasite.

Among the properties described for galectins, it has been shown in the last years that galectins are able to bind to several human pathogens, from fungi (Fradin, Poulain et al. 2000; Jouault, El Abed-El Behi et al. 2006), bacteria (John, Jarvis et al. 2002; Fowler, Thomas et al. 2006), helminths (van den Berg, Honing et al. 2004) and protozoan like *Leishmania major* (Pelletier and Sato 2002; Pelletier, Hashidate et al. 2003) and *Trypanosoma cruzi* (Moody, Ochieng et al. 2000; Kleshchenko, Moody et al. 2004). Those findings suggest that galectins could act as pathogen recognition receptors for the immune system (Rabinovich and Gruppi 2005), not only recognizing pathogens, but also regulating the immune response after the first encounter (Rabinovich, Toscano et al. 2004). Much work has been done with the best studied galectins, galectin-1 and galectin-3, but very little is known about other family members. For this reason, it was studied whether different galectins were able to bind *T. cruzi* or not, by purifying recombinants human galectins and using them for *in vitro* experiments by means of a systematic study regarding several galectins of the three groups and all life cycle stages of the parasite.

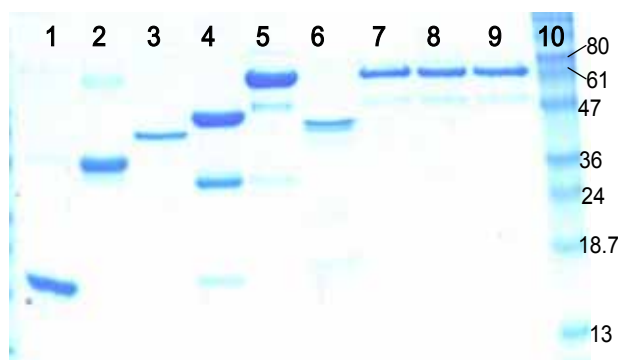
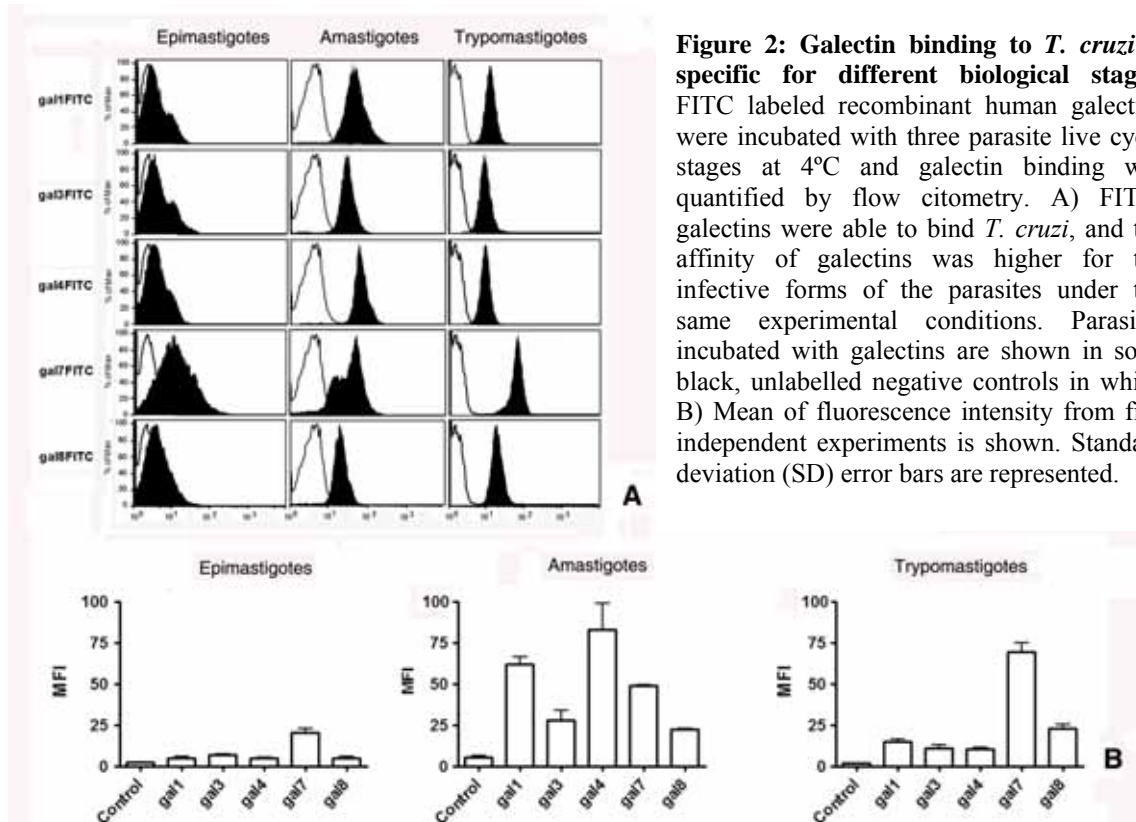


Figure 1: Purified galectins are shown after SDS-PAGE running and Coomassie Blue Staining. Lines, 1: Gal-1, 2: Gal-3, 3: Gal-4, 4: Gal-7-GST, 5: Gal-8-GST, 6: Gal-9, 7: Gal-8R69A-GST, 8: Gal-8R233A-GST, 9: Gal-8R69A;R233A-GST, 10: Molecular weight markers, MW is indicated in KDa.

First, recombinant human galectins were purified. Monomeric galectins 1 and 7, tandem repeat galectins 4, 8 and 9, and the quimera type galectin-3 were purified (Figure 1), and then conjugated to fluorophores, FITC or Alexa-488, in order to be used in flow cytometry and fluorescence microscope.

The first experimental approach to evaluate the ability of recombinant human galectins FITC labelled to bind the parasite was to incubate these proteins in solution with the parasite using the Y strain, that belongs to the TcII *T. cruzi* subgroup. It has already been reported that different parasite life cycle stages expose different glycan composition on their surface, and this could influence the galectin binding. Three parasite biological stages were analysed separately, and it was demonstrated a specific and differential binding profile regarding binding intensity and specificity. Galectins could bind much better to the infective parasite forms, the intracellular amastigotes and the bloodstream trypomastigotes, than the non-infective epimastigotes, present only in the insect vector. Epimastigotes showed to have more exposed ligands for Gal-3 and Gal-7, meanwhile Gal-7 and Gal-8 showed to have more exposed ligands in the case of trypomastigotes, and Gal-1 and Gal-4 in the case of amastigotes (Figure 2).



In order to establish subcellular location of galectin ligands in *T. cruzi*, the binding of galectins by the parasites was examined under confocal microscopy, using FITC labelled galectins (Figure 3). Parasites were fixed and permeabilized. The binding of Gal-3 and -7 to epimastigotes was seen as granular, not restricted to a specific subcellular location. The binding pattern was observed both in cytoplasmic areas and in the cell surface. The same pattern was observed for the strong binding of Gal-7 to trypomastigotes, except for the lack of labelling in the parasite flagellar pocket. However, when Gal-8 was incubated with trypomastigotes, the binding was restricted to the parasite membrane. The binding of galectins by amastigotes was also restricted to ligands present on the cell surface. FITC labelled Peanut Lectin Agglutinin was used for a positive staining of parasite surface galactosides. This is a lectin that specifically recognizes Gal- β (1-3)-GalNAc carbohydrates present on the parasite surface.

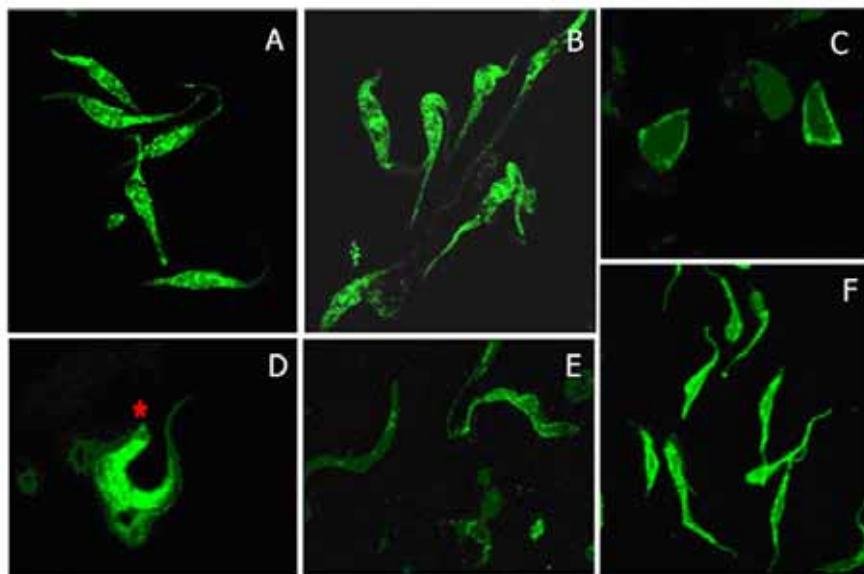


Figure 3: Subcellular location of galectin ligands in *T. cruzi*. Parasites were fixed and permeabilized with ice-cold methanol, and subsequently stained with the indicated FITC-galectins. Images were acquired by confocal microscopy. **A-** epimastigotes, gal-3FITC stained, **B-**epimastigotes, gal-7FITC stained, **C-**amastigotes, gal-1FITC stained, **D-** trypomastigotes, gal7FITC stained. Flagellar pocket is indicated by a red asterisk, **E-**trypomastigotes, gal-8FITC stained, **F-** epimastigotes, surface staining with PNA-FITC

Recombinant galectins were labelled with biotin in order to identify parasite ligands. Total protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After incubation with biotin labelled galectins and streptavidin horse peroxidase, galectin ligands were revealed. Proteins from 10 to 100 KDa were detected. The main ligand was a band around 80KDa for every galectin tested, but the number of bands detected was different for each one (Figure 4A). Galectin immobilized columns were prepared in order to isolated those soluble galectin ligands. Soluble ligands were subjected to proteomic analysis, MALDI-TOF and mass spectrometry, but we failed to identify any parasite molecule, only *T. cruzi* tubulin. However, using specific antibodies again parasite mucins, it was demonstrated the presence of the mucin AgC10 between the galectin ligands in the case of Gal-7 and Gal-8 (Figure 4B).

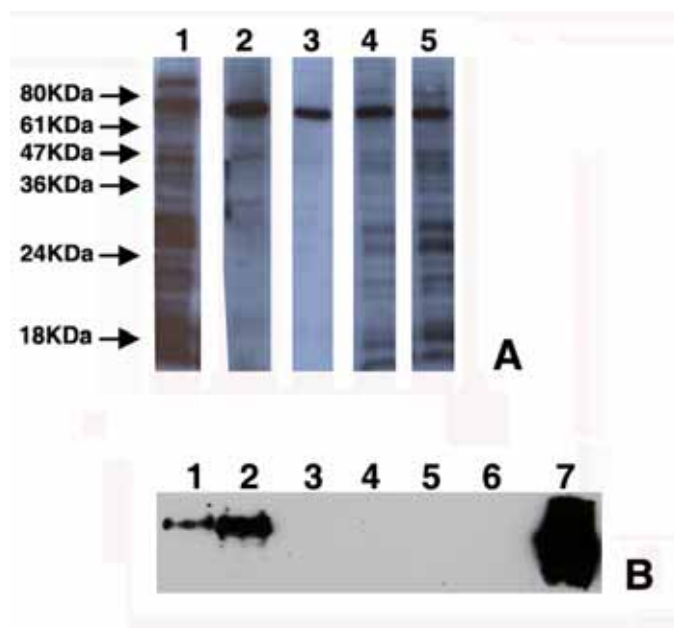


Figure 4: Identification of parasite galectin ligands D) Epimastigotes protein extracts were transferred to nitrocellulose membrane and western blotted with biotin labelled rgalectins. Subsequently, galectin ligands were revealed by streptavidin-HRP. Lines are, 1: Gal-1, 2: Gal-3, 3: Gal4, 4: Gal-7, 5: Gal-8 E) Gal-7 and Gal-8 were immobilized to Glutathion-sepharose, and parasite extracts were applied. GST was used as a negative control. After extensive washing, sepharose was eluted with 100mM lactose. Eluted fractions were subjected to western-blot using specific antibodies for the parasite mucin AgC10. 1:Gal-7 lactose elution, 2:Gal-8 lactose elution, 3: GST lactose elution, 4: Sepharose elution in the absence of rGals, 5,6:washing elutions, 7:purified AgC10

1.2. Galectins- *T. cruzi* interaction is glycan-dependent

Once shown the specific binding, it was necessary to define its nature. The ability of galectins to bind β -galactosides is one of features that defined the galectin family. Nevertheless, galectins can interact with other proteins by a sugar independent way. In order to define the nature of the galectin binding to *T. cruzi*, the binding assays were done in the presence of lactose as inhibitory hapten to block carbohydrate dependent interactions. Galectin binding to *T. cruzi* was totally inhibited in the presence

of 50 mM lactose, indicating that galectins are recognizing glycoconjugates in the parasite surface (Figure 5). In addition, due to the fact that monomeric galectins suffer concentration-dependent association affecting their valency, several galectin concentrations were tested, as it is known that the concentration is a critical factor to induce oligomerization of galectins in solution, and the affinity constant for many natural galectin ligands differs from the monomeric to the oligomeric status. Galectins were used at 0.2, 2 and 20 μ M. An important difference was observed when Gal-7 concentration was increased, but no difference was observed with other galectins tested (Figure 5).

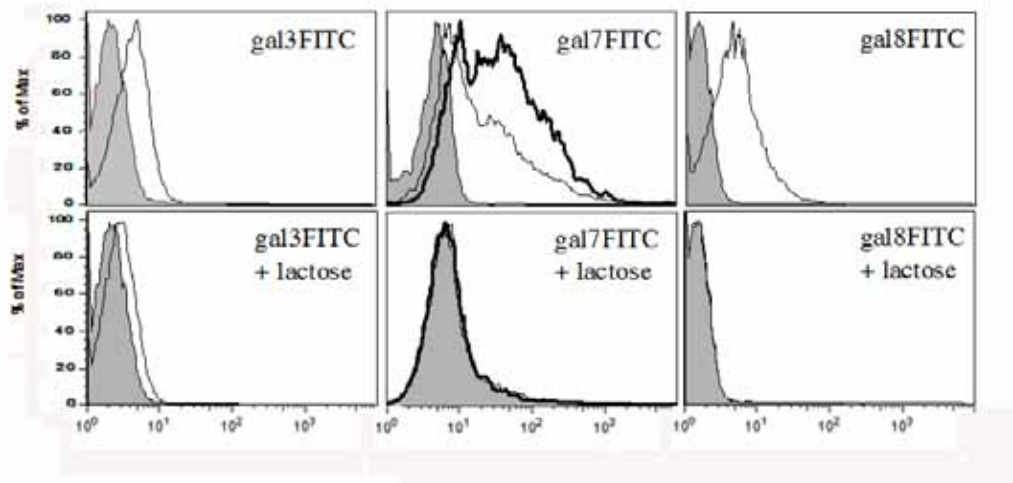


Figure 5: Galectin binding to trypomastigotes were totally inhibited when lactose (50 mM) was added. Negative controls are in solid grey, thin black line refers to parasites after incubation with FITC-galectins, 2 μ M, for 15 minutes at 4°C. Parasite-associated fluorescence was quantified by flow cytometry. In the case of galectin-7, the thicker black line (-) corresponds to a galectin concentration of 20 μ M, and the thin line (-) to 2 μ M.

The fact that the binding of galectins to the parasites was dependent on the carbohydrate recognition was confirmed by the use of recombinant galectin-8 proteins carrying punctual mutations in the carbohydrate recognition domains that abolish the carbohydrate binding. The mutants used were galectin-8R69, galectin-8R233 and the galectin-8R69R233, that have a non-functional N-terminal CRD, C-terminal CRD and both CRDs, respectively. The parasites used were metacyclic trypomastigotes, differentiated *in vitro* using a poor medium, named Grace medium (Castanys, Osuna et al. 1984). Grace medium imitates the acid environment of the final portion in the insect guts. This induces parasite differentiation, resulting into infective metacyclic trypomastigotes. Single Gal-8 mutants showed a reduced binding by metacyclic

trypomastigotes, and an absolute lack of binding for the double mutant galectin-8 (Figure 6). This indicates that the binding of Gal-8 is mediated by the CRD, cooperating the two CRDs to increase binding affinity.

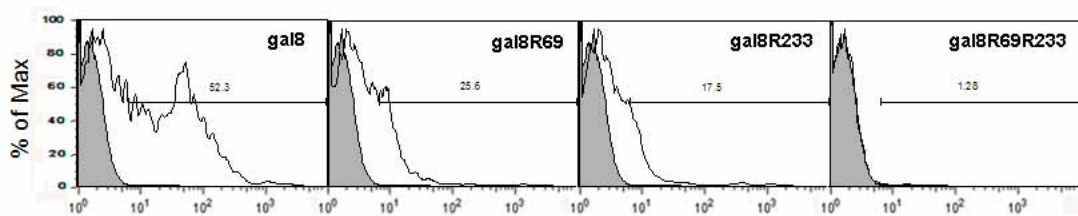


Figure 6: The two carbohydrate recognition domains present in galectin-8 are needed to get an efficient binding to the parasite. Metacyclic trypomastigotes were incubated with FITC labelled galectin-8, and with the FITC labelled mutants R69, R233 and R69R233 for 15 minutes at 4°C. The mean of fluorescence intensity was measured by flow cytometry. All the mutants showed a reduced binding capacity to *T. cruzi*.

1.3 Galectin binding to *T. cruzi* promotes parasite adhesion to host cells

It has been reported that galectin-3, and also galectin-1 and -9 play an important role during host-pathogen interactions, the initial steps leading to infection, suggesting that galectins can be one of the receptors that are involved in the adhesion of the parasite to the host cells (Rabinovich and Gruppi 2005). Once that it was seen that galectins were able to bind specifically to the infective forms of the parasite, we next tried to address whether this interaction could promote parasite adhesion to host cells. *In vitro* adhesion experiments were performed to test this hypothesis. Cell lines susceptible to be infected were used as a model, the murine monocyte cell line THP-1 and the epithelial cell line LLcMK2. Those cells express natural ligands for galectins. Fluorescence trypomastigotes were incubated with cells in the presence or in the absence of recombinant galectins, and parasites attached to the cells were counted as described in Material and methods. When the parasites were incubated with the host cells in the presence of recombinant galectins, the adhesion of parasites was significantly higher than the controls in the absence of exogenous galectins (Figure 7). The incubation time was limited to 10 minutes at 4 °C to be sure that we are looking at parasite adhesion and not cell penetration, blocking parasites proteases that can influence over the recombinant galectin, as it will be discussed below. Not surprisingly,

not every galectin was able to promote adhesion with the same strength. The better that galectin bound to Trypomastigotes, the better the galectin was able to promote parasite adhesion. So, in concordance with galectin binding assays to *T. cruzi*, Gal-8, Gal-7 and Gal-3 promoted the adhesion to THP-1 cells. Besides, the adhesion was increased when galectin concentration used was higher. When lactose was included during the incubation the adhesion was abolished, and in the case of galectin-8, CRD mutations abolished this effect too (Figure 7).

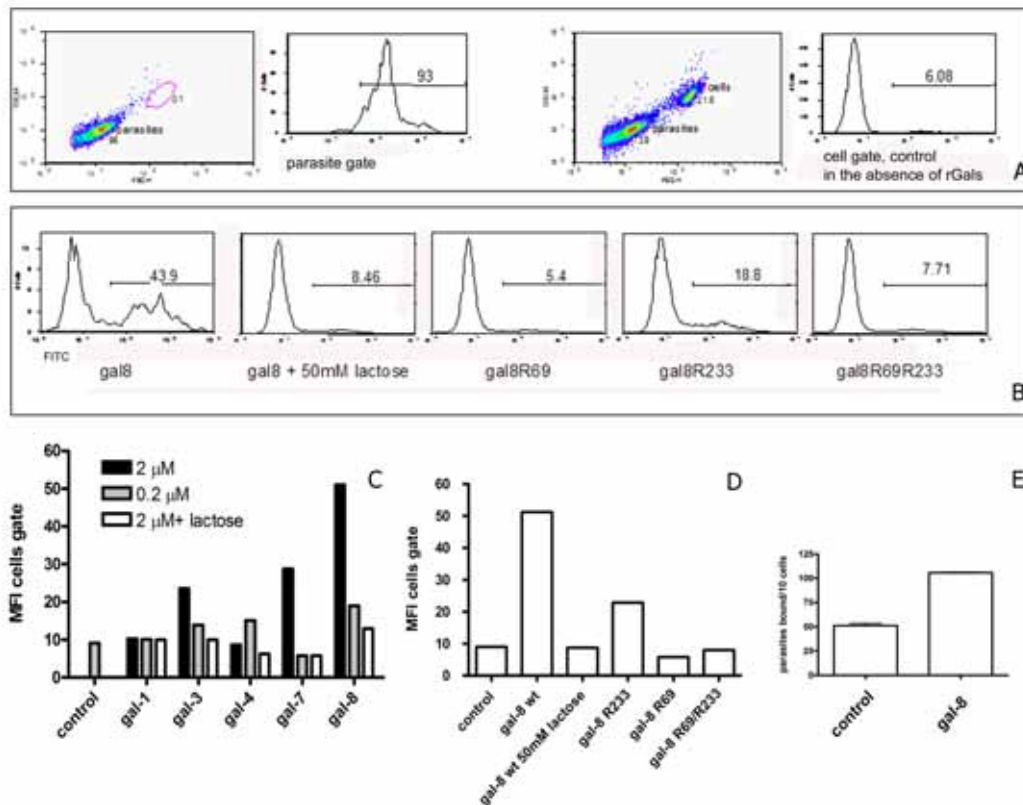


Figure 7: Parasite adhesion to host cells was increased when recombinant galectins were present. A) parasites were labeled with CFSE, and incubated with non-labeled cells. Parasites and cells were resolved by flow cytometry due to their different size, and the fluorescence intensity associated to the cell gate (THP-1) was quantified. Almost 100% of the parasites presented high fluorescence intensity, and only 6% of the cells showed fluorescence intensity when incubated with parasites in the absence of rGals. B) galectin-8 were added to the mixture of cells and parasites for 10 minutes at 4°C, and the rate of positive cells increased to 43.9% of the total. This effect disappeared when 50 mM was included, or when galectin-8 R69, R233 and R69R233 were used instead of the wild type galectin. C) mean fluorescence intensity is shown using the same approach with gal-1,-3,-4,-7 and -8, at 0.2 μM and 2 μM, and in the presence of lactose. Result is significant from two independent experiments performed. D) Mean fluorescence intensity values for galectin-8 and mutants, significant from two independent experiments performed. E) parasites were incubated with a LLcMK2 monolayer for 10 minutes at 4°C, and attached parasites were counted after extensive washing in the presence or absence of 2 μM recombinant galectin-8.

1.4 The Incubation of Galectins with *T. cruzi* Results in the Formation of Truncated Galectins

In order to test if *T. cruzi* could modify the galectin structural integrity during the parasite-galectin interaction, soluble recombinant human galectins were incubated with live parasites for different times, from 5 to 120 minutes at 37°C. It was seen that this led to the proteolitic processing of galectins by the parasite. Parasites were pelleted, and the cell-free supernatant was analyzed by SDS-PAGE. A time dependent effect was observed when Gal-3, -4 and -8 were incubated with live parasites. Gal-3 was processed by *T. cruzi*, and three different bands appeared in a sequential way. The same effect was seen in the case of the tandem-repeat type Gal-4 and -8, a cleavage of these galectins was seen. No differences were seen after incubation with the parasite regarding Gal-1 and Gal-7, both monomeric galectins (Figure 8).

Parasite-free supernatants after the incubation were subjected to western blotting analysis with appropriated anti-galectin antibodies. As the antibodies used were monoclonal, they detected only the full-length galectin, and some of the new bands that appeared over time were detected depending on the presence of the specific epitope for the antibody in the resulting fragment. The recognition of the resulting smaller bands by specific anti-galectin antibodies confirmed that at least the recognized bands were truncated galectins as the result of parasite cleavage (Figure 8).

The kinetics of the cleavage was specific for each galectin tested, as it was defined by densitometry of the resulting bands at each time point. Half of the total recombinant galectin-3 and galectin-4 was processed after 10 minutes, and the time required for galectin-8 was around 40 minutes (Figure 8).

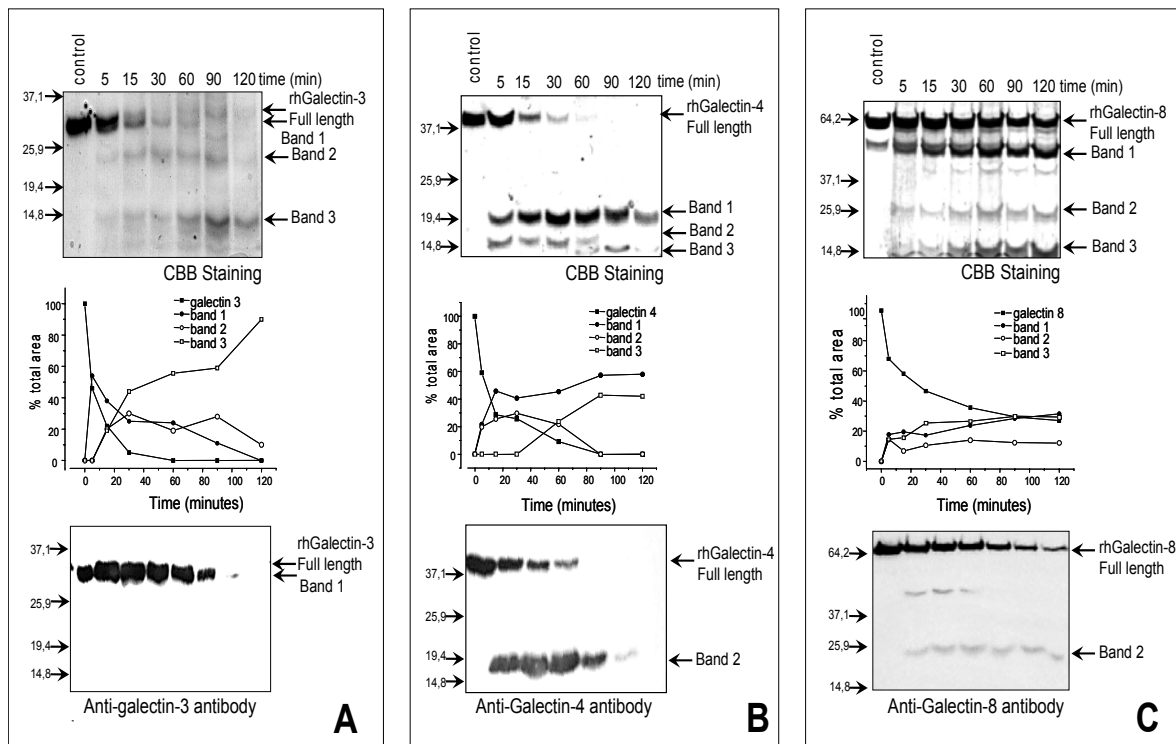


Figure 8: Time course of galectin-3,-4 and -8 cleavage by *T. cruzi*. Galectin-3 (A), -4 (B) and -8 (C) were incubated with parasites for given times and parasite-free supernatants were subjected to SDS-PAGE followed by CBB staining, showing the appearing bands. Integrated intensities of full length galectins and the new bands were obtained by densitometric scanning of CBB-stained SDS-PAGE gel. The cleavage of galectin-3, -4 and -8 was analyzed by Western blotting with monoclonal anti-galectins antibodies.

In order to gain insight on the nature the processing, the resulting bands were subjected to N-terminal peptide sequencing. The aminoacid sequences where the cleavages take place on the galectin-3 are P³⁰AGAGGY, band 1, A⁶²YHGAPGAY, band 2 and G¹⁰⁵PYGAPAG, band 3, all of them on the N-terminal collagen-like domain, out of the CRD (Figure 9). The first cleavages that appeared in time for Gal-4 and -8 were in the sequences Q¹⁷⁵LNSLPTME¹⁸³ and Q¹⁶⁰ASSLELTE¹⁶⁸ respectively, both located in the peptide link between the two CRDs (Figure 10).

This allowed us to establish that neither of the galectins was being processed on the carbohydrate recognition domain, but at the linker peptide (Gal-4 and -8) and on the N-terminal domain (Gal-3). Interestingly, Gal-4 and -8 were cleaved by the parasite in a related sequence, sharing the aminoacids serin-leucin in the cleavage site. Thus, it can be establish a consensus sequence for this parasite protease, QXXSLXXE.

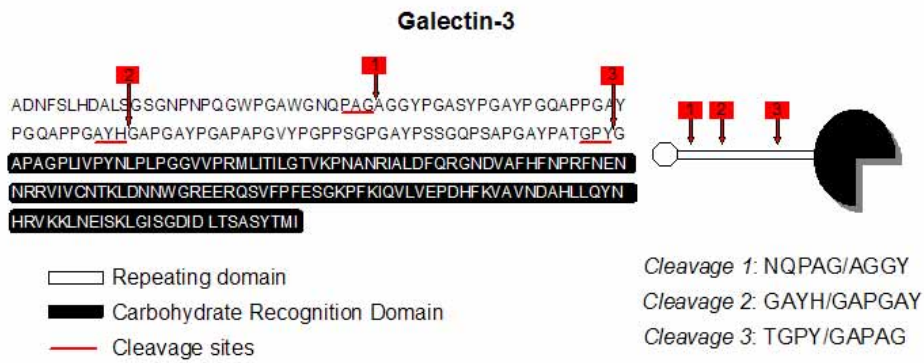


Figure 9: Cleavage sites of galectin-3. *T. cruzi*-truncated galectin-3 was subjected to N-terminal protein sequencing. Cleavage sites of bands 1, 2, and 3 are indicated in the peptide sequence of human galectin-3, and the sequences obtained by N-terminal protein sequencing are underlined.

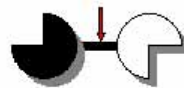
Galectin-4

MAYVPAPGYQPTYNPTLPYYQPIPGGLNMGMSVYIQGVA SEHMKRFFVNFVVGQDPGSDVAFHFNPRFDGWDKV
VFNTLQGGKKGWSEERKRSMFPFKGAFAFELVFVLAEHYKVVVNGNPFYEGHRLPLQMVTHLQVDGDLQLQSINF
 GGQPLRPQGPPMPPYPGPGHCHQQLN**SLPTMEG**PPTF NPPVYFGRLQGGLTARRTIIIKGYVPPTGKSFAINFK
 VGSSGDIALHINPRMGNNGTVVRNSLLNGSWGSEEKKITHNPF GPGQFFDLSIRCGLDRFKVYANGQHLLDF AHR
 LSAFQRVDTLEIQGDVTLSYVQI

Galectin-8

MLSLNNLQNIYNPVIPIYVGTIPDQLDPTLVICGHVPSDADRFOVDLQNGSSVKPRADVAFHFNPRFKRAGCIVCNTLI
NEKWGREEITYDTPFKREKSFEIVIMVLKDKFQAVVNGKHTLLYGHRIGPEKIDTLGIYGVNIHSIGFSSSDLQSTQAS
SLELTEISRENVPKSGTPQLSLPFAAR LNTPMGPGRTVVVKGEVNANAKSFNVDLLAGKSKDIALHLNPRLNKAFVVRNS
 FLQESWGEERNITSPFSPGMYFEMIIYCDVREFKVAVNGVHSLEYKHRFKELSSIDTLEINGDIHLEVRSW

CRD 1
 CRD 2
 Cleavage site



Galectin-4 QQLN/**SLPTMEG**
 Galectin-8 TQAS/**SLEL**TEISR

1.4.1 Cleavage of Galectin-4 and -8 is Inhibited by the Specific Protease Inhibitor orto-Phenantroline.

The cleavage of Gal-4 and -8 by *T. cruzi* was shown to be sequence specific and related in both galectins. This implies that at least one parasite protease was implicated in such cleavage. In order to know the kind of protease that was processing the recombinant galectins, the assay was done in the presence of different protease inhibitors covering all known protease families as listed in material and methods. Of all inhibitors tested, only one was effective to prevent the cleavage of the recombinant galectins, the compound 1,10-ortho-phenantrolin (OPA), a Zn-metalloprotease inhibitor (Figure 11A).

1.4.2. Cleavage of Galectin-3 is Inhibited by the Specific Proteases Inhibitors orto-Phenantroline and Mercuric Chloride.

There were detected three different cleavage sites within the galectin-3 sequence, with at least two of them unrelated. Two shared a common sequence, GAP, and the other one was quite different, AGGY. The same protease inhibitors used previously were employed to inhibit galectin-3 cleavage by *T. cruzi*. Only OPA resulted efficient to inhibit two of the three cleavages observed in the N-terminal domain of Gal-3, the cleavage 2 and 3 placed at the common GAP sequence (Figure 11). Thus, it was likely that more than one protease were implicated. Since the cleavage within galectin-3 took place exclusively in the N-terminal domain, domain that shares homology with collagen, a set of collagenases inhibitors were tested. Of all collagenases inhibitors assayed, mercuric chloride, TLCK and TPCK, just mercuric chloride was able to inhibit the cleavage left, in the sequence AGGY, the first one to occur. The combination of magnesium chloride and OPA kept the full length galectin-3 (Figure 11). It is also known that both proteases work independently, because the presence of OPA does not affect the activity of the other protease susceptible to mercuric chloride, and vice versa.

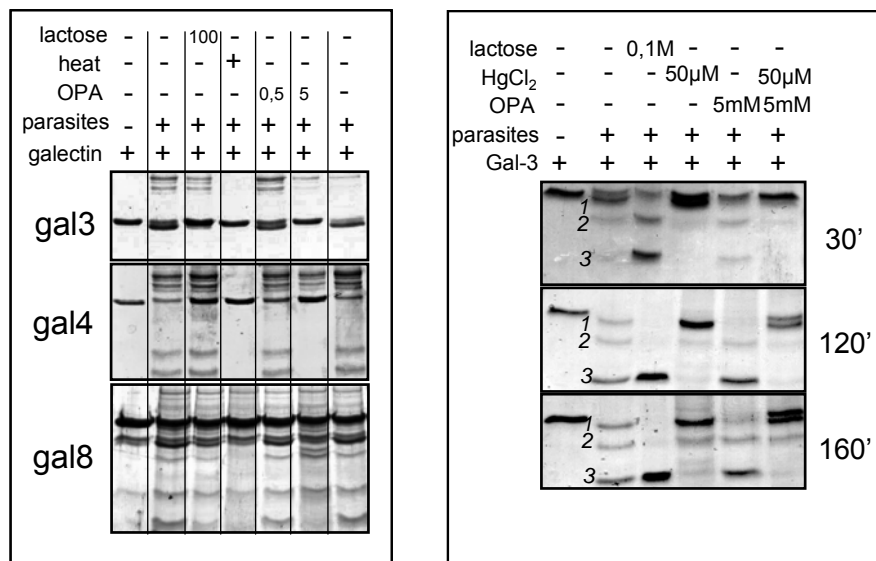


Figure 11. The cleavage of galectins by *T. cruzi* is inhibited by specific protease inhibitors. A). Galectin-3, -4 and -8 (2μM) were incubated with parasites in the presence or absence of the indicated protease inhibitors, or with heat-killed (100 °C for 10 min) *T. cruzi*. The zinc chelator, 1,10-ortho-phenanthroline (OPA) was able to inhibit galectins cleavage by the parasite. Concentrations are indicated at mM range. B) Galectin-3 (2μM) was incubated with parasites at given times and in the presence of OPA, mercuric chloride or the two compounds at the same time. The cleavage of galectin-3 was analyzed by CBB staining. Mercuric chloride inhibited the cleavage in the band B and C, and OPA in the band A. Only a combination of both inhibitors kept intact the full length galectin-3, even after 160 minutes in contact with *T. cruzi*.

1.4.3 The Protease Involved in the Cleavage of Galectins is Composed of a Complex of Different proteins.

In order to know if the proteases responsible of the cleavage of galectins were secreted to the extracellular medium, parasites were *in vitro* cultured and supernatants were collected to test if these supernatants could process recombinant galectins. In addition, parasites were treated with phosphatidylinositol phospholipase C (PIPLC), enzyme that releases GPI anchored proteins from the membrane. The PIPLC treated and untreated parasites and the resulting supernatants were incubated with recombinant galectins. This approach will indicate whether the protease that processes galectins is secreted by the parasites, and/or whether the protease is anchored to the membrane by GPIs. Recombinant galectins were submitted to SDS-PAGE and coomassie blue staining after their incubation with parasites and supernatants (Figure 12). The protease that cleaves Gal-3 at the sequence AGGY is not secreted, and is not anchored through GPI. The one specific for GAP was secreted, since the supernatants of cultured parasites

produced the cleavages 2 and 3 in the Gal-3. Gal-4 was cleaved by other protease, not secreted but GPI-anchored, because the conditioned supernatant after PIPLC treatment was able to process Gal-4. Gal-8 was processed by an actively secreted protease.

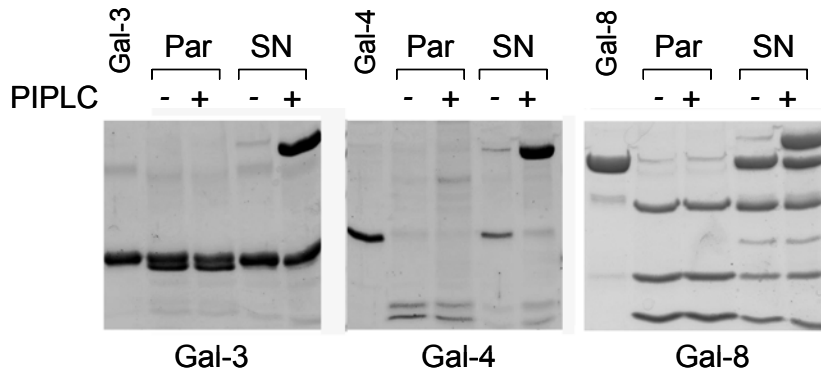


Figure 12: Gal-3, -4 and -8 are processed by different parasite proteins. Parasites were treated with PIPLC in order to release GPI-anchored proteins to the medium. rGals were incubated 15 minutes at 37°C with parasites (Par) or supernatants (SN). After incubation, mixtures were centrifuged and parasite-free supernatants were resolved by SDS-PAGE following Coomassie Blue staining.

1.4.4 Galectin Binding is needed for the Cleavage of Galectin-8 by T. cruzi.

The availability of the galectin-8 mutants allow us to answer different questions regarding the interaction and processing. It has already been shown, that this mutants are unable to bind to ligands present on the parasite surface. However, those proteins still have the sequence where the cleavage in the recombinant Gal-8 occurs, the QASSLELTE situated in the linker peptide. Mutants in only one CRD were slightly processed, but in a lesser extent than the wild type Gal-8 (Figure 13). It was observed that *T. cruzi* was unable to cleave Gal-8 R69R233 mutant, even after a long incubation of 120 minutes, when the wild type Gal-8 was totally processed. This is in concordance with the lack of binding for galectin-8R69R233, and the slightly binding of R69 and R233 mutants showed before (Figure 6).

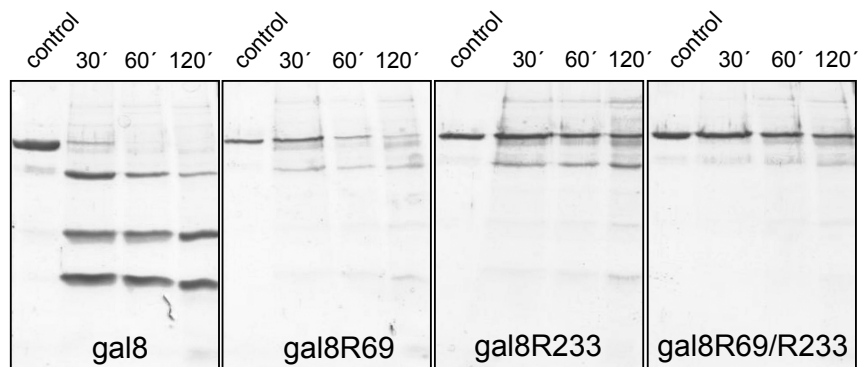


Figure 13: Functional carbohydrate recognition domains are needed by *T. cruzi* to cleave the protein. Gal-8 and punctual mutants (2 μ M) were incubated at given times with parasites at 37°C. Meanwhile mutants lacking glycan binding activity were not processed by *T. cruzi*, Gal-8 was cleaved as expected;

1.4.5 Blocking the Proteolysis Leads to Galectin Induced Killing of Parasites.

T. cruzi as a parasite has developed during the evolution some molecules that could lead to the modulation of host responses to the infection. As described above, it has been shown a novel mechanism that the parasite develops to induce cleavage of galectins. The question that arises now is what the functional meaning of this phenomenon can be. It is clear that the parasite proteases are able to disable the galectin multivalency, and in that way, many of their functions. Now it is proposed the matter in terms of the possible effects of the galectin over the parasite itself. In order to address this question, parasites were incubated with galectins in the presence of OPA, or in other words, when the parasite is not able to process the galectin.

Parasites were incubated with Gal-3, -4 and -8 in the presence of OPA, and then they were stained with propidium ioide (Figure 14). The death was induced by the presence of galectins, because parasites in the presence of OPA alone kept live over the time of the experiment. 50% of the parasite population was killed in 5 μ M Gal-8 in the presence of OPA, meanwhile it was necessary 20-25 μ M of Gal-3 or Gal-4 plus OPA to reach the 50% of dead parasites. Gal-1 was used as a control, showing no effect on the parasite viability.

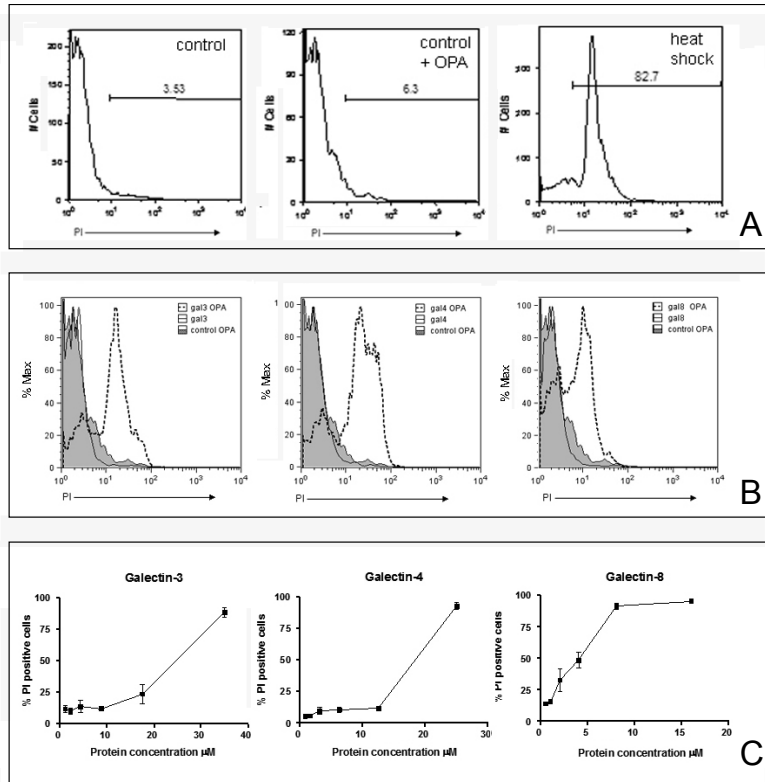


Figure 14: Parasites are killed when they are incubated with galectins in the presence of orto-phenantroline. A) Parasites were treated with propidium iodide (PI) to check cell viability. Live parasites were not stained by PI, neither parasite after OPA treatment, (10 minutes, 37°C) but parasites killed by heat shock (100°C, 10 min) did. B) Galectin incubation leads to parasite killing when OPA (5mM) was added (dotted line). No dead parasites were seen when parasites were treated only with OPA (grey tinted) or with galectins alone (thin black line) C) Concentration needed to induce parasite killing for each galectin was quantified. Parasites were incubated with galectins at given concentrations and OPA for 10 minutes at 37°C. Results represent the mean of three independent experiments showing SD.

2. GALECTIN EXPRESSION ASSOCIATED TO IMMUNE CELLS DURING MURINE EXPERIMENTAL INFECTION

The *in vitro* experiments with recombinant human galectins pointed out that galectins can bind to glycans exposed on the *T. cruzi* surface, and that this binding could play a role during parasite adhesion to host cells. The initial interaction could promote galectin induced parasite killing as a first defensive system of the innate immune response against *T. cruzi*, event that made *T. cruzi* to develop specific proteases through the evolution.

These findings give us little information about the global role of galectins though, and whether they have a real role in the immune response during Chagas disease. Our next goal was to define the pattern expression of different galectins during the murine experimental infection with *T. cruzi* in spleen and heart, as a first step to understand their roles. Different galectins associated to different immune cell types were analyzed in spleen cell subpopulations. The spleen is an immune organ where the whole repertoire of immune cells meet, thus is a good example of the immune response outcome that it is occurring during the infection. In addition, it was addressed the role of galectins during the immune response that takes place in the heart in the acute phase of Chagas disease. Results are shown below.

2.1 Profile of Galectin Expression and Galectin Ligands, and Changes during Infection in Spleen Cells

First, it was described the surface galectin profile in the major spleen cell populations by flow cytometry, B cells (Anti-mouse CD45R B220), T cells (anti-mouse CD3), dendritic cells (DCs, anti-mouse CD11c) and macrophages (anti-mouse F4/80). Spleens from control mice and spleens from infected animals were compared. It was seen some of the immune events linked to *T. cruzi* infection, a marked splenomegaly as direct consequence of polyclonal leukocyte activation and an increased number of antigen presenting cells like dendritic cells and macrophages.

Resting B cells exhibited on their surface Gal-1 and -8, but galectin profile changed during infection. Gal-1 was constitutively expressed, and Gal-3 expression was highly up-regulated during infection. In contrast, Gal-8 was down-regulated on B cells from infected animals (Figure 15).

Regarding T cells, it was seen that spleen cells from control mice did not express none of the galectins evaluated, but during infection, T cells showed Gal-1, Gal-3 and Gal-9 expression (Figure 16). The same study was performed in antigen presenting cells, DCs and macrophages. Both cell types were increased in spleen during the infection in mice, and they expressed high levels of Gal-1, Gal-3 and Gal-9, both in control and infected mice, but they kept the same galectin expression profile during infection, varying the total cell number (Figure 17).

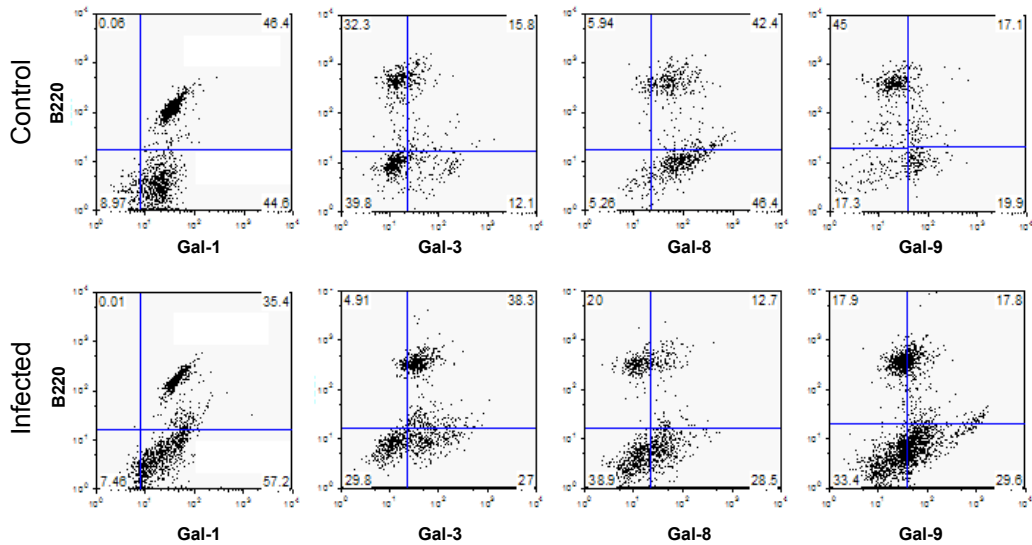


Figure 15: Surface associated gal-1, gal-3, gal-8 and gal-9 in B cells from control and infected mice. C57BL6 were infected with 2000 *T. cruzi* trypomastigotes from Y strain. Non-infected mice and mice at day 14 post infection were sacrificed, spleen cells were isolated and B220 in conjunction with galectins presence were checked by the use of specific antibodies. In B cells, Gal-1 is constitutively expressed; Gal-3 and Gal-9 are up-regulated during infection, in contrast to Gal-8, that is down-regulated during infection.

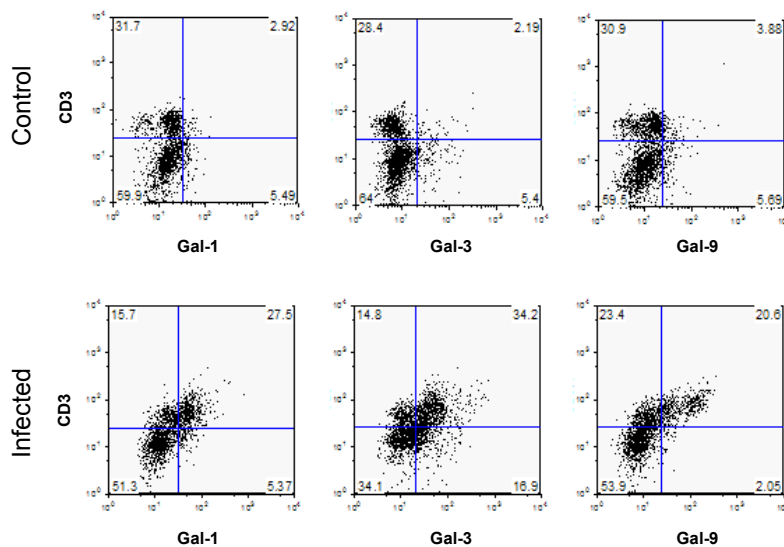


Figure 16: Surface associated gal-1, gal-3, and gal-9 in T cells from control and infected mice. C57BL6 were infected with 2000 *T. cruzi* trypomastigotes from Y strain. Control and infected mice at day 14 post infection were sacrificed, spleen cells were isolated and CD3 and galectins expression were tested by FACs.

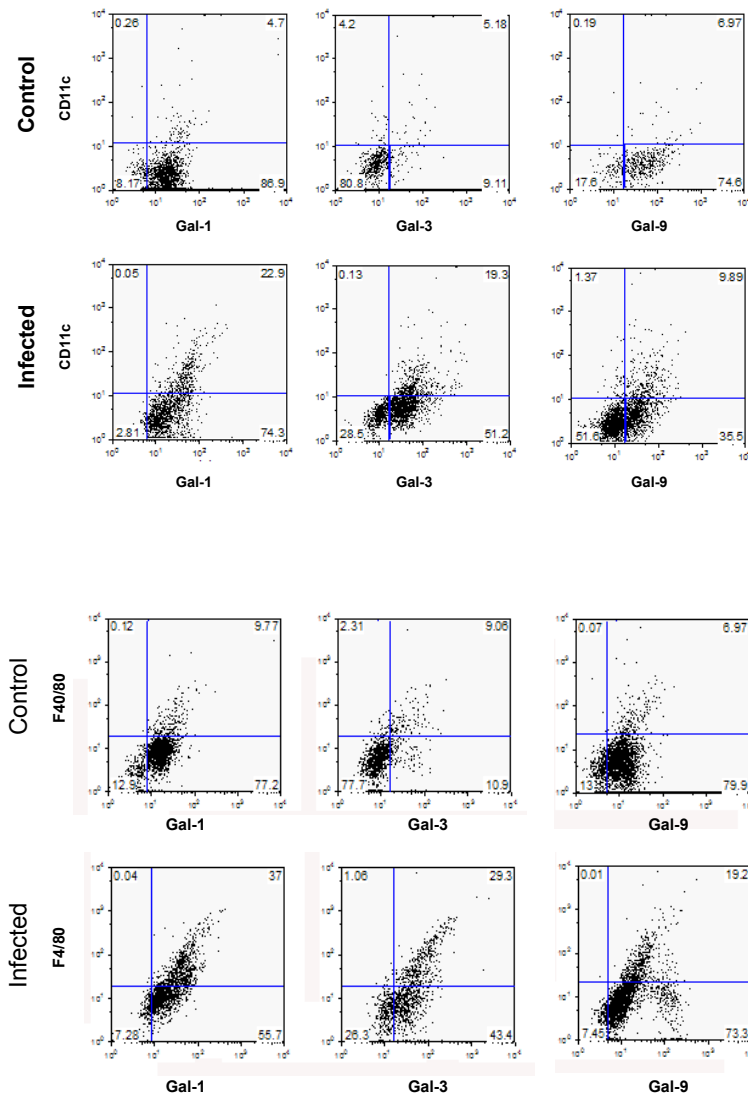


Figure 17: Surface associated gal-1, gal-3, and gal-9 in dendritic cells and macrophages from healthy and infected mice. C57BL/6 were infected with 2000 *T. cruzi* trypomastigotes from Y strain. Non-infected mice and mice at day 14 post infection were sacrificed, spleen cells from C57BL/6 mice were isolated and surface associated galectins were detected by flow cytometry. CD11c expression was used to label dendritic cells and F4/80 pan marker was used for macrophage labelling.

It was characterized the endogenous galectins that immune cells express in control mice and during infection. However, it is also important to understand the role of galectins if the exposure of endogenous ligands changes during infection. This could be a consequence of the parasite trans-sialidase activity, enzyme that uptakes terminal sialic acid from the host exposing the subjacent galactosyl residues, affecting so the galectin ligands structure and accessibility. To investigate whether galectin ligands change during infection, spleen cells were incubated with recombinant Gal-1 and Gal-3 FITC labelled, and then fluorescence associated to the cells were quantified by flow cytometric analysis. Cells showed a highly increase of galectin binding ability at 14 dpi, and at 28 dpi it returned to standard conditions (Figure 19). This indicates a higher presence of accessible galectin ligands on the spleen cell surface at the peak of parasitemia compared to cells from control animals.

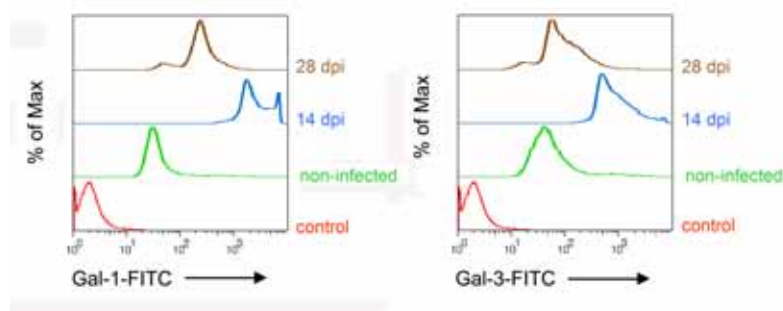


Figure 18: Galectin ligands on the spleen cell surface change during infection. Spleen cells from control and infected mice (14 and 28dpi) were incubated with recombinant Gal-1 and Gal-3 FITC labeled for 15 minutes at 4°C. Galectin binding was quantified by FACs. Cells in the absence of rGals were used as negative control.

2.2. Profile of Galectin Expression and Changes during Infection in Heart.

The heart is one of the major targets of *T. cruzi*, and the parasite settles down for long time in this tissue. During the acute phase, a great number of immune cells infiltrate the organ. Infiltrating immune cells persist in the cardiac tissue despite a reduction in the parasite burden. To gain insight into the galectin functions regarding immune response in heart during infection, we tried to attempt to identify the cells that are expressing galectins in the heart of infected mice.

We first checked galectin genes expression by RT-PCR in heart of control, and infected mice.

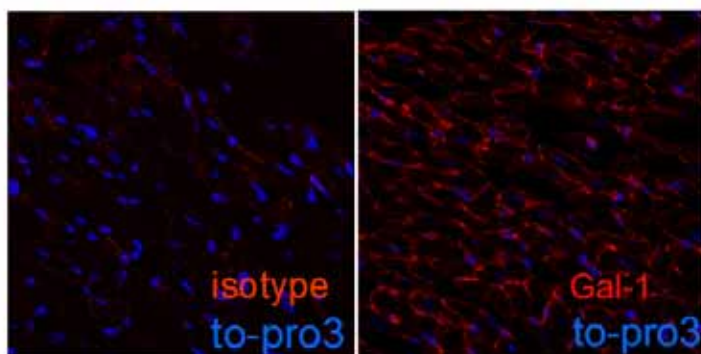
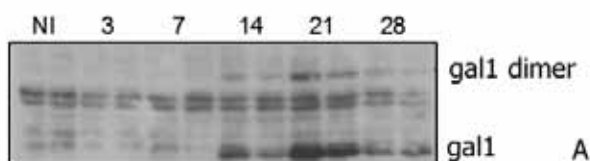


Figure 20. Galectin-1 expression is up-regulated in Hearts during *T. cruzi* infection. A) Infected C57BL/6 mice were sacrificed at indicated days after infection, hearts were removed and total protein extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Two independent mice are shown at every time point and non-infected mice were used as control. B) Heart frozen slides were stained with anti-mouse galectin-1 and to-pro3 for nucleic acids detection. Slides were analyzed by confocal microscopy. Galectin-1 expression was found to be present in all cardiac cells.

The gal-1 mRNA levels were not up-regulated over infection time, but a significant increase of the protein levels were observed by Western blotting, reaching the highest expression at day 21 post-infection (Figure 19A). Tissue sections from infected animals were stained with anti-mouse Gal-1 antibodies and nucleus were stained with TO-PRO[®]-3 iodide. This showed that Gal-1 was being expressed in cardiomyocytes cells (Figure 19B), with a broad distribution with no focalized expression.

Regarding gal-3 mRNA, there was a strong increase in hearts of infected animals compared with controls. Expression was up to 40 fold increase at 14 days post-infection, and 20 fold increase at 28 days post-infection (Figure 20A). This gal-3 mRNA increase was in concordance with the high protein expression observed by western blotting (Figure 20B). The western blot revealed the presence of monomeric and dimeric forms of Gal-3, and it can be observed the presence of a smaller band than the monomeric form, that might correspond to the truncated Gal-3 by parasite proteases, as it was observed *in vitro*. Heart sections were stained with anti-mouse CD68 and anti-mouse Gal-3 to identify the cells responsible of the Gal-3 expression. CD68 is a intracellular macrophage marker, which co-localized with the Gal-3 expression in infected mice (Figure 20C). Thus, meanwhile cardiomyocytes were expressing Gal-1 in hearts of infected mice, Gal-3 is expressed by infiltrating macrophages.

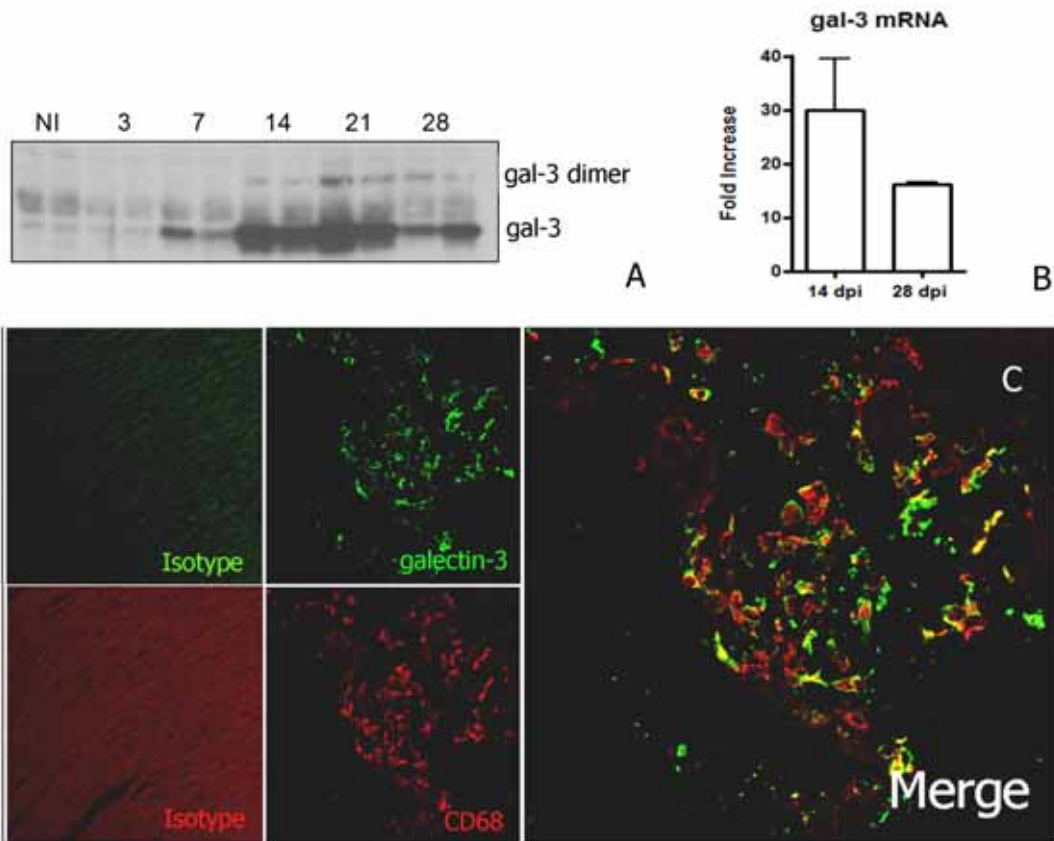


Figure 20: Galectin-3 expression is up-regulated in Hearts during *T. cruzi* infection. A) Infected C57BL/6 mice were sacrificed at indicated days after infection, hearts were removed and total protein extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Two independent mice are shown at every time point and non-infected mice were used as control. Gal-3 expression was detected using a specific anti-Gal-3 antibody. Gal-3 expression was found markedly increased at 14 and 21 days post infection. B) Total heart RNA was isolated and mRNA gal-3 was found to be up-regulated at 14 and 28 dpi as seen by RT-PCR. Results are expressed as the fold increase values (\pm standard deviation) of pooled RNA from 5 different mice done in duplicate, comparing infected tissue at given times with the non-infected control C) Heart frozen slides were stained with anti-mouse Gal-3 and anti-mouse CD68 for macrophage detection. Slides were analyzed by confocal microscopy and Gal-3 expression was found to be associated mostly to infiltrating CD68⁺ cells. Isotype control antibodies were used for negative controls.

3- ROLE OF GALECTIN-3 DURING INFECTION: EXPERIMENTAL INFECTION IN THE GALECTIN-3 DEFICIENT MODEL

The ability of galectins to recognize *T. cruzi in vitro*, together with the regulation of galectin expression that was seen *in vivo* in immune cells, suggested that galectins might play a pivotal role for the initiation, maintenance and resolution of the immune response upon infection. The experimental approach was to use a deficient galectin-3 mouse model. Knock-out mice were infected in the same way that wild type mice and parasitological and immunological parameters were measured over time of infection.

3-1. Galectin-3 $-/-$ mice show increased parasitemia.

Both wild type mice and deficient galectin-3 mice were infected intraperitoneally with 2000 blood trypomastigotes, Y strain. Blood parasitemia was measured every 2 days by direct counting of parasites. Gal-3 $^{-/-}$ mice showed a significant higher parasitemia compared with their wild type genetic background C57BL/6 mice, with no difference on the time course, presenting the peak at the same day after infection (Figure 21).

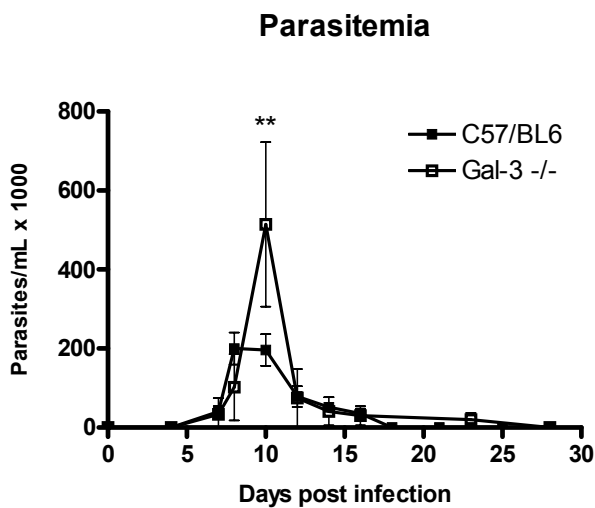


Figure 21: Galectin-3 deficient mice show a significantly higher parasitemia than wild type mice. Parasite burden was quantified by direct counting under optical microscopy after tail vein bleeding. Data show the mean of 15 mice counted in 5 independent experiments (\pm SD). Parasite burden in blood was quantified every 2 days during 28 days. Galectin-3 deficient mice showed a significantly higher parasitemia at day 11 post infection. The kinetics of the infection were the same for wild type and deficient mice. ** $P < 0.01$

3.2. Humoral Response in gal-3 deficient mice.

3.2.1 The IgG1/IgG2a balance is not altered in gal-3^{-/-} mice

The serum levels of anti-parasite IgG1 and IgG2a were measured throughout acute infection, since IgG1/IgG2 balance is shown to be related to a Th2 or Th1 immune response. As shown in Fig. 22, the amount and kinetic of specific IgG1 and IgG2a antibodies seemed to be similar in the two strains of mice, wild type and gal-3^{-/-}. Both strains attained similar levels of specific antibodies over the time of infection, independently of the Gal-3 expression.

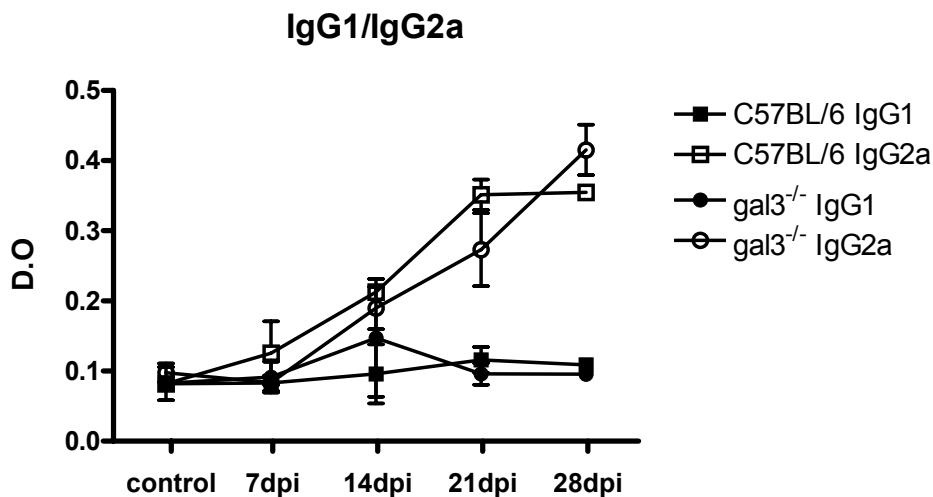


Figure 22: IgG1 and IgG2a antibody responses in infected C57BL/6 and gal-3^{-/-} mice. ELISA plates were coated with 20 µg/ml of *T. cruzi* total protein extract, and pooled serum samples from five mice were analysed at a 1:100 dilution. IgG values are presented as mean ±SD of OD (450 nm). Results are significant from three independent experiments performed.

3.2.2. Glycan Binding Specificity of Antibodies in Mice Serum.

The *in vivo* qualitative production of specific IgG antibodies was also investigated for a better evaluation of the host response. It was performed a screening of glycan-binding specificity of the antibodies present in non infected and infected serum of control and gal-3^{-/-} mice, since most of the glycoconjugates present in *T. cruzi* are highly antigenic.. A library of natural and synthetic glycans with amino linkers is printed onto slides to form printed glycan array. The array has 406 glycan targets, covering the most common glycans structures present in the nature. Array was incubated with serum from wild type and gal-3^{-/-} mice, non-infected and infected. Immunoglobulins present in mice serum recognized some glycan structures, and Alexa-488 conjugated antibody raised against mouse immunoglobulins was used for detection.

Results are summarized in table 1. Non-infected wild type mice have antibodies that recognized small glycans structures and other more complex glycans that are associated to a natural innate immune response. There are not important differences in the anti-glycans antibodies in the case of Gal-3 deficient mice under non pathological conditions. However, after infection with *T. cruzi*, anti-glycan antibodies speificities are totally different when we compare wild type and Gal-3 deficient mice. As a consequence of infection, much more glycans are recognized by murine antibodies in wild type mice, meanwhile many of these glycans are not immunogenic in the absence of galectin-3. Between the glycans that are recognized by serum from infected wild type animals but not from Gal-3^{-/-} mice can be found monossaccharide and dissacharides (glycan numbers 220,16,10,14), fucosylated glycans (glycan numbers 67,57) and sulfated glycans (glycan numbers 32,26,284,25,28). Antibodies from serum of infected Gal-3^{-/-} mice bound to the common structure Galb1-3GlcNAcb1-3Galb1-X, and this structure were not immunogenic for the wild type mice.

Wild Type Non Infected Controls

ID	Glycan Structure	RFU	STDEV	%CV
12	<i>a-L-Rha-Sp8</i>	24968	2076	8
16	<i>b-D-Gal-Sp8</i>	13711	2181	16
227	<i>Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	10037	575	6
67	<i>Fuca1-2Galb1-4(Fuca1-3)GlcNAcb-Sp8</i>	8200	1208	15
65	<i>Fuca1-2Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	7480	971	13
216	<i>NeuAca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb Sp0</i>	6303	529	8
135	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	5986	641	11
136	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	4027	1224	30

Gal3⁺ Non Infected Controls

ID	Glycan Structure	RFU	STDEV	%CV
12	<i>a-L-Rha-Sp8</i>	21175	860	4
227	<i>Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	7723	436	6
16	<i>b-D-Gal-Sp8</i>	5966	1351	23
135	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	4328	237	5
170	<i>(GlcNAcb1-4)5b-Sp8</i>	3415	356	10
297	<i>Galb1-4GlcNAcb1-6Galb1-4GlcNAcb-Sp0</i>	3354	201	6
306	<i>GlcNAcb1-4GlcNAcb-Sp12</i>	2973	99	3
67	<i>Fuca1-2Galb1-4(Fuca1-3)GlcNAcb-Sp8</i>	2772	263	9

Wild Type 28dpi Infected Controls

ID	Glycan Structure	RFU	STDEV	%CV
220	<i>Neu5Aca2-3Galb-Sp8</i>	36696	2647	7
67	<i>Fuca1-2Galb1-4(Fuca1-3)GlcNAcb-Sp8</i>	34639	5306	15
16	<i>b-D-Gal-Sp8</i>	27694	4342	16
184	<i>GlcAb1-6Galb-Sp8</i>	27202	1132	4
12	<i>a-L-Rha-Sp8</i>	17405	4787	28
57	<i>Fuca1-2Galb1-3GalNAca-Sp8</i>	16742	1776	11
218	<i>Neu5Aca2-3Galb1-3[6OSO3]GalNAca-Sp8</i>	16396	1146	7
227	<i>Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	15429	1697	11
135	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	12311	251	2
304	<i>GlcNAcb1-3Man-Sp10</i>	12277	859	7
136	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	12116	924	8
55	<i>Fuca1-2Galb1-3GalNAcb1-3Gala1-4Galb1-4Glc-Sp9</i>	11517	745	6
167	<i>GlcNAcb1-4(GlcNAcb1-6)GalNAca-Sp8</i>	11060	609	6
32	<i>[3OSO3]Galb1-3GlcNAcb-Sp8</i>	10220	1156	11
26	<i>[3OSO3][6OSO3]Galb1-4GlcNAcb-Sp0</i>	9503	2364	25
10	<i>a-L-Fuc-Sp8</i>	9042	2157	24
284	<i>[3OSO3]Galb1-4[6OSO3]GlcNAcb-Sp0</i>	8344	971	12
24	<i>GlcNAcb1-3(GlcNAcb1-4)(GlcNAcb1-6)GlcNAcb-Sp8</i>	7975	588	7
158	<i>GlcNAcb1-3(GlcNAcb1-6)Galb1-4GlcNAcb-Sp8</i>	7827	187	2
25	<i>[3OSO3][6OSO3]Galb1-4[6OSO3]GlcNAcb-Sp0</i>	7439	1946	26
274	<i>Galb1-3(GlcNAcb1-6)GalNAc-Sp14</i>	7080	126	2
14	<i>a-Neu5Ac-Sp11</i>	7033	2164	31
28	<i>[3OSO3]Galb1-4(6OSO3)Glc-Sp0</i>	6581	988	15
364	<i>Fuca1-2Galb1-3GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-6Galb1-4Glc-Sp21</i>	6016	1401	23

Gal3⁺ 28dpi Infected Controls

ID	Glycan Structure	RFU	STDEV	%CV
129	<i>Galb1-3GlcNAcb1-3Galb1-4GlcNAcb-Sp0</i>	37794	2011	5
277	<i>Galb1-3GlcNAcb1-3Galb1-3GlcNAcb-Sp0</i>	34816	1179	3
130	<i>Galb1-3GlcNAcb1-3Galb1-4Glc-Sp10</i>	34459	589	2
114	<i>Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4GlcNAcb-Sp0</i>	34198	295	1
136	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	10956	588	5
227	<i>Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	8450	794	9
12	<i>a-L-Rha-Sp8</i>	7848	223	3
132	<i>Galb1-3GlcNAcb-Sp8</i>	7098	2663	38
184	<i>GlcAb1-6Galb-Sp8</i>	6581	793	12
67	<i>Fuca1-2Galb1-4(Fuca1-3)GlcNAcb-Sp8</i>	5284	722	14
135	<i>Galb1-4(Fuca1-3)GlcNAcb1-4Galb1-4(Fuca1-3)GlcNAcb-Sp0</i>	4536	256	6
131	<i>Galb1-3GlcNAcb-Sp0</i>	3745	2134	57

3.3 Serum Cytokines Profile is Drastically Altered in gal-3 Deficient Mice During Infection

It is well known that the cytokine balance is important to address the immune response against *T. cruzi*, and that galectin-3 is able to regulate cytokine production by different immune cell types. Considering the higher parasite burden that Gal-3 knock-out mice showed, we next tested whether Gal-3^{-/-} mice showed an altered cytokine serum levels. Cytokines were measured in serum of non-infected control and Gal-3^{-/-} mice, at 14 days post infection, time point in the acute phase close to the peak of parasitemia, and 28 days post-infection, when no parasites can be observed in blood, and might be considered like the resolution of the acute phase.

Most of the cytokines measured were absent or at very low levels, both in wild type and gal-3^{-/-} mice in non-infected animals. However, non-infected wild type mice showed high IL-1 β and IL-10 levels in sera, although control gal-3^{-/-} mice showed reduced IL-10 level and absence of IL-1 β . As expected, at 14 dpi wild type mice showed an elevation of IL-2, of Th-2 cytokines, IL-10 and IL-5, and pro-inflammatory cytokines like IFN- γ , TNF- α , IL-6 and IL-1 β , going down at 28 dpi (Figure 23), when parasites disappear from the blood stream. In contrast, Gal-3^{-/-} mice showed a lack of pro-inflammatory cytokine production like IL-2 and IL-1 β at 14 dpi, although the IL-1 β boost was observed at 28 dpi, suggesting a delay in the initiation of the immune response.

It was observed an induction of IFN- γ production both in wild type and Gal-3^{-/-} mice in the infected animals, in coincidence with the highest parasite burden, but surprisingly, the total amount of this important cytokine during the infectious process was 5 times lower than in the Gal-3^{-/-} mice than in the wild type animals.

TNF- α was also diminished in the Gal-3^{-/-} mice at 14 dpi, but the major differences were found at 28 days post infection, when no TNF- α could be detected in gal-3^{-/-} mice, in contrast to the wild type mice that still showed a significant amount of TNF- α in serum. IL-6 and IL-10 were also down-regulated in deficient mice during infection compared to wild type animals.

IL-5 was not detected in non-infected mice, neither in deficient nor in wild type, but it was observed a strong induction in the wild type mice at 14 dpi. Nevertheless, IL-

5 was almost absent in gal-3^{-/-} mice at this infection time point. However, at 28 dpi, IL-5 is not detected in wild type mice, but in the case of Gal-3^{-/-} mice, IL-5 are higher than those detected at 14 dpi (Figure 23), even when the levels were still very low.

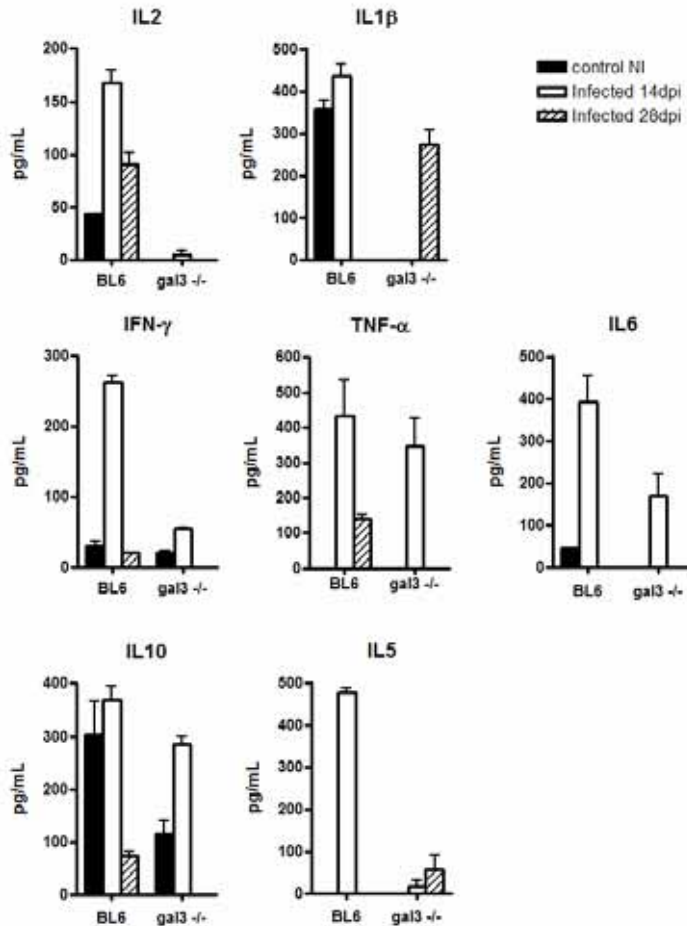


Figure 23: Cytokine seric levels during infection were altered in galectin-3 deficient mice during infection. Analyzed cytokines are indicated on the top for every graph. The levels of cytokines were measured in serum from non-infected and infected mice at 14 and 28 days post-infection, in wild type and galectin-3 deficient mice. A protein array from FASTQuant® Microspot Assays was used for cytokine detection. Results are the mean of two independent experiments done in triplicate. In each experiment, serum were collected and pooled together from a 5 mice group at every time point. Results are given in pg/mL and bars represent standard deviation. Solid black columns represent the non-infected controls, white columns infected mice at 14 dpi, and striped columns infected mice at 28 dpi.

3.3. Spleen cell subpopulations and gene expression.

Once it was observed that infected Gal-3^{-/-} mice presented higher parasitemia, and it was shown that the serum cytokine levels were down-regulated, our aim was to gain some insight on the mechanism underlying these alteration. In order to do this, spleen of infected animals were studied quantitatively and qualitatively. More precisely, we studied whether the cells type and quantity are the same in wild type mice and in Gal-3^{-/-} mice, and then gene expression by RT-PCR of selected genes related to the evolution of the disease was examined.

3.3.1. Spleen cells and nature number of cell sub-populations.

To get an overview of what is happening in the Gal-3 deficient model we decided to study a major immune organ like the spleen. First at all, it was evaluated the total cell number present in the spleen of wild type and Gal-3^{-/-} animals during the infection, at 14 and 28 days post infection, and in control animals. The total cell number was counted and no significant differences were found (Figure 24). Next, the percentages of the major spleen cell subpopulations were quantified by flow cytometry. B cells, CD4 and CD8 positive cells, T cells, macrophages and dendritic cells were quantified by using specific antibodies, and no significant differences were found (Figure 24). In wild type animals, it was observed some of the known events expected during *T. cruzi* infection. B and T cells suffer a polyclonal expansion, total number of cells increase, and an important amount of macrophages and dendritic cells can be found in the spleen, normally almost absent in spleen of healthy animals. Despite our previous findings in cytokine production, no differences were observed in Gal-3^{-/-} mice, neither in healthy mice nor in infected mice at any time point of infection measured, compared to the genetic C57BL/6 background, so Gal-3 is not relevant for spleen physiology.

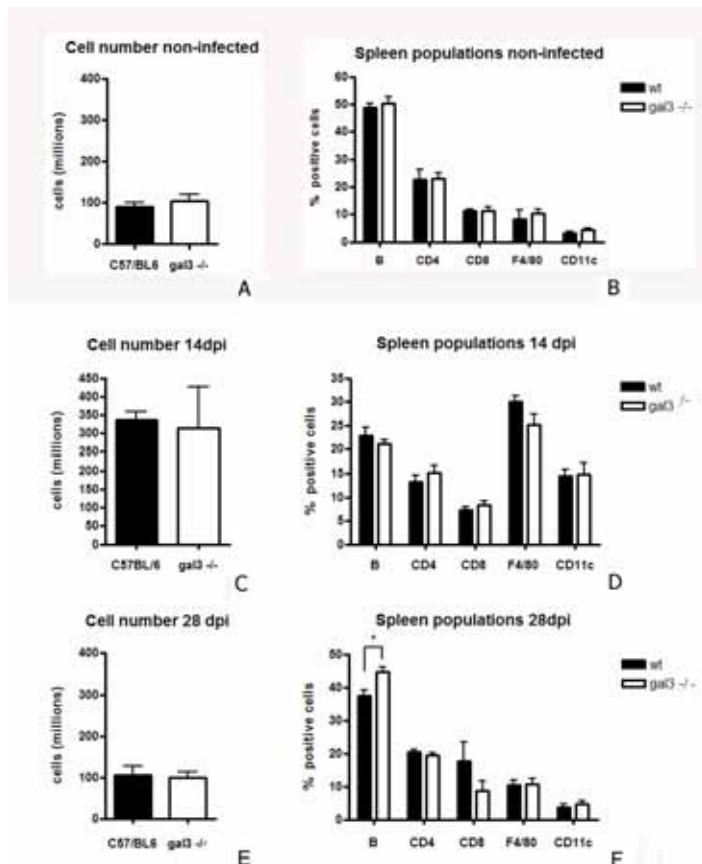


Figure 24: No significantly differences were found neither in the number nor in the nature of spleen cells of galectin-3 deficient mice during the infection course. Total cell number were equal in spleens of wild type and Gal-3^{-/-} non infected mice (A), and infected at 14 dpi (C) and 28 dpi (E). Percentages of different cell types present in spleen from infected animals were measured by the use of specific antibodies and flow cytometric analysis: non infected (B) at 14 dpi (D) and at 28 dpi (F). B220 positive cells were considered B cells; CD4 and CD8 were used to differentiate T cell subtypes, F4/80 labelled macrophages and CD11c dendritic cells. Results are the mean of 10 individually analyzed mice from 3 independent experiments. Error bars show SD. Significant differences are shown by *P<0.05

3.3.2. Spleen Gene Expression during T. cruzi Infection

Once it was seen that the spleen cell composition of the Gal-3 deficient mice was similar to the wild type animals both in control and in infected mice, we studied the expression of several genes that had previously been related to processes that take place during the immune response. Chosen genes were implicated in cell migration, cell adhesion, inflammation and immune response. Galectin genes expression was also quantified, because there is the possibility that the lack of galectin-3 could be compensated by the over-expression of other members in the galectin family. The full gene list is shown in material and methods.

The table 2 shows the genes that were found to be regulated during infection. First at all, spleens from non-infected wild type and knock-out animals were compared, showing no differences in the selected gene expression profile. Infected animals at 14 and 28 days post infection were compared to their non-infected controls. In accordance with our previous results, galectin genes were up-regulated during the infection in the wild type model. In addition to gal-1, and gal-3 mRNA, also gal-2 and gal-9 mRNA were strongly induced at 14 days post infection, and in a lesser extend at 28 days post infection, with no up-regulation of gal-9 at this time point. It is common that knock-out animal models develop some compensatory mechanism to supply the lack of a particular gene, this is what homeostasis is. Thus, in the galectin-3 deficient mice could happen that other galectins assume the role of the missing galectin-3. When galectin mRNA expression in Gal-3 $-/-$ mice was compared with their genetic background during infection, we did not found major differences. gal-2, and gal-9 expression were not altered, and gal-1 was 3 times lower in the gal-3 $-/-$, but still up-regulated in regard to its non-infected control. The profile of galectin genes changes during infection is similar in wild type and gal-3 $^{-/-}$ mice. That means that at least in spleen it is not likely a functional complementation by other galectins.

Surprisingly, TLRs were more up-regulated in the Gal-3 $^{-/-}$ mice at the time points observed, specifically TLR1, TLR4, and TLR6, although differences were highly variable.

Regarding genes implicated in the immune response, IFN- γ was 8 and 15 times up-regulated at 14 and 28 days post infection respectively in the wild type model. This was expected, as IFN- γ is one of the most important mediators to mount an effective

response against *T. cruzi*, but it is something new that the *gal-3^{-/-}* mice showed a fault of IFN- γ expression, up to 4 times lower than the wild type. This is in concordance with the lower amount of IFN- γ present in serum of infected *Gal-3^{-/-}* mice. Other genes that showed lower up-regulation in the *Gal-3* knock-out mouse were iNOS and COX-2, both implicated in inflammation, and *Ccl2*. Other genes showed the same kind of regulation in both animals, like *Itgb1*, *Ccr5* or *Gcnt*. (Table 2)

Table2. Spleen. Regulated genes during *T. cruzi* infection in mice.

<i>Gene</i>	<i>BL6 14dpi</i>	<i>BL6 28dpi</i>	<i>Gal-3^{-/-} 14dpi</i>	<i>Gal-3^{-/-} 28dpi</i>
<i>gal-1</i>	8.2 ± 4.2	2.9 ± 0.35	2.46 ± 0.1	2.86 ± 0.49
<i>gal-2</i>	7.06 ± 5.5	2.59 ± 0.81	6.20 ± 2.05	1.99 ± 0.125
<i>gal-3</i>	18 ± 6.02	8.93 ± 4.93	x	x
<i>gal-4</i>	-6.64 ± 2.4	-	-1.9 ± 0.1	3.55 ± 1.79
<i>gal-7</i>	-	-	-	-
<i>gal-8</i>	3 ± 0.6	1.84 ± 0.19	-	3.33 ± 0.9
<i>gal-9</i>	7.82 ± 2.81	-	7.11 ± 2.11	-
<i>TLR1</i>	-	-	10.29 ± 6.1	10.98 ± 1.49
<i>TLR2</i>	-	-	-	-
<i>TLR4</i>	2.3 ± 1.2	-	11.35 ± 7.65	5.8 ± 2.1
<i>TLR6</i>	-	-	22.8 ± 10.2	16.50 ± 3.2
<i>IFN-γ</i>	8.2 ± 4.2	15.65 ± 0.6	2.68 ± 0.7	6.65 ± 2.25
<i>Cox-2</i>	3.53 ± 2.03	-2.15 ± 0.14	-	-
<i>iNOS</i>	34.08 ± 7.92	-	20.25 ± 10.2	-
<i>Itgb1</i>	-	4.23 ± 1.23	-	3.25 ± 0.95
<i>Ccr5</i>	4.7 ± 3.0	3.87 ± 0.1	2.05 ± 0.55	3.2 ± 0.8
<i>FoxP3</i>	-3.65 ± 0.38	-	-	-
<i>Jak1</i>	-7.5 ± 3.05	-	-5.49 ± 1.55	-
<i>Gcnt</i>	2.52 ± 0.33	-	2.97 ± 0.57	-
<i>Ccl2</i>	6.0 ± 3.9	-	-	-4.89 ± 2.18

Table 2: Quantification of gene expression in the spleen of infected C57BL/6 mice and *Gal-3^{-/-}* mice. RNA was isolated from spleen on the indicated days after infection; RT-PCR was performed to quantify expression of the genes listed in Material and Methods. Results are expressed as the fold increase values (\pm SD) of pooled RNA from 5 different mice done in duplicate, comparing infected tissue at given times with the non-infected control. Values in red typing represent more than five times up-regulated genes, and in blue are shown genes more than five times down-regulated genes.

3.4. Galectin-3 Knock-out Mice Show a Deficient Immune Response in the Heart that Does not Affect to the Parasite Burden.

The data that have been exposed till now show the outcome of the systemic immune response in the deficient galectin-3 mouse model. Nevertheless, due to the great importance of the heart, like one of the main target of Chagas disease, we decided to study further the immunological processes that could be associated to galectins functions in the cardiac tissue. The goals of this section were to evaluate the parasite burden in heart, the nature and composition of the cellular infiltrate in the heart during the acute phase and the gene expression that is taking place in the organ.

3.4.1 Galectin-3 deficient Mice Present the Same Parasite Burden in Heart, but Have a Deficient T cell and Macrophage Migration.

The parasite DNA in heart was measured by qPCR during the acute phase of infection using specific probes after total heart DNA purification. No parasite DNA was detected in the non-infected controls, and no significant differences were observed between the Gal-3^{-/-} mice and the wild type, neither at 14 days post infection nor at 28 days post infection (Figure 25).

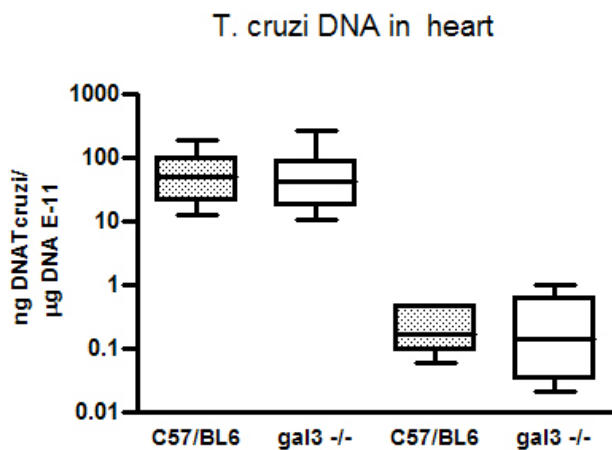


Figure 25: Quantification of *Trypanosoma cruzi* DNA in the heart tissue of infected C57BL/6 mice and galectin-3 deficient mice. DNA from ten independent mice was isolated from heart tissue on the indicated days after infection, and quantitative polymerase chain reaction (PCR) was performed as described in Materials and Methods to detect parasite DNA. *T. cruzi* DNA is expressed as the number of nanograms of parasite DNA per microgram of total DNA obtained from a heart tissue sample. Empty boxes denote values for C57BL/6 mice, and filled rhombi denote values for gal-3^{-/-} mice. Results express values from 10 different mice. No differences between the parasite loads in C57BL/6 and gal-3^{-/-} mice were found to be statistically significant.

It is thought that the cardiac pathology during Chagas disease has to do with two factors; one is the parasite burden in the heart, and the other one is the composition and strength of the immune cell infiltrate in heart, leading to some autoimmune events. Parasite burden was no significantly different in Gal-3^{-/-} mice, but that alone is not an image on what is happening in the tissue. Next, the nature and composition of infiltrating cells in infected hearts were evaluated. Anti-mouse CD4 and CD8 were used to count both T cells subtypes by confocal microscopy, and anti-mouse CD68 was used as a macrophage marker (Figure 26). No infiltrating cells were detected in hearts from not infected animals, neither in the wild type nor in the Gal-3^{-/-}. Slides from ten individual mice were used to count infiltrating T cells and macrophages (Figure 27). It was observed a diminished presence of T cells at day 14 post infection, and a significant reduction of infiltrating CD68⁺ cells at 14 and 28 days post infection in Gal-3^{-/-} compared with wild type controls. In addition, the tendency of cell migration was quite different. T cells reached the highest number at 14 dpi, and at 28 dpi are disappearing from the infected heart in wild type animals. In contrast, macrophages are increasing the total cell number at 28 dpi. Gal-3^{-/-} mice showed no reduction of T cells at 28 dpi, it is likely that T cells do not leave the tissue like the wild type model, and showed no increase of macrophage infiltration at 28 dpi.

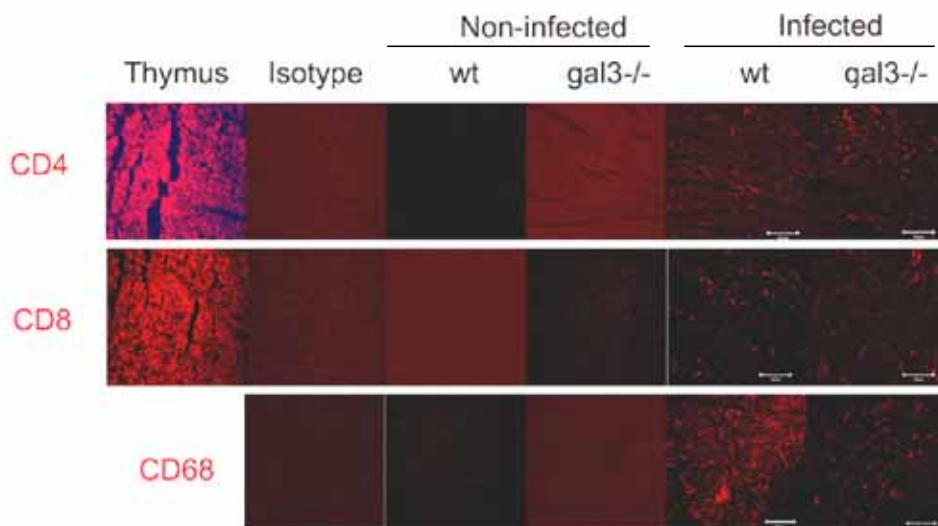


Figure 26: Immunohistochemical staining of infiltrating cells in infected hearts. CD4, CD8 and CD68 specific antibodies were used to mark specifically T CD4 and CD8 cells and macrophages in tissue sections. Thymus was used as positive control for CD4 and CD8 staining, and isotype antibodies for negative controls. Sections from non-infected and infected (28dpi) wild type and gal3^{-/-} mice were used.

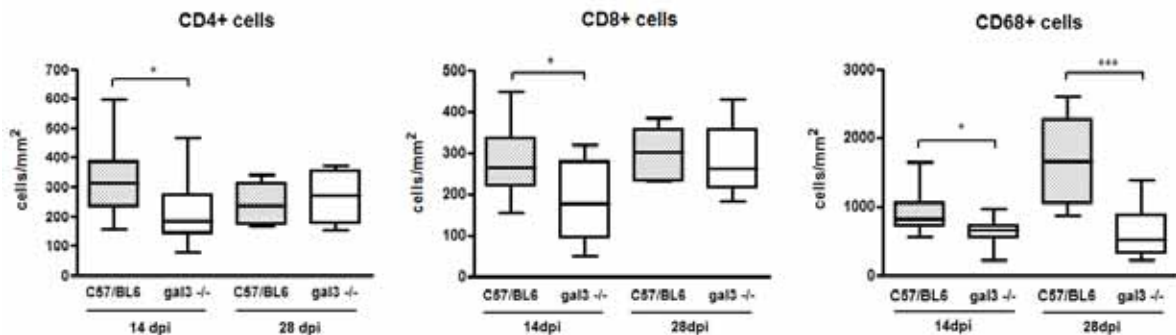


Figure 27: Cell infiltrates in infected hearts of wild type and galectin-3 deficient mice. The numbers of infiltrating CD4+, CD8+, and CD68+ cells into the heart of *T. cruzi*-infected wild type and Gal-3^{-/-} mice at 14 and 28 days after infection were counted by confocal microscopy. Cells were counted by immunohistochemistry, and data represent the mean of 10 individual mice from 2 independent experiments. Data from non-infected animals are not shown, as infiltrating cells are almost undetectable in healthy hearts. At least 20 different fields were counted for each individual heart. Bars represent SD, *P<0.05, ***P<0.001

3.4.2. Heart Gene Expression during *T. cruzi* Infection

Considering that the CD4 and CD8 T cell number were lower in the heart from Gal-3^{-/-} mice at 14 dpi, and the macrophage cell number was lower at 14 and 28 dpi, it was tested the gene expression in the same way that it was done for the spleen. The expression of the same genes was quantified by RT-PCR. This would provide us information about the nature of subjacent immune response that occurs in the heart. The number of immune cells in infiltrating cells is not high in control non infected hearts, and no differences have been yet reported for Gal-3^{-/-} mice under normal conditions. As expected, there were no significant differences in gene expression between non-infected wild type and Gal-3 deficient mice.

We next analyzed gene expression at 14 and 28 days post-infection, comparing again infected tissue versus non-infected tissue. Results are summarized in table 3. When galectin gene expression was quantified, it was observed in wild type mice that gal-3 mRNA was highly up-regulated during infection, but no significant changes were seen regarding other galectins. However, although no changes for mRNA gal-1 were detected, Gal-1 is up-regulated in infected hearts, as it was seen by western blotting. In

the gal-3 deficient model, gal-7 mRNA was up-regulated, and increase of Gal-1 protein expression was also detected.

TLRs expression was increase in wild type mice as expected, because it is known that TLR2,-4 and -9 are implicated in *T. cruzi* recognition, but nothing has been reported about TLRs and immune response in the heart during Chagas disease. TLRs expression was much more up-regulated in the Gal-3^{-/-} mice, although due to the high variability that was found between animals regarding TLRs expression, only the change in TLR2 was significantly different.

Consistently with the current knowledge about immune response in Chagas disease, some important immune genes were up-regulated, both in wild type mice and in Gal-3^{-/-} mice, like IFN- γ , IL-12, TNF- α , iNOS, Arg I or COX-2, genes related to quimiotaxis and cell migration, like Ccr2, Ccr5, Ccl2, selectins and integrins. However, some of these genes were differentially regulated in the Gal-3^{-/-} mice. There was a 200old increase of IFN- γ expression observed in wild type mice, in contrast to the 22 fold increase in the knock-out mice. IL-12, TNF- α and iNOS were not so up-regulated in the Gal-3 mice like in the wild type. Their mRNA expression levels were around one half compared with the wild type model. Even the lower number of infiltrating cells does not explain such lower induction folds, when the cell number difference was as much as two fold. Taken together, all the data pointed out to a diminished immune response in the gal-3^{-/-} mice. Indeed, we found some differences regarding expression of genes implicated in migration. Ccr2, Ccr5, Sel-e and Sel-p showed a significant diminished overexpression in the Gal-3 ^{-/-}mice at 14 days post infection, meanwhile Ccl2 and integrin-alpha showed no difference. At 28 days post infection, significant differences were observed only for Ccr2.

Table 3. Heart. Regulated genes during T. cruzi infection in mice.

Gene	BL6 14dpi	BL6 28dpi	Gal-3 ^{-/-} 14dpi	Gal-3 ^{-/-} 28dpi
gal-1	-	-	3.41 ± 0.38	-
gal2	-	-	-	-
gal-3	42.86 ± 23.60	28.25 ± 32.50	x	x
gal4	-	-	-	-
gal7	-	-	3.16 ± 0.75	10.93 ± 5.77
gal-8	-	-	-	-
gal-9	2.46 ± 0.6	-	5.12 ± 2.86	2.91 ± 0.02
TLR1	46.43 ± 53.93	51.65 ± 70.07	79.15 ± 75.68	118 ± 20.01
TLR2	6.94 ± 5.38	4.57 ± 3.69	13.23 ± 0.98	10.89 ± 1.93
TLR4	2.56 ± 1.99	-	270.02 ± 208.19	289.56 ± 135.17
TLR6	16.23 ± 14.05	18.74 ± 24.84	53.83 ± 57.27	56.76 ± 24.00
IFN- γ	205.67 ± 49.07	37.82 ± 3.55	22.93 ± 3.66	5.91 ± 0.47
Cox-2	8.58 ± 2.21	-	6.49 ± 1.77	2.03 ± 0.72
Arg I	22.00 ± 22.09	-	21.67 ± 3.44	-
iNOS	12.97 ± 6.27	-	5.28 ± 0.96	-
IL12b	54.21 ± 22.81	14.71 ± 2.66	19.07 ± 3.43	7.28 ± 0.80
TNF	17.96 ± 2.52	4.21 ± 0.37	9.16 ± 0.06	4.06 ± 0.75
FasL	5.12 ± 0.36	2.55 ± 0.41	6.65 ± 0.76	7.56 ± 1.71
Stat1	7.89 ± 0.52	10.96 ± 9.40	3.81 ± 0.86	4.05 ± 2.78
Ccl2	46.39 ± 31.41	-	41.16 ± 4.72	-
Ccr2	20.54 ± 12.55	15.62 ± 4.44	8.04 ± 1.42	5.20 ± 1.47
Ccr5	15.11 ± 12.40	6.46 ± 0.29	8.96 ± 2.49	4.92 ± 1.87
Itgam	10.79 ± 3.27	2.26 ± 1.71	28.12 ± 19.94	5.85 ± 4.45
Icam-1	8.54 ± 3.54	3.32 ± 1.41	8.58 ± 1.45	4.39 ± 0.28
Sel-e	16.65 ± 2.24	-	12.56 ± 10.27	-
Sel-p	24.08 ± 15.47	-	23.87 ± 25.03	-

Table 3. Quantification of gene expression in the heart tissue of infected C57BL/6 mice and gal-3^{-/-} mice. RNA was isolated from hearts on the indicated days after infection; RT-PCR was performed to quantify expression of the genes listed in Material and Methods. Results are expressed as the fold increase values (\pm SD) of pooled RNA from 5 different mice done in duplicate, comparing infected tissue at given times with the non-infected control. Values in red typing represent more than five times up-regulated genes.

4- GALECTIN-3 DEFICIENT DENDRITIC CELLS

We have shown that galectin-3 is up-regulated in B cells and are constitutively expressed in antigen presenting cells that migrate to the heart during infection. The role of galectin-3 seems to be important and global during the initiation and establishment of the immune response, because galectin-3 knock-out mice showed an impaired cytokine serum levels, a deficient immune cells migration and deficient expression of genes related to inflammation and immune response. Looking for the mechanism underlying this lack of response, we focused our attention in the cells responsible of initiate the immune response, the dendritic cells. These cells are known to express and secrete galectin-3, and are the most powerful antigen presenting cells, so they are a good candidate to find out an explanation that could explain the phenotype that we had described in the Gal-3 $-/-$ mice.

4.1. Gal-3 $-/-$ Dendritic Cells Differentiated from Bone Marrow Precursors Show a Normal Surface Marker Expression.

We decided to work with DCs in order to explain the altered response observed in the gal-3 deficient mouse. It has been published that galectin-3 is expressed on DCs and that this protein is important for dendritic cells functions, like cytokine production (Bernardes, Silva et al. 2006), or migration (Brustmann 2006). Gal-3 does not play any role in differentiation and maturation (Breuilh, Vanhoutte et al. 2007), but there are some reports involving other galectins in DCs maturation (Dai, Nakagawa et al. 2005; Fulcher, Hashimi et al. 2006). However, there is no evidence on the relevance of galectin-3 in DCs differentiation *in vitro* from bone marrow cells. To rule out the possibility that gal-3 $-/-$ bone marrow precursors could not differentiate to viable DCs, we incubated cells with GM-CSF for 9 days, and then we checked first the presence of galectin-3 (Figure 28,A-D), and then the expression of CD11c, MHC II and the costimulatory molecules CD80 and CD86, in non-stimulated DCs and LPS-matured DCs. Wild type DCs are supposed to be CD11c⁺ cells, have high MHCII surface expression, low CD80 and CD86 expression of immature DCs, but after maturation with LPS these co-stimulatory molecules are up-regulated. This phenotype is exactly what we found when we looked at these markers, both on wild type DCs and gal-3 $-/-$

DCs, and both increased CD80 and CD86 expression after LPS treatment, confirming thus the nature and viability of gal-3^{-/-} DCs. (Figure 28, D-L)

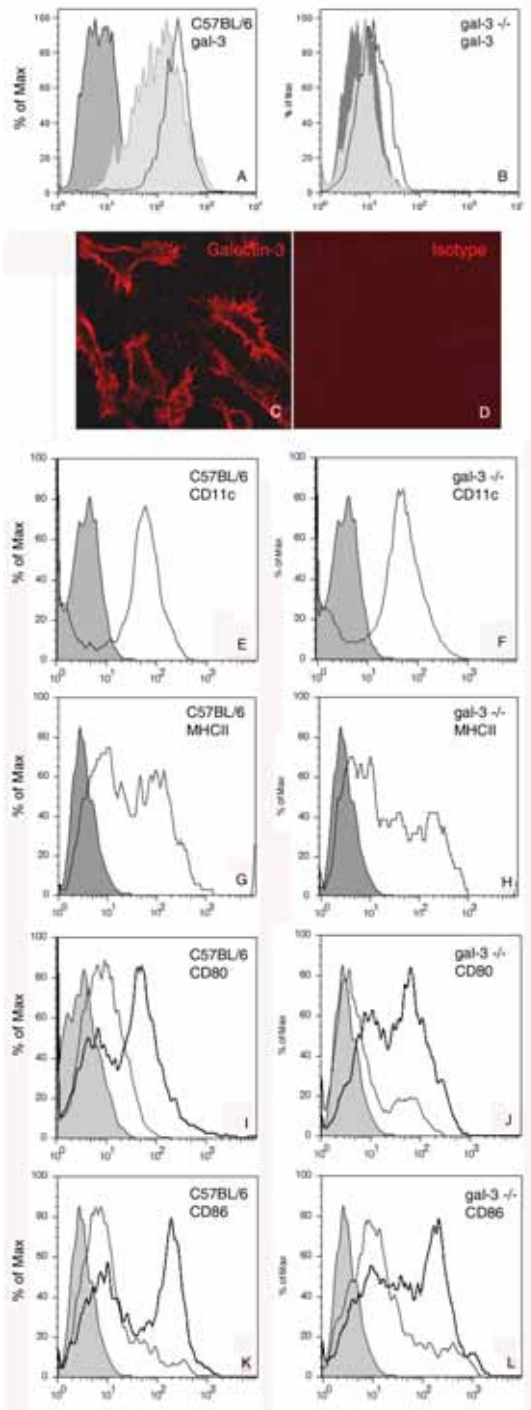


Figure 28. Galectin-3^{-/-} dendritic cells showed a normal expression of surface markers. A) Galectin-3 expression was tested by flow cytometry on differentiated DCs. Isotype antibody control is shown in dark grey solid histogram, immature DCs in dark grey solid histogram, and DCs after over night LPS incubation in black line. It is shown the presence of galectin-3 in DCs and the up-regulation after LPS incubation B) Gal-3^{-/-} DCs were stained with antiGal-3 antibodies too, and no signal was detected. C) Gal-3 expression was observed by confocal microscopy in non-infected wild type DCs. D) Isotype controls were used for negative controls. E, F) Immature Gal-3^{+/+} or Gal-3^{-/-} DCs were analyzed for the surface expression of CD11c by flow cytometry (black line). Isotype controls antibody was used as negative control (solid grey) G, H) Immature Gal-3^{+/+} or Gal-3^{-/-} DCs were analyzed for the surface expression of MHCII by flow cytometry (black line). Isotype controls antibody was used as negative control (solid grey). I-L) Gal-3^{+/+} or Gal-3^{-/-} DCs were analyzed for the surface expression of CD80 and CD86 by flow cytometry. Immature DCs (thin black line) and cells after over night LPS treatment (thick black line) were analyzed, and isotype control were used as control (solid grey). Both co-stimulatory molecules were expressed at low levels in immature DCs, and both were up-regulated after LPS incubation, in wild type and in Gal-3^{-/-} DCs.

4.2. Parasite Adhesion and Invasion are Increased in gal-3^{-/-} Dendritic Cells.

Because galectin-3 plays a role during *T. cruzi* recognition, parasite adhesion and invasion of DCs could be the first events altered due to the lack of the protein. To check out this possibility, adhesion assays were done in the same way that it was done for the *in vitro* adhesion experiments. Surprisingly, we did not find any decrease in parasite adhesion as could be expected for a DC lacking Gal-3, a protein that it is known to play a role during parasite adhesion to host cells as it has been shown before. Even more, what it was observed is that parasites bound better to gal-3^{-/-} DCs than wild type DCs. (Figure 29A). In concordance with an increased adhesion, we observed an increased invasion, because the number of trypomastigotes produced after 72 hours post-infection was much higher in gal-3^{-/-} DCs (Figure 29B). To further evaluate this phenomenon, we checked expression of other galectins on DCs surface looking for a possible molecular compensation. It was shown that gal-3^{-/-} mice showed higher expression of galectin-8 on the cell surface (Figure 29E-F) This could be related to the higher parasite adhesion to the gal-3^{-/-} DCs because Gal-8 was found to be the most strong adhesion mediator when it was added exogenously.

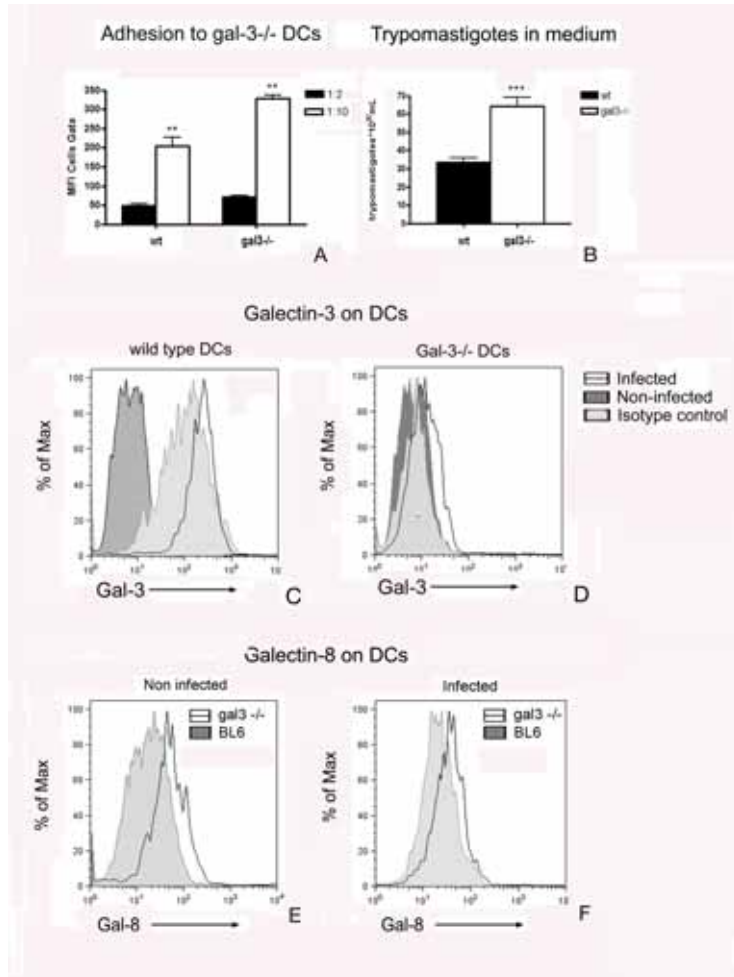


Figure 29. *T. cruzi* binding and invasion of DCs were higher in gal3^{-/-} due to an up-regulation of Gal-8 expression. A) CFSE labelled parasites were incubated at 1:2 and 1:10 cell:parasite ratio with wild type and Gal-3^{-/-} DCs and fluorescence attached to the cell gate was quantified by FACS. B) Trypomastigotes present in cell culture medium of infected wild type and Gal-3^{-/-} DCs were counted 72 hours after infection. C) Gal-3 surface expression was analyzed in control and infected wild type (C) and Gal-3^{-/-} DCs (D). Non-infected DCs and (E) infected DCs (F) were checked for Gal-8 surface expression by FACS. Results are the mean of three independent experiments in duplicate. Error bars means SD, **P<0.01,***P<0.001

4.3. Galectin-3 $-/-$ Dendritic Cells Showed a Significantly Diminished Production of the Pro-inflammatory Cytokines TNF- α and IL-1 in Response to LPS and *T. cruzi* Infection

DCs are a key mediator between innate and adaptive immunity, in part because after pathogen recognition, they produce a set of cytokines that will influence the outcome of the later response. Depending on the pathogen, different recognition receptors will be involved, and therefore different cytokines will be produced.

Thus, if galectin-3 can act as a potential *T. cruzi* receptor, its recognition by galectin-3 could trigger a cytokine response that will be altered in the deficient DC, and this could lead to global systemic consequences. To test this hypothesis, wild type and gal-3 $-/-$ DCs were treated with LPS or exposed to *T. cruzi* trypomastigotes overnight, and cytokines levels were measured in the supernatants. (Figure 24) Due to the great importance of the Th1/Th2 cytokine balance during the infection, we measured the most common cytokines associated to both kinds of responses. GM-CSF used for DCs differentiation was removed prior to the assay, because it has been described a direct toxic effect of this cytokine to *T. cruzi* (Olivares Fontt, De Baetselier et al. 1998).

In the absence of LPS or parasites, we did not detect almost any cytokine in DCs culture medium. Only small amounts of IL-10 were detected on the supernatant of wild type and gal-3 $-/-$ DCs. Not surprisingly, after LPS or parasite exposition, cytokine levels resulted strongly increased in culture supernatants of wild type DCs. LPS maturation of wild type DCs, or incubation with trypomastigotes led to the production of the following cytokines: IL-1, TNF- α , IL-12, IL-6, IL-10, and IL-17. Some cytokines were measured but not detected, like INF- γ and GM-CSF. This cytokine production by DCs in response to pathogenic stimuli can be considered normal, but several important differences were observed in the case of gal-3 $^{-/-}$ DCs (Figure 30), and the differences were not restricted to LPS or parasite induced response. The up-regulated production of TNF- α and IL-1 α in response to LPS and *T. cruzi* was significantly lower in gal-3 $^{-/-}$ DCs than in wild type DCs, as much as six times lower. Both cytokines are pro-inflammatory, are defined like Th1 cytokines and play an outstanding role during the immune response initiation.

On the other hand, increased levels of IL-10 after *T. cruzi* infection were observed in the gal-3 $^{-/-}$ DCs compared to wild type DCs. IL-10 levels were increased in response to LPS too, but no difference was seen between gal-3 $-/-$ and wild type DCs. IL-12 and

IL-6 were strongly induced in response to LPS or *T. cruzi*, but there were no significant differences between wild type and deficient DCs.

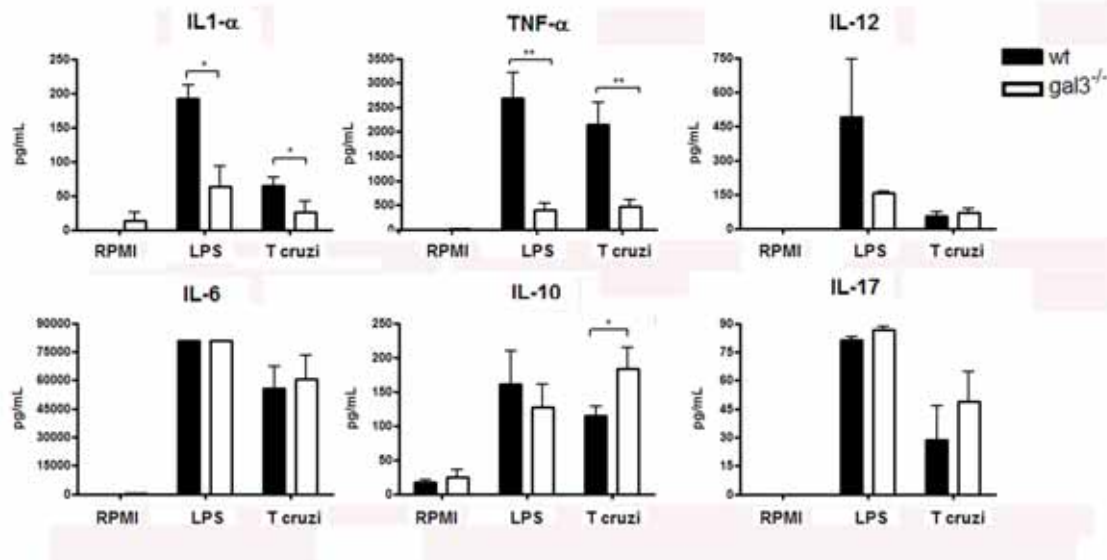


Figure 30. Cytokine production by dendritic cells in response to LPS and *T. cruzi*. The amounts of GM-CSF, IFN- γ , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF- α in the culture supernatant of gal-3 ^{+/+} DCs (black columns) and gal-3 ^{-/-} DCs (white columns) were determined by flow cytometry. IL-12p70 was determined by ELISA. Supernatants culture from immature DCs without treatment, from DCs incubated over night with LPS (1 μ g/mL) and after incubation with *T. cruzi* trypomastigotes (10 parasites per cell) over night were measured. GM-CSF and IFN- γ were not detected. Data are the mean of three independent experiments (\pm SD) performed in duplicate, and results are given in protein picograms per millilitre. *P<0.05, **P<0.01

4.4. Galectin-3 Deficient Dendritic Cells Present an Altered Surface Expression of Toll Like Receptors when they are Infected by *T. cruzi*.

To evaluate further the diminished TNF- α and IL-1 α produced by gal-3^{-/-} DCs upon stimulation, we focused our attention in TLRs expression by those cells. It is well known, that *T. cruzi* is recognized by TLR2, TLR4 and TLR9, and this recognition led to an efficient cytokine production. After LPS treatment, TLR2 and TLR4 recognized the bacterial product and through well established intracellular signals involving MyD88, TRAF and NF κ B activation, DCs and macrophages initiate an immune response again the pathogen, leading to cytokine production, IL-12 and TNF- α in the case of *T. cruzi*. Thus, if gal-3^{-/-} DCs are not producing pro-inflammatory cytokines in response to *T. cruzi* and LPS, the question at this point arises clearly: Do TLRs work

properly in the gal-3^{-/-} DCs? In order to find out whether the cytokine deficient production was due either to a defect in TLRs expression or to the lack of galectin-3 itself, TLRs surface expression was measured. TLR1, TLR2, TLR4, TLR6 and TLR9 expression were measured.

Surface expression of the mentioned TLRs was measured on wild type and gal-3^{-/-} DCs, in non-infected and in *in vitro* infected cells (Figure 31). There were not detected any differences regarding TLRs surface expression between wild type and gal-3^{-/-} DCs in the absence of parasites. Nevertheless, after *T. cruzi* infection, all of the TLRs tested were up-regulated in the wild type DCs, as it was expected, but some TLRs were not expressed on the surface of gal-3^{-/-} DCs. TLR2 and TLR6 maintained the same surface expression levels than the wild type DCs, but on the other hand, TLR1 was almost absent. TLR4 was in an intermedial status, the expression was lower than the wild type, but not so low like the TLR1.

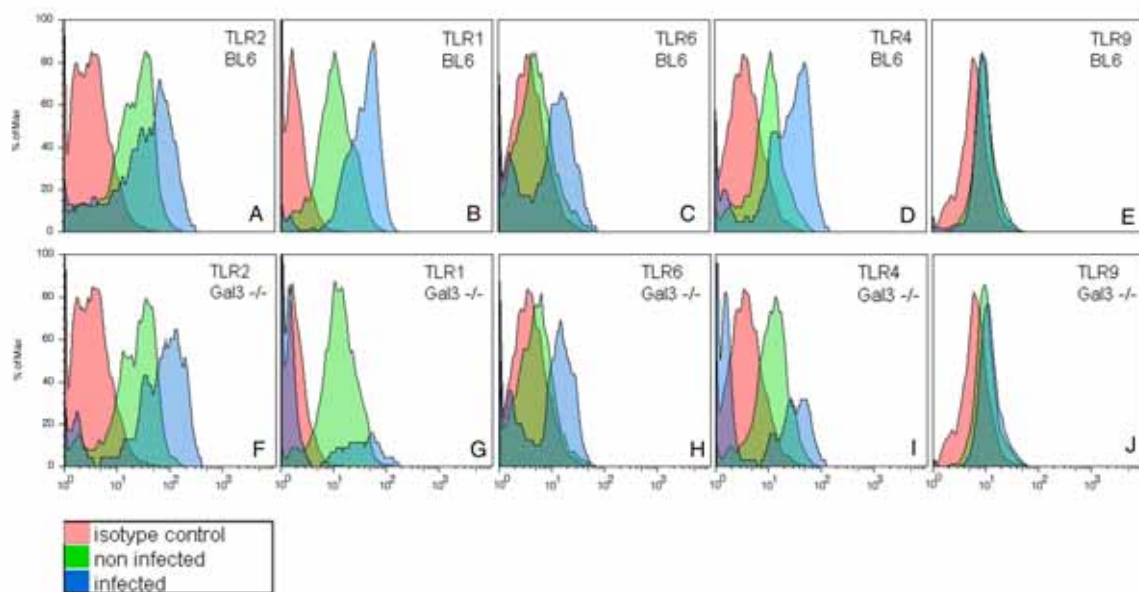


Figure 31. TLRs surface expression was impaired on gal-3^{-/-} DCs after *T. cruzi* infection. TLR-1, -2, -4, -6 and -9 surface expression were measured by flow cytometry. Wild type DCs (A-E) and gal-3^{-/-} DCs (F-J) were cultured over night in the presence of ten trypomastigotes per cell (blue histograms) or in the absence of parasites (green histograms). Specific TLR expression measured is indicated in each box. Appropriated isotypes antibodies were used in each case as negative controls (pink histograms). TLR1 was almost absent when gal-3^{-/-} DCs were infected (G) compared to wild type DCs (B). TLR-4 was also down-regulated in deficient DCs (I) compared to wild type (D).

DISCUSSION

The central paradigm of modern molecular biology is that biological information flows from DNA to protein. But Genome and Proteome are not the only factors in life functions of an organism, and carbohydrates play an outstanding role in biology. Glycosylation is the most common post-transductional modification, highly conserved through the evolution. In mammals, 50% of total proteins are glycosylated, 80% of the membrane proteins. This reflects its importance as biological information containers, far from the simplistic and classical point of view of sugars as energy source or scaffold proteins. Hence, the almost unlimited structural diversity of complex glycans, mainly due to the possibility to create branched structures, make them the perfect way to contain information in biological terms. On the other hand, this complexity, the difficulty in easily determine glycan sequences, and the fact that their biosynthesis could not be directly predicted from the DNA template makes the study of glycans one of the most complicated challenge in biology. These problems have held back the development of the glycobiology and the glycomics, when compared to other biomolecules. However, situation is changing nowadays due to the development of a variety of new technologies for exploring the structures of sugar chains.

Natural ligands of galectins are galactosides, sugars that are of outstanding relevance in *T. cruzi* biology, due to the unique enzyme that this protozoon possesses, the trans-sialidase, which is able to modify galactosyl exposition in glycans. Thus, it is an attractive possibility that *T. cruzi* could modify the galectin ligands. On one hand in the parasite, altering the pathogen recognition and interaction with host cells, and on the other hand in the vertebrate host, altering the immune response mounted against the parasite. This conceptual framework prompted us to define the role of galectins during the infection with *T. cruzi*. Some studies have been done, linking galectins and *T. cruzi*, but always in a very specific context. The aim of this work is to study the role of galectins during *T. cruzi* infection in a more broad sense. In order to do this, first we did *in vitro* experiments with recombinant galectins, taking several members of the three different subgroups, some of them not ever studied in the context of a parasite disease. Then, we studied the expression and role of galectins in a mouse model, and once we defined it, deficient mice in galectin genes were infected to confirm their biological role by means of characterising its phenotype. Finally, attempting to precise the molecular and cell events that galectins are modulating, their role were studied in differentiated dendritic cells, master initiators of the immune response.

1-In vitro interaction of galectins and *T. cruzi*

1-1 *T. cruzi* binds galectins in vitro

The protective and immune response against pathogens exposure is initiated by host recognition of specific Pathogen Associated Molecular Patterns (PAMPs), which are defined structures present in microorganisms, pathogenic or not, but not in host cells. The nature of the PAMPs described so far is variable, including linear or ramified structures, glycans, proteins, lipids or nucleic acids, and regarding *T. cruzi* one of those PAMPs might be glycoconjugates on *T. cruzi* surface, as it has been described in the case of GPI-anchored members (Campos, Almeida et al. 2001). Specific interactions between galectins and their glycoconjugates ligands are considered to be critical determinants in pathogen recognition (Rabinovich and Gruppi 2005). Hence, our first goal was to define whether galectins from the host were able to recognize specifically glycans exposed on the surface of the pathogen *T. cruzi*, and we found that galectins from the host were able to recognize glycans exposed on the surface of the pathogen *T. cruzi*. It is known that the surface glycoconjugates are extremely different in every biological stage. Our results show that galectins bind the three biological forms *in vitro*, however trypomastigotes and amastigotes, infective forms present in the host, are recognized by galectins with more avidity than the non-infective epimastigotes. There are several reasons to explain this, and reflects the changes that take place in the membrane composition, accessibility and exposure of the infective forms. Mucins of trypomastigotes are longer, contain additional α -galactopyranosyl residues and unsaturated, instead of saturated, fatty acids in the sn-2 position of the alkylacylglycerolipid component (Almeida, Camargo et al. 2000). These changes are supposed to arise through the evolution in order to survive in the new environment, the vertebrate host, where *T. cruzi* will encounter several galectins and other immune proteins. Whether the parasite evolved to display galectin ligands in infective forms, or the host developed galectins with high affinity towards parasite structures is difficult to predict over the common evolutive pressure of both species. In a more specific way, the affinity of different members of the galectin family was quite different considering the same parasite biological forms, indicating not only different parasite surface composition, but different fine specificity for each galectin. Although all galectins bind the common glycan core Gal β GlcNAc, other sugar units flanking this core could modify the affinity for each particular galectin. Amastigotes are the parasite form that binds

galectins with highest affinity and Gal-7 and Gal-8 present the highest affinity for trypomastigotes. Amastigotes are mainly intracellular, and this location suggests that they could modify intracellular function of galectins, in a variable way depending on the cell type and on the galectins expressed intracellularly. It has been described an alternative infection cycle inside the host, where intracellular amastigotes release from lysate cells, and then infect adjacent cells. As the amastigote is an intracellular non motile form, the higher affinity for galectins could favour the entry in new live cells, promoting adhesion and invasion. The fact that galectins can bind to *T. cruzi* is in agreement with recent reports that show the ability of galectins to bind to different pathogens. It has been shown that Gal-1 is able to bind several human viruses (Levroney, Aguilar et al. 2005; Ouellet, Mercier et al. 2005). Gal-3 has been shown to interact with pathogens like *Helicobacter pylori* (Fowler, Thomas et al. 2006), *Mycobacterium tuberculosis* (Barboni, Coade et al. 2005), *Klebsiella pneumoniae* (Mey, Leffler et al. 1996), *Candida* sp., (Kohatsu, Hsu et al. 2006) and the helminth *Schistosoma mansoni* (van den Berg, Honing et al. 2004). Gal-3 and Gal-9 have been reported to bind *Leishmania major*, although the biological implications for each galectin binding seem to be very different, since only Gal-9 but not Gal-3 promotes adhesion of the parasite to macrophage (Pelletier and Sato 2002; Pelletier, Hashidate et al. 2003). Regarding *T. cruzi*, Gal-3 had already been indirectly described to bind *T. cruzi* surface (Moody, Ochieng et al. 2000; Kleshchenko, Moody et al. 2004). Our data confirm that Gal-3 bind to *T. cruzi* surface and show for the first time that other galectin members can do it as well. The relevance of this *T. cruzi*-galectins interaction might vary depending on the individual galectin and on the cell type or matrix component that the parasite interacts with. Galectins are present in immune cells, and this interaction galectin-parasite could modulate their response, as it will be discussed below regarding galectin deficient models. The interaction between galectins and *T. cruzi* could be considered as a mediator mechanism of cell-parasite adhesion as well, during the encounter parasite-host cell, and could mediate interactions with extracellular matrix components.

Although it was demonstrated the interaction between galectins and *T. cruzi*, it was not clear whether it was carbohydrate-dependent. There was not binding for any galectin when assays were done in the presence of lactose, a natural inhibitory hapten for galectins. This indicates that *T. cruzi* recognition is mediated by the CRD in a sugar-dependent way. Moreover, this was supported by the results obtained in assays using

galectin-8 mutants. The lack of carbohydrate binding activity in one CRD showed a very diminished affinity and the mutant with no sugar binding activity (both active CRDs are mutated) showed no binding at all.

Location of the main galectin ligands on the surface of *T. cruzi* was seen under confocal microscopy. Many of the ligands were located all over the parasite surface with some intracellular diffuse staining. For amastigotes only surface ligands were seen in the case of amastigotes for all galectins tested, and for galectin-8 with trypomastigotes. Further work will be needed to identify the nature of particular parasite galectin ligands. Using a classical approach of affinity chromatography with immobilized recombinant galectins we could not identify any *T. cruzi* ligand. These preliminary attempts failed most probably to the inherent low affinity interactions, since galectins covalently linked to immobilized columns do not present multivalency. Using an alternative approach like western blot using soluble streptavidin-labelled galectins, in which the multivalency of galectins is preserved, we could observe many more unidentified ligands. A band around 75KDa was the main *T. cruzi* ligand of all the galectins tested, but its nature remains unknown.

We could identify one potential ligand of Gal-3, Gal-7 and Gal-8, the mucin AgC10. Moody et al showed a 45KDa parasite mucin that was detected as a Gal-3 ligand (Moody, Ochieng et al. 2000), and indeed, it could be AgC10. AgC10 is a highly glycosylated mucin, that has been shown to exert several immunomodulatory effects, leading to T cell immunosuppression, inhibiting IL-2 effects (Kierszenbaum, Fresno et al. 2002; Alcaide and Fresno 2004). Host molecules that may interact with AgC10 could be implicated in the immunosuppressor activities attributed to the mucin, and galectins are reasonable candidate, since it is known that Gal-3 modulate T cell responses by control of TCR clustering at the immune synapse (Demetriou, Granovsky et al. 2001) and other galectins might be implicated because different galectins could bind common receptors as it has been described (Patnaik, Potvin et al. 2006), although the biological functions must not be necessarily the same. However, further investigations need to be done in order to confirm this hypothesis.

1.2- Galectins promote parasite-cell adhesion *in vitro*.

As galectins are multivalent proteins, they can cross-link receptors mediating cell-cell adhesion and/or cell-extracellular matrix, processes that make the galectin family important markers during tumour progression (Liu and Rabinovich 2005). Thus, *in vitro* experiments were done in order to evaluate the ability of galectins to promote parasite adhesion to host cells. Two cell lines were used in the experiment, THP-1 and LLcMK2. Both are susceptible of being infected, and express β -galactosides that can interact with extracellular galectins. Adhesion experiments were done with infective tissue trypomastigotes, as this biological stage is the more frequent way of infecting new cells during the disease. Experiments were done at 4°C to block penetration, because at this temperature it is known that parasite invasion are almost inhibited, so we can assume that we are measuring only parasite adhesion to host cell surfaces.

In clear correlation with the previous binding assays, the galectins that showed higher affinity to trypomastigotes, Gal-3, Gal-7 and Gal-8, were the galectins that promoted adhesion to monocytes. The galectin induced adhesion was concentration dependent, and the CRD was implicated, since the effect was abolished in the presence of lactose. When Gal-8 mutants were used instead of the wild type protein, adhesion was not promoted, implicating an active CRD as a required element to favour parasite adhesion to host cells. However, the Gal-8 mutant that has a non-functional N-terminal domain, Gal-8R233, is still able to promote parasite adhesion to host cells, although in a lesser extent than the wild type. Gal-8R233 is supposed not to cross-link glycosylated receptors, as only one terminal domain of the protein can. It could be argued that the mutated CRD interact with some host molecules in a sugar independent way. However, that is not occurring in the parasite binding because lactose totally inhibits wild type galectin-8 binding to the parasite. Another possible explanation, is that Gal-8, although it is a tandem repeat galectin, form homodimers through the N-terminal CRD, as it has been published recently (Stowell, Arthur et al. 2008), allowing the Gal-8 dimer to be bivalent even when one of the two CRDs displays no sugar binding activity. In that way Gal-8 could still bind with relative high affinity to cognate ligands.

1-3 Galectin processing by *T. cruzi*

The association of Gal-3 with *T. cruzi* results in the formation of truncated form of Gal-3, which contains the intact CRD but lacks the N-terminal collagen-like domain. This truncated Gal-3 is unable to oligomerize. The cleavage sites within the N-terminal domain were identified in three specific sequences that can be associated in two groups, suggesting the action of at least two parasite proteases. The nature of the proteases implicated in Gal-3 cleavage was defined as a Zn-metalloprotease and a collagenase. In a systematic analysis of all protease families inhibitors, just OPA, a specific Zn-metalloprotease inhibitor, and HgCl₂, inhibited Gal-3 processing, confirming the previous idea of two proteases. There is a report of HgCl₂ as an inhibitor of a *T. cruzi* collagenase (Santana, Grellier et al. 1997) that could be the protease responsible of one of the cleavages observed in Gal-3. The N-terminal domain of Gal-3 might be an appropriate substrate for a collagenase, because the domain of Gal-3 shares a high degree of homology with collagen. The inhibition of just one protease did not affect the activity of the other one, indicating that both proteases work independently. Cruzipain is the more abundant protease described in *T. cruzi* so far, but it is not implicated in Gal-3 processing, because a specific inhibitor (E64) was unable to inhibit Gal-3 cleavage. Our data suggest that Gal-3 is cleaved mainly by non-secreted surface proteases. It is known that Gal-3 forms lattices on cell surfaces that can affect many of the innate immune responses triggered by Gal-3. Lateral mobility of receptors such as TCR on T cells or polarization of Th1 responses are controlled by Gal-3 lattice formation. Thus, destruction of the N-terminal domain of Gal-3 might lead to modulate innate host immune responses depending on Gal-3 oligomerization. *T. cruzi* could affect even more to Gal-3 functions, if the intracellular amastigotes are able to process intracellular Gal-3, as it is known that the pleiotropic effects of galectins depend on their subcellular location. In this context, the first cleavage would affect to the phosphorylation site that control the translocation of the protein from nucleus to cytosol (Tsay, Lin et al. 1999), and it is unclear how this change could affect to the regulation of cell apoptosis or cell growth. Further experiments must be done in order to explain the consequences of Gal-3 cleavage *in vivo*. The control of Gal-3 biological activities is not new, and it has been reported before the existence of proteases implicated in Gal-3 processing, either by the mammalian matrix metalloproteases 2 and 9 (Ochieng, Fridman et al. 1994; Nangia-Makker, Raz et al. 2007), and by the pathogen *Leishmania major* (Pelletier and Sato

2002). The first one is an example of how cleavage of Gal-3 could modulate endogenous functions of the protein, and the second one exemplifies how a pathogen could use a similar system to modulate endogenous galectins to promote its survival and replication, in this case *L. major*, in a way resembling to what we have reported for *T. cruzi*. Gal-3 is also processed by an unidentified metalloprotease like *L. major* does, but the implication of collagenases had not been reported for any other microorganisms so far.

We report here for the first time that there are other members of the galectin family that experiment proteolytic processing after *T. cruzi* interaction. Both Gal-4 and Gal-8 are cleaved by *T. cruzi* in the linker peptide rendering to monomeric galectin forms. This might inhibit the inherent functions depending on the natural Gal-4 and Gal-8 bivalency. Cleavage of these two galectins was inhibited by OPA, indicating the action of the same or different Zn-metalloprotease. Gal-8 is being nowadays associated to the immune system, for instance controlling neutrophil functions, adhesion and superoxide production (Nishi, Shoji et al. 2003; Yamamoto, Nishi et al. 2008). Thus, the cleavage of Gal-8 by *T. cruzi* might represent a direct advantage for the outcome of the infection. Gal-4 is reported to be restricted to the epithelial tract, situated in-between enterocytes in the epithelial lumen, although its expression in other tissues can not be ruled out. Another point is that galectins processing could be necessary for the advance of the parasite through extracellular space in order to reach the host cells.

1.4 Inhibition of parasite proteases leads to parasite killing by recombinant galectins

During Chagas disease, the parasite *T. cruzi* must evade complex defensive systems that are present in the host. Some of them include molecules that are intended to induce direct parasite killing. The complement system is one of the first innate mechanisms that target the parasite. However, *T. cruzi* is able to escape from the complement because the calreticulin of *T. cruzi* binds specifically to recognition subcomponents of the classical and lectin activation pathways of the human complement, preventing parasite killing (Ferreira, Valck et al. 2004). Glycoconjugates are major targets of different innate defensive systems. Among them it can be found collectins, defensins, dectins or anti-glycans antibodies, that are innate microbicides. For example, collectins are soluble CBPs able to induce microbial clearance (van de

Wetering, van Golde et al. 2004) and sera of patients with chronic Chagas' disease contain elevated levels of anti- α -galactosyl antibodies that are lytic to *T. cruzi*. (Milani and Travassos 1988; Gazzinelli, Pereira et al. 1991). Galectins could induce parasite death in a similar way to the anti-galactosyl antibodies, since both groups of proteins may bind the same *T. cruzi* ligands. It has been reported that the smallest unit that was reactive with anti-galactosyl antibodies was Gal α 1-3Gal β 1-4GlcNAcol (where GlcNAcol is N-acetyl-glucosamininitol), isolated from a mucin-like protein from *T. cruzi* termed F2/3 (Almeida, Ferguson et al. 1994). Galectins might be able to bind to this mucin-like protein, and could exert an effect similar over *T. cruzi* to that produced by lytic anti-gal antibodies by means of cross-linking parasite glycans. Moreover, it is known that Gal-1 and Gal-3 are quickly released from cells after *T. cruzi* interaction as part of the innate immune response, and it is likely to be the same for other members.

Our results show that indeed galectins are able to induce direct parasite killing, when the protease processing galectins is inhibited by OPA. The galectins that bind with high affinity to the parasite, Gal-3, Gal-4 and Gal-8, induced direct parasite death when the galectin processing was inhibited. Thus, functional cross-linking of crucial glycoconjugates of *T. cruzi* can induce direct parasite death. A vegetal lectin, Euonymus Europeus Agglutinin (EEA), with related binding affinity to the shown by galectins and anti- α -gal lytic antibodies can induce killing as well (Almeida, Krautz et al. 1993). Therefore, we can not rule out that anti-gal antibodies, galectins and EEA are interacting with the same molecules. The F2 fraction recognized by most of the anti-gal antibodies corresponds mostly to a 74KDa mucin-like protein (Almeida, Krautz et al. 1993), and this is close to the molecular weight of the major *T. cruzi* ligand of the galectins tested, as we have seen by western blot using recombinant galectins as a probe. This F2 fraction could be the galectin ligand on *T. cruzi* surface responsible of galectin mediated death, and further work must be done to confirm this hypothesis.

It is likely that *T. cruzi* has developed protective mechanisms to avoid the protective lytic role of galectins and limit or block their effector functions, in this case using specific proteases able to process galectins. After this processing, galectins are not able any more to cross-link receptors on *T. cruzi* surface, preventing parasite death. Interestingly, the resulting truncated galectins still have a functional CRD. We can speculate that galectin fragments around the parasite might provide some advantages in the host, like galactosyl epitopes hiding or interaction with host cells promotion. It has

been published that Gal-3 can induce death of *Candida albicans* (Kohatsu, Hsu et al. 2006), in agreement with the hypothesis that galectins are direct microbicidal agents. The specific mechanism has not been described, but in contrast to the reported affinity of Gal-3 to β -galactosides, Gal-3 binds to mannose complex present on *Candida* sp. This is the only reference as galectins as direct microbicidal agents, and our data is the first demonstration of a pathogen mechanism able to prevent this action. Thus, identification of the proteases implicated in galectin processing might represent a promising candidate in the search for molecular targets seeking effective chemotherapeutic agents to treat Chagas' disease

The fact that galectins bind to infective parasites can increase parasite adhesion to host cells thanks to their multivalency seems to be paradoxical regarding to the ability of galectins to kill the parasite on the basis of the same multivalency, because a truncated galectin is unable to promote parasite adhesion to the cell. So, does *T. cruzi* use galectins like molecular bridges to the host cells, or does it cleave the bridges that galectins form in its own surface to kill the parasite? The experimental conditions of our *in vitro* experiments allowed us to look at both events independently. Adhesion experiments were performed at 4°C. This reduce the rate of enzymatic activity of the proteases, meanwhile processing experiments were done at 37°C, temperature of the host where the parasite should encounter galectins. Most importantly, the cleavage of galectins seems to be a process that needs more time than the passive interaction of parasite-galectins. It could be postulated that first *T. cruzi* interacts with full length galectins-*T. cruzi*, and this promotes parasite adhesion to host cells, at the same time that many other parasite and host molecules are interacting. Adhesion of parasite to host cells is a fast event, and once the parasite is attached to cell membrane, it starts cell penetration and galectin processing, preventing the microbicide action.

However, this simple scenario may be much more complicated *in vivo* due to several factors. Different glycans in different cell types present variable affinity for galectins, competition between host glycans and parasite glycans, presence of more than one galectin at the same time, depending on the infected cell type, galectin concentration, glycan tissue profile, or even the existence of parasite CBPs that could compete with host galectins, both over parasite or endogenous host ligands.

2- Galectin expression in the murine model of experimental infection

The data obtained from experiments *in vitro* suggest that galectins might play an important role during the outcome of Chagas' disease in the infection establishment, playing a role in parasite recognition, parasite adhesion to host cells and parasite killing. In addition, galectins interact not only with *T. cruzi*, but with host receptors to modulate immune responses, regulating subsequent events like inflammation, cell migration, cytokine production, activation of the innate immune response and subsequent linking to the adaptive one. Many studies have demonstrated that galectins are related to the mentioned events, but they have been done in a particular galectin, in a specific environment. Our goal was to perform a systematic study of different galectin expression in different immune cell types. The context of Chagas disease and its study under the murine experimental infection provide us the opportunity to get insight on the role of different galectins under the same pathogenic circumstance.

Indeed, the fact that galectins may be of outstanding relevance during Chagas disease was confirmed by the data obtained from infected mice. Galectins were differentially regulated in spleen, a peripheral lymphoid tissue, and in immune cells migrated to a target organ of *T. cruzi*, the heart.

2.1- Galectin expression profile in immune cells

In the immune system, galectins are mostly expressed by activated but not resting T and B cells, and they are significantly up-regulated in activated macrophages and regulatory T cells (Liu and Rabinovich 2005; Rabinovich, Toscano et al. 2007). The course of *T. cruzi* infection is not an exception, and it is known that some galectins are up-regulated and secreted in several cell types during the disease, Gal-1 in B cells and macrophages (Zuniga, Gruppi et al. 2001; Zuniga, Rabinovich et al. 2001), and Gal-3 in DCs and B cells (Vray, Camby et al. 2004). Our results confirm these reported observations, and provide for the first time a global scenario of galectin expression in T cells, B cells and antigen presenting cells.

T cells showed an increase in Gal-1, -3 and -9 surface expression after *T. cruzi* infection. Crosslinkage of T cell surface receptors by galectins can trigger different transmembrane signalling through which different processes such as survival and activation are modulated (Toscano, Ilarregui et al. 2007). Our results demonstrate the

presence of these galectins on T cell surface subsets, but it is not clear if these galectins are expressed by the T cells or by other surrounding cell types, interacting extracellularly with exposed ligands on the T cell surface. Since galectins are actively secreted by many immune cells, they can work in an autocrine or paracrine way. Thus, it has been suggested that the induction of apoptosis by Gal-1 in T cells is dependent on the Gal-1 secreted by B cells during *T. cruzi* infection (Zuniga, Rabinovich et al. 2001), and it is possible that other galectins present on T cells surface could come from other cells like B cells, macrophages or DCs.

Regarding B cells, although it has been described an increase in Gal-1 levels in B cells infected with *T. cruzi*, we have not seen such increase. Nevertheless, it is known that Gal-1 is mainly secreted by B cells, so we could hypothesized that Gal-1 secreted by B cells are bound to receptors in other cell types like T cells. It was found an up-regulation of Gal-3 expression, as it has been reported before showing that Gal-3 mediates IL-4-induced survival and differentiation of B cells towards a memory cell phenotype (Acosta-Rodriguez, Montes et al. 2004). It can not be ruled out that other galectins could be implicated in this process as well. In this regard, we have observed down-regulation of Gal-8 and up-regulation of Gal-9 at mRNA levels during the experimental infection. The precise role of each galectin over B cell fate still remains unclear, but it is likely that competition and/or cooperation between different galectins are implicated to control B cell responses.

The presence of Gal-1, -3 and -9 was demonstrated on the surface of APCs (macrophages and DCs) on spleen of control and infected mice. The amount of these galectins on the surface of APCs was up-regulated in response to the infection. In concordance, splenic DCs from infected animals increase Gal-3 expression, affecting so their migration (Vray, Camby et al. 2004). Compelling evidence has implicated galectins in macrophages and DCs immune responses. As we have shown that Gal-1 and -3 binds to *T. cruzi in vitro*, we could speculate that these proteins might favor parasite phagocytosis or activation of the immune response, suggesting that galectins could work as PRRs. Gal-1 has been described to lead macrophage activity towards resistance or susceptibility to *T. cruzi* depending on its concentration (Zuniga, Gruppi et al. 2001).. This author shows that Gal-1 concentration address the macrophage activity *in vitro* after *T. cruzi* challenge. Low Gal-1 concentrations decrease IL-12 and NO production, favoring parasite replication. High concentration induces apoptosis, killing phagocytosed parasites and controlling the spread of the infection. An interesting issue

would be to compare Gal-1 produced by immune cells from different mice strains associated to resistance or susceptibility to the disease, to confirm this effect *in vivo*.

Galectins work as versatile molecules capable of modulating the physiology of different immune cells. We have shown that after *T. cruzi* infection, spleen cells change their galectin expression profile. An extra regulation point consists in modulating the glycan structure of their ligands. This is done by changes on galactosyltransferases under pathological conditions, which in turn will modify glycan structures. In addition, in the case of *T. cruzi* infection, host glycan composition can be modified by the enzyme trans-sialidase, unique to *T. cruzi*, by exposition on galactosydes residues. According to this, our results show that spleen cells during infection present the highest affinity for galectins at day 14 after infection, correlating with the highest parasite burden, and then with the highest amount of circulating trans-sialidase. By means of higher exposure of galactosyl ligands, host galectin ligands might be modified, increasing the affinity for galectins, and modifying the cellular responses triggered by galectins. How desialylation affects to the carbohydrate binding specificity of galectin-1 has been recently demonstrated (Toscano, Bianco et al. 2007). This work shows that Th2 T cells are protected from Gal-1 mediated apoptosis through differential α 2,6- sialylation of cell surface glycoproteins, meanwhile Th1 and Th17 effector cells express low sialylation levels, that in turn allows the formation of Gal-1 lattices on the cell surface triggering apoptotic signals to the cell. As *T. cruzi* retires terminal sialic acids from exposed glycoconjugates, it could be hypothesized that Gal-1, and most likely other galectins, would not form lattices on the cell surface, affecting so clustering membrane receptors and signal transduction.

2.2- Galectin expression in heart of infected animals.

Gal-1 and Gal-3 were up-regulated in heart tissue during the infection time. We analyzed the presence of other galectins during the course of infection, but only a slightly increase in gal-9 mRNA was detected. Although Gal-8 is normally expressed in heart tissue, we failed to detect the protein by western blot, but we did not observed any difference in gal-8 mRNA levels during infection, so we can assume that the role of Gal-8 is not so relevant as the one seen for Gal-1 and Gal-3, both up-regulated upon infection.

Neither Gal-1 nor Gal-3 expression was detected in hearts from non-infected animals, but were highly up-regulated during the infection, reaching the maximal expression at day 21 post infection, when the highest parasite burden occurs in heart tissue.

Most of the Gal-1 was expressed by cardiac cells, but it is likely that infiltrating immune cells are expressing Gal-1 as well. Gal-1 was clearly up-regulated, in accordance with previous results observed in humans (Giordanengo, Gea et al. 2001), but we did not observed any gal-1 mRNA increase, so other posttranscriptional regulatory systems should be responsible of the protein up-regulation like mRNA stabilization or an decrease in protein turn-over. Unlike the anti-inflammatory Gal-1, Gal-3 is not expressed by cardiac cells; infiltrated CD68- macrophages are the main source of Gal-3 in infected hearts. These cells are expressing arginase I, a marker of alternative macrophage activation (Cuervo, Pineda et al. 2008). Supporting this, Gal-3 has been described as a new alternative activation marker (MacKinnon, Farnworth et al. 2008). The induction of Gal-3 reached maximal values at 14 days post infection, when it was already observed high macrophage heart infiltration.

So, galectins are highly regulated in lymphocytes and antigen presenting cells, and it is likely that they participate in immune response through cell activation and cell migration to the heart after *T. cruzi* invasion. But it is the use of galectin-1 and specially galectin-3 deficient mice the most valuable tool that we had to define the role of those galectins in the immune system. Thus, supporting on the galectin expression profile over time of infection in the wild type model, the results obtained by use of recombinant galectins, and the reported data, it can be proposed the following scenario during *T. cruzi* murine infection. Gal-3 could participate in *T. cruzi* recognition, leading to the production of effector molecules able to activate the immune system. The up-regulation of Gal-3 would induce pro-inflammatory responses, Th1 responses activating the protective axis IFN γ -iNOS-NO that control parasite replication and spread. As a potent chemotactic agent, Gal-3 could favor inflammation promoting recruitment of immune cells to the sites of infection. Gal-1 could compete with Gal-3 ligands counteracting its pro-inflammatory actions, skewing the Th1 response to a Th2 one and inducing apoptosis of activated Th1 but not of Th2 effector T cells. In heart, macrophages expressing Gal-3 would be targeted by Gal-1 controlling the inflammatory responses initiated previously. Role of Gal-1 as a suppressive mediator is supported by its ability to induce apoptosis to

activated but not resting T cells, and by its expression in organs of immune privilege, where the cellular immune response should be quickly eliminated.

The galectin knock-out models served to confirm the roles of Gal-1 and Gal-3 during the outcome of the immune response upon infection. It is noteworthy to mention the importance of the genetic background of the mice used in this study. Control wild type and the deficient models possess a C57BL/6 background. This strain is characterized by a high resistance to parasite infections. Those mice develop reduced parasitemia compared with other susceptible mice like Balb/c, show almost an absence of clinical symptoms and do not show any mortality rate. The causes that make one strain resistant and other susceptible remains unclear and it represents a promising field of study in order to find host parameters associated to natural resistance to the infection. A Th-1 polarized response seems to be linked to the resistant strains, but it is still unclear. The results obtained in this study could give many differences in a model using a susceptible strain. Actually, an important problem lies in Chagas disease research. Host genetic factor together with the intrinsic genetic variability found between different *T. cruzi* polyclonal strains might be the cause of some apparently contradictory reported observations in the literature.

3- Study of the Immune Response in Deficient Galectin Mice

Infection of Gal-1^{-/-} mice showed no major differences during the course of infection compared to wild type in clear contrast to what could be expected, due to the large number of immune processes that Gal-1 is affecting, and because of the strong up-regulation of the protein in the wild type murine model. However, neither the parasitemia nor the immunologic parameters evaluated were significantly different compared to the wild type mouse. This could be due to the functional compensation of other galectin members not evaluated, like the galectin-2, a monomeric galectin structurally closely related, but functional redundancy between different galectins has not been demonstrated so far. Another possibility is that the main actions of Gal-1 up-regulation is related to control the mounted immune response, which could lead to potentially damage the host, and then the lack of Gal-1 could not generate any abnormality in the early stages of the infection.

On the other hand, deficient galectin-3 mice presented many immune alterations, as it was evidenced by the higher parasitemia found, confirming the importance of Gal-3 during the host response against *T. cruzi*. Results regarding Gal-3 deficient mice will be discussed below.

3-1 Study of humoral response in galectin-3 deficient mice

In the case of *T. cruzi* infection, polyclonal lymphocyte activation has been proposed as the hallmark of immunological dysregulation during the acute phase of Chagas disease (Minoprio, Itohara et al. 1989). Several studies indicate the importance of Abs for host survival and parasite clearance (Krettli and Brener 1976; Rodriguez de Cuna, Kierszenbaum et al. 1991). In addition, immunoglobulin IgG1/IgG2a balance is associated to a Th1 or Th2 response. High levels of IgG1 are linked to Th2 responses, and predominance of IgG2a to the protective Th1 response. The higher parasitemia observed in Gal-3^{-/-} mice was not due to a different IgG1 or IgG2a production, where no differences were observed.

However, we investigated further the nature of the antibodies produced over the time of infection regarding glycoconjugates epitopes. Gal-3 is able to bind to *T. cruzi* glycans, molecules highly antigenic. So, the humoral response raised against glycan epitopes could be altered in the Gal-3^{-/-} mice. Analysis of the specificity of antibodies to different glycans showed interesting differences. Serum from non-infected healthy mammals showed a set of natural antibodies able to recognize specific glycan structures, and no differences were found in this regard between wild type and Gal-3^{-/-} mice. However, the number of glycan structures recognized by serum from infected mice was highly increased in wild type mice, suggesting the recognition of new glycosylated *T. cruzi* antigens, but most of these glycans were not detected by serum from infected Gal-3^{-/-} mice, and moreover, the most antigenic glycan structures in the case of Gal-3 conserved the core Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc, what is indeed the basic structure of the natural Gal-3 ligands. It is difficult to explain now the origin of this phenomenon. One possibility could be that during infection, host Gal-3 binds to *T. cruzi* glycoconjugates, hiding this sugar epitopes. In the absence of Gal-3, Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAc containing sugars would be exposed, and would generate immunogenic responses. However, many other glycan structures resulted invisible for

the B cell repertoire in the Gal-3^{-/-} mice, suggesting that Gal-3 is a necessary factor for this glycan recognition. We cannot rule out the possibility that some of these glycan structures are exclusively present in the parasite, and it is possible that they could be found in the host glycoconjugates, leading to the suppression or appearance of autoimmune processes. Further studies should be done to explain the nature of this phenomenon, and the possible implications for the resistance against *T. cruzi* infection. The glycan array used is composed by 406 glycans, and this represents a modern technique to evaluate the glycan binding profile of any carbohydrate binding protein. It will be available soon a pathogen glycan array, consisting in a microarray of 96 specific pathogenic bacteria polysaccharides derived from dozens of Gram-negative bacteria, thus allowing the use of this glycanarray for therapeutical diagnosis.

3-2 Study of the Cellular Response in Gal-3^{-/-} Mice

Analysis of the cytokine levels in serum from infected mice clearly shows that during *T. cruzi* infection Gal-3^{-/-} mice mount a misbalanced cytokine profile expression in response to the parasite. Decreased proinflammatory cytokines levels were present in serum from Gal-3^{-/-}. IL-2 was almost absent, suggesting that Gal-3 is necessary to induce IL-2 by T cells. This is in agreement with reported observations, showing that the inhibition of Gal-3 expression by anti-sense oligonucleotides decreases the proliferation of activated T lymphocytes inhibiting IL-2 synthesis (Joo, Goedegebuure et al. 2001). This could be a cause of the diminished levels of T cell-dependent cytokine levels, including Th1 and Th2, IFN- γ , IL-5 and IL-10. However, this cannot be the only explanation, because in *T. cruzi* infection, other cell types appear to be important for this cytokines synthesis, and although a deficient T cell activation could occur in the Gal-3^{-/-} mice, we cannot rule out implications of other cell types, as NK cells and CD4-CD8- T cells, both producers of IFN- γ (Zhang and Tarleton 1996). Similarly, macrophages and B cells are important producers of IL-10, apart from T cells, so any could present a deficient in IL-10 production. However, although is virtually impossible to attribute the decreased seric cytokine levels to a particular immune cell type, the deficient in IL-2, IL-6, IL-5 and IFN- γ suggests T cell abnormalities in the Gal-3^{-/-} mice. However, the observed decrease in cytokines levels is not restricted to any polarized response or cytokine, showing a reduction in the strength of the response instead of its

nature. Since Gal-3 plays important roles in the synapse formation between APCs and T cells (Demetriou, Granovsky et al. 2001; Seminario and Bunnell 2008), phagocytosis and antigen uptake and presentation (Sano, Hsu et al. 2003), Gal-3^{-/-} APCs could show a limited T cell activation capacity, correlating with the absence of IL-1 in serum, a key regulator of the initiation of inflammatory processes produced by APCs during the early stages of infection. Supporting this, Gal-3 potentiates IL-1 production in human monocytes (Jeng, Frigeri et al. 1994) and our results showed that isolated Gal-3^{-/-} DCs presented a strongly decreased IL-1 and TNF- α production after incubation with LPS or *T. cruzi* in the absence of other immune cell types. The late IL-1 boost observed in Gal-3^{-/-} mice at 28 days after infection is likely to depend on other mechanisms apart from the lack of Gal-3, maybe as a consequence of the fully activation of the immune system and network of multiple cytokines.

The dramatically altered seric cytokine levels of Gal-3^{-/-} upon infection could be due to an abnormal immune response, resulting in different total numbers of immune cells subtypes. However, at least in spleen we could not see any difference, neither in total cell number nor in cell composition. Gal-3 mice showed the normal chagasic splenomegaly, lymphocyte activation and proliferation and increase in the number of APCs, so we can conclude that Gal-3 is not related to the changes in spleen physiology related to the infection.

Gene expression profile in spleen confirmed the idea that Gal-3^{-/-} mice develop a reduced response against *T. cruzi*, and it corroborated the changes in galectins expression at protein levels observed by flow cytometry in C57BL/6 mice. In Gal-3^{-/-} mice this regulation was quite similar, only gal-1 mRNA expression was lower in deficient mice at 14 days post infection. This could indicate that no other galectin is assuming the role of the missing Gal-3 in the knock-out model.

In addition, changes in chemokines receptors and integrins were observed both in wild type mice and in Gal-3^{-/-}, with no significant differences between strains, indicating that these processes are independent of Gal-3 or compensated in some way. However, some other genes resulted in an altered expression in the Gal-3^{-/-} mice, mainly related to inflammation and protective immune responses. This is the case of IFN- γ , COX-2 and iNOS. This prompted us to define Gal-3 as a positive regulator of *T. cruzi* mediated inflammation. The deficient inflammatory response observed in Gal-3^{-/-} mice is in agreement with several works showing important inflammatory deficiencies related to Gal-3 like lower granulocyte migration after artificial induction of peritonitis (Colnot,

Ripoche et al. 1998), lower number of infiltrating macrophages (Hsu, Yang et al. 2000), deficient Gal-3 mast cells lower amount of active mediators released by mast cells (Chen, Sharma et al. 2006) and reduced phagocytosis (Sano, Hsu et al. 2003).

Inflammation is of great relevance in Chagasic pathology, because cardiac lesions are consistent with a process of inflammation and myocardial remodeling, which includes T cell/macrophage-rich myocarditis, hypertrophy, and fibrosis with heart fiber damage (Higuchi Mde, Benvenuti et al. 2003). Since inflammation is one of the hallmarks of cardiac lesions, one might speculate about its role in the disease outcome. Study of infected heart in Gal-3^{-/-} mice showed that the parasite burden was similar during the acute phase of the infection, indicating that Gal-3 is not responsible of heart invasion by *T. cruzi*. However, the number of infiltrating host cells in heart tissue was markedly lower in the absence of Gal-3, consisting in lower number of CD4, CD8 T cells and macrophages. In contrast to wild type mice, whose number of infiltrating macrophages goes up at 28 days post infection, in Gal-3^{-/-} mice it is stabilized, and keep at low levels during the infection, showing that Gal-3 is necessary for the recruitment of macrophages to the heart.

Our results are in concordance with other models of study using Gal-3^{-/-} mice. When they have been infected with *S. pneumoniae*, a defect in immune cells migrations to the sites of infection has been reported (Nieminen, St-Pierre et al. 2008), colitis was reduced after *C. albicans* colonization (Jawhara, Thuru et al. 2008) and presented less inflammation in a murine model of asthma (Zuberi, Hsu et al. 2004). Discrepancies have been described by several authors relating the pro-inflammatory role of Gal-3 during schistosome infection (Bickle and Helmbly 2007; Breuilh, Vanhoutte et al. 2007; Oliveira, Frazao et al. 2007).

Our results regarding gene expression in hearts from *T. cruzi* infected mice confirmed the deficient inflammatory response in mice lacking Gal-3. In Gal-3 deficient mice IL-12, TNF- α , IFN- γ and iNOS, outstanding Th1 cytokines were repressed compared to the wild type mice. Controversially, parasite burden was not significantly different from wild type mice. Reduced expression of the chemokine receptors Ccr2 and Ccr5 correlate with the lower number of infiltrating immune cells. However, the differences in the total immune cell number could not be enough to justify the differences observed in Ccr2 and Ccr5 or cytokines gene expression. It is possible that Gal-3^{-/-} mice express less chemokine receptors, what would lead to a deficient cell

migration, but this issue should be study in isolated immune cells. The relevance of Ccr2 and Ccr5 to mount an effective response against *T. cruzi* in heart has been shown by the fact that both knock-out models result to present higher parasite burden in heart (Machado, Koyama et al. 2005; Hardison, Kuziel et al. 2006).

Data from murine models show that inflammatory cytokines play a central role in *T. cruzi* infection. The innate and adaptive immune responses triggered by the parasite and its derived surface molecules, such as GPI anchors, during the acute phase lead to exacerbated production of inflammatory cytokines, including IL-12, TNF- α , and IFN- γ , and chemokines such as CCL3 (MIP-1a), CXCL10 (IP-10), and CCL5 (RANTES) (Teixeira, Gazzinelli et al. 2002). Now, Gal-3 might be considered as an inductor of this processes, and the lack of Gal-3 results in a decreased cardiac inflammation. Further experiments to evaluate cardiac damage will help to explain whether the origin of Chagas cardiopathy is produced by host self-targeted responses or by the persistence of the parasite, taking advantage of a system with the same parasite burden, but a strongly decreased inflammatory process.

The origin of this defective inflammation could be related to the ability of Gal-3 to bind glycans present on the *T. cruzi* surfaces. Acting as a PRR, Gal-3 could trigger activation signals in immune cells in order to mount the immune response. Thus, APCs responsible of initiate the immune response would be less efficient due to the lack of one of their PRR. This could explain why TLRs are highly up-regulated in Gal-3^{-/-}, as a possible compensation process to cover the lack of a functional related protein, in this case Gal-3. Unfortunately, the anti-TLRs available antibodies did not work properly for protein detection in western blotting, and we do not know whether the increase in mRNA of TLRs correlates with higher protein expression.

3.3- Deficient Galectin-3 Dendritic Cells

DCs possess special features that allow them to act as APCs and are central in the initiation and development of immunity and tolerance. Immature DCs capture and process Ag via their high endocytic activity and undergo a process of maturation and activation after recognition of PAMPs present in microorganisms by PRRs. In addition to the well known TLRs, a vast array of extracellular and intracellular non-TLR innate receptors exists in DCs (Sabatte, Maggini et al. 2007), each one adapted to recognize specific pathogens and therefore lead to an appropriate immune response. Besides, it has

been reported that Gal-3 and its ligands are up-regulated in DCs after *T. cruzi* infection with functional consequences on their capacities of adhesion and migration (Vray, Camby et al. 2004), and changes in glycosylation patterns after maturation have been confirmed (Bax, Garcia-Vallejo et al. 2007), highlighting the importance of glycans and CBPs in DCs mediated responses. Thus, the lack of Gal-3 in DCs could lead to insufficiencies related to *T. cruzi* recognition, and related to intracellular signaling into the DCs. Thus, DCs are a reasonable candidate to explain the altered immune response observed previously in Gal-3^{-/-} mice.

Gal-3^{-/-} DCs showed a normal differentiation process from bone marrow precursors, so it seems that Gal-3 is not necessary for DC differentiation *in vitro*. It is not probably neither that Gal-3 is implicated in DCs maturation, because LPS treatment and *T. cruzi* infection induced properly the expression of the activation markers CD80 and CD86. We observed that *T. cruzi* was able to infect the wild type DCs *in vitro*, supporting in intracellular replication, as previously reported (Van Overtvelt, Vanderheyde et al. 1999). However, we expected that Gal-3^{-/-} DCs were more resistant to infection, because it was shown that Gal-3 mediates interaction of *T. cruzi* with host cells *in vitro*. Surprisingly, Gal-3^{-/-} DCs showed higher parasite adhesion and led to an augmented trypomastigotes release to the medium. This contradictory data can be easily explained, because of the higher expression of Gal-8. Experiments *in vitro* showed that Gal-8 is a much stronger adhesion promotor than Gal-3, what can explain the enhanced adhesion of *T. cruzi* to Gal-3 knock-out DCs. However, this does not imply that Gal-8 can compensate the functions of Gal-3. We have seen *in vitro* that ligands of Gal-8 in *T. cruzi* are different from those of Gal-3, and meanwhile Gal-3 forms lattices, Gal-8 tends to cross-link receptors. So, intracellular signals triggered by Gal-3 and Gal-8 may be completely different. In fact, Gal-3^{-/-} DCs showed a strong deficiency in the production of IL1 and TNF- α in response to LPS and *T. cruzi*. This could initiate an inadequate immune and inflammatory response, as it was observed in the *T. cruzi* infection of Gal-3^{-/-} mice. Secretion of other pivotal cytokines were not affected by the lack of Gal-3, like IL-12 and IL-6, meanwhile the anti-inflammatory IL-10 production was significantly increased after *T. cruzi* infection. It seems that Gal-3^{-/-} DCs show a Th-2 polarized phenotype, and the increase of IL-10 together with the decreased TNF- α levels reminds to the function of recently described regulatory DCs. What it is clear is that the absence of Gal-3 affects surface receptors expression somehow, because DCs result unresponsiveness to LPS and *T. cruzi*. It is quite surprise that Gal-3^{-/-} do not

produce TNF- α after LPS-challenge. Our data are contradictory to other reports, showing that Gal-3^{-/-} DCs secrete normal amounts of TNF- α when they were incubated with LPS (Breuilh, Vanhoutte et al. 2007). Differences could be attributed to different LPS serotype or concentration.

In an attempt to find out the cause of the lack of TNF- α production, we checked TLRs expression on DCs surface, since it is well reported that LPS is recognized by TLR2 and 4, and that TLR2 and TLR9 recognize GPI anchors and parasite DNA, triggering MyD88 and TRIF-dependent intracellular signals (Tarleton 2007)

Wild type DCs showed an up-regulation of TLRs after *T. cruzi* infection as expected. In the case of Gal-3^{-/-} DCs, TLR2 and TLR9 were up-regulated in a similar way to the wild type DCs, but contrary to the wild type model, surface expression of TLR1 was almost missing in Gal-3^{-/-}. TLR1 has not been implicated in *T. cruzi* recognition, but it is relevance because, in order to trigger intracellular signals, TLR2 may work as a dimer, either with TLR1 or TLR6. In our model, TLR2 could form dimmers with TLR6, but the heterodimer TLR1-TLR2 is not present. This could explain the absence of TNF- α and IL1 production, meanwhile IL-12 or IL-6 is produced normally by Gal-3^{-/-} DCs and IL-10 resulted increased. This points out that in DCs Gal-3 is modifying but not suppressing the profile of immune receptors.

We still do not know much about the relation between Gal-3 and TLR1-TLR2 complex. It is possible that the problem is related to internalization and recycling to the membrane of receptors. Lattice formation following the binding of galectins to complex N-glycans on the cell surface effectively traps glycoprotein receptors on the cell surface, preventing their endocytosis, and regulating distribution of cell surface receptors as well as the cell responsiveness to receptor agonists. This could be the case of Gal-3^{-/-} DCs and TLRs. TLRs are glycosylated proteins, thus potential ligands of Gal-3. It has been reported a direct interaction between Gal-9 and TLR3, but no other one has been demonstrated so far. This would allow getting insight in the process. Other possibility is that Gal-3 collaborates with TLR2 and TLR1 to form a complex, all of them recognizing *T. cruzi*. This would support the role of Gal-3 as a danger molecule. Gal-3 is released in response to pathogens, and its interaction with *T. cruzi* and TLRs complex would indicate to the innate system the pathogenic nature of the microbe. In the absence of Gal-3, there is a lack of the danger signal that keeps the innate system in an anergic

status. It can be possible that another unknown molecules link Gal-3 to TLRs. It has been shown the existence of a Gal-9 ligand termed Tim-3 (T cell immunoglobulin mucin-3) that synergizes with TLRs to promote TNF- α on DCs (Anderson, Anderson et al. 2007). Whether Tim-3, or other Tim member, work together with Gal-3 to induce TNF- α production is an interesting question that may be answered by further experiments. Finally, Gal-3^{-/-} mice reduced TNF- α production in response to *C. albicans* in a way dependent of TLR2 (Jouault, El Abed-El Behi et al. 2006), in agreement with our observations.

4- Final remarks

Understanding the sugar code is a major challenge for immunologists and will be critical to support the design of rational therapeutical approaches aiming at manipulate inflammatory or autoimmune disorders. These therapeutic approaches will be aimed at blocking the formation of specific glycans, controlling the expression of specific glycan processing enzymes or interfering with glycan-CBP interactions. .The glycomic era should emerge close to the development of effective tools to understand the glycobiology.

The present work shows how galectins can control and address the relation between the parasite and the host, acting as decoders of the information contained in *T. cruzi* and host glycans in a bidirectional way, and dictating the fate of the innate and subsequent immune response. However, this is only the first step towards the complete understood of the relevance of galectins during Chagas disease, and by extrapolation, during other infectious, autoimmune or tumorigenic processes.

The advance in the field of glycobiology may mark the beginning of a new era of therapeutic strategies, and it could be possible the use of sugar-binding proteins or their antagonists for the treatment of inflammatory and immunological disorders.

CONCLUSIONS

- 1- Human recombinant galectin-1,-3,-4,-7 and -8 are able to bind *T. cruzi* in a carbohydrate recognition domain dependent way. The affinity of galectins for *T. cruzi* surface ligands is much higher for infective forms, amastigotes and trypomastigotes, than for the non-infective form. The interaction of galectins with *T. cruzi* promotes parasite adhesion to host cells *in vitro*.
- 2- *T. cruzi* expresses proteases that are able to process galectin-4, -8, and -3; Zn-metalloproteases cleave galectin-4 and -8 in the linker peptide region, and galectin-3 is processed by the action of two different proteases, a Zn-metalloprotease and a collagenase.
- 3- The inhibition of the proteases present in *T. cruzi* responsible of galectin processing leads to direct killing of the parasite by recombinant human galectins.
- 4- During experimental infection with *T. cruzi* in C57BL/6 mice, presence of galectin-1,-3 and -9 were increased in T cells, B cells increase galectin-3, and-9 and decrease galectin-8 expression, and galectin-1,-3, and -9 were constitutively expressed in macrophages and dendritic cells. Cardiac cells increase the expression of galectin-1, and galectin-3 is strongly expressed by infiltrating CD68+ macrophages.
- 5- In murine the model genetically deficient for galectin-3, the infection courses with higher parasitemia than wild type controls, correlating with impaired seric IL-2, IL-1, IL-5, IFN- γ and IL-10 levels.
- 6- The profile of anti-glycan antibodies in response to *T. cruzi* is totally different in galectin-3 deficient mice compared to the wild type model. The main difference is related to the generation of antibodies able to recognize the glycan Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc in the deficient model, structure that results not immunogenic for wild type infected mice.
- 7- Galectin-3 deficient mice show a deficient inflammatory and immune response during *T. cruzi* infection, show a diminished expression of IFN- γ , COX-2 and iNOS genes in spleen and heart in response to the infection and reduced a ability to recruit macrophages and T cells to the heart, with no significant changes in the parasite burden in heart compared to wild type animals.
- 8- The absence of galectin-3 does not affect dendritic cell differentiation and activation *in vitro*, but it is responsible of the reduction of TNF- α and IL-1 in response to LPS and *T. cruzi*, through a mechanism involving TLR2-TLR1 heterodimer surface formation.

CONCLUSIONES

- 1- Galectinas recombinantes humanas 1,3,4,7 y 8 son capaces de unir *T. cruzi* de modo dependiente del dominio de reconocimiento a carbohidrato. La afinidad de las galectinas recombinantes es mucho mayor por formas infectivas del parásito, amastigotes y trypomastigotes, que por las formas no infectivas. La interacción de las galectinas con *T. cruzi* promueve adhesión a células hospedadoras in vitro.
- 2- *T. cruzi* expresa proteasas capaces procesar galectina-2, 4 y 8; Zn-metalloproteasas cortan las galectinas 4 y 8 en la región del linker peptídico, y la galectina-3 es procesada por la acción de dos proteasas diferentes, una Zn-metalloproteasa y una colagenasa
- 3- La inhibición de las proteasas presentes en *T. cruzi* responsables del procesamiento de galectinas conduce a la muerte del parásito por galectinas recombinantes
- 4- Durante la infección experimental por *T. cruzi* en ratones C57BL/6, se incrementó la presencia de galectinas 1,3 y 9 en células T, células B incrementaron expresión de galectina-3 y 9 mientras que redujeron la expresión de galectina-8, y las galectinas 1,3 y 9 son constitutivamente expresadas en macrófagos y células dendríticas. Células cardíacas aumentan la expresión de galectina-1 durante la infección, y galectina-3 es expresada por macrófagos infiltrados en el corazón de ratones infectados
- 5- En el modelo murino genéticamente deficiente en galectina-3, la infección cursa con una elevada parasitemia que en los ratones salvajes, correlacionándose con una producción desajustada de IL-2, IL-1, IL-5, IFN- γ and IL-10 en suero.
- 6- El repertorio de anticuerpos anti-glicanos generados en respuesta a *T. cruzi* es totalmente diferente en ratones deficientes en galectina-3 comparado a ratones control salvajes. La principal diferencia está relacionada con la generación de anticuerpos capaces de reconocer el glicano Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc en el modelo deficiente, estructura que no resulta antigénica en el modelo salvaje.
- 7- Ratones genéticamente deficientes en galectina-3 muestran una respuesta inmune e inflamatoria deficiente durante la infección por *T. cruzi*, una expresión disminuida de los genes IFN- γ , COX-2 and iNOS en hígado y corazón en respuesta a la infección y una capacidad reducida para reclutar macrófagos y células T al corazón, sin diferencias significativas en la carga parasitaria
- 8- La ausencia de galectina-3 no afecta a la diferenciación y activación de células dendríticas in vitro, pero es responsable de la reducción de TNF- α y IL-1 en respuesta a LPS y a *T. cruzi*, a través de un mecanismo que implica la formación de heterodímeros TLR1-TLR2

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