January 2017

Immune Regulatory Mechanism And Myeloid Cells Functions In Brain Parasite Infection

Fredice Quenum Zangbede

IMMUNE REGULATORY MECHANISM AND MYELOID CELLS FUNCTIONS IN BRAIN PARASITE INFECTION

by

Freddie O. Quenum Zangbede, PhD
Bachelor of Science, North Dakota State University (Fargo), 2010

A Dissertation
Submitted to the Graduate Faculty

of the

University of North Dakota

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota
December
2017

This dissertation, submitted by Fredice O. Quenum Zangbede, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Bibhuti Mishra, Chairperson

David Bradley

Othman Ghribi

Jyotika Sharma

John A. Watt

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Grant McGimpsey, PhD
Dean of the Graduate School

December 5, 2017
Date

PERMISSION

Title: Immune regulatory mechanism and myeloid cells functions in brain parasite infection.

Department: Microbiology and Immunology

Degree: Doctor of Philosophy

In presenting this dissertation in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my dissertation work, or in his absence, by the Chairperson of the department or the dean of the School of Graduate Studies. It is understood that any copying or publication or other use of this dissertation or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my dissertation.

Fredice O. Quenum Zangbede
December, 2017

Table of Contents

LIST OF FIGURES

Figures

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my adviser and the other members of my committee for the guidance and support during my time in the doctorate program at the University of North Dakota

ABSTRACT

Neurocysticercosis (NCC) is the most common parasitic infection of the central nervous system (CNS) caused by the helminth parasite *Taenia solium* (*T. solium*) and affects 50-100 million people worldwide. Its clinical symptoms include headache, seizures, hydrocephalus, epilepsy and stroke. These clinical symptoms can have life-long detrimental effects. As many as half of the total adult-onset seizures and 10% of the stroke cases in the endemic areas are attributed to NCC, whereas 20% to 30% of symptomatic patients display hydrocephalus. Treatment of NCC remains a major challenge as the severity of the symptoms is thought to be elicited by the degenerating larvae resulting from therapeutic treatment or normal attrition. Specifically, the inflammatory response in the CNS detected during symptomatic phase is thought to be responsible for the neuropathology and underlining cause of clinical symptoms. Interestingly, NCC has a long asymptomatic phase, typically lasting 3-5 years before the onset of the symptomatic phase. This asymptomatic phase is characterized by little or no signs of inflammation detected around the live cysts. Thus it is commonly believed that viable cysticerci induce immune suppressive effects, and loss of these effects when the parasite dies likely leads to activation of uncontrolled inflammatory response and neuropathology. Therefore, treatments with anti- parasitic drugs combined immunosuppressive/ anti-inflammatory factors such as

corticosteroids are predominantly used to control the host immune inflammatory response. However, their longtime use can cause other problematic side effect. The characterization of the immunoregulatory mechanisms in the CNS during helminth parasitic infection is critical to develop novel therapeutic targets for NCC, which can aid in finding treatment of a plethora of chronic neuro-inflammatory diseases as inflammation plays central role in development of majority of the chronic CNS inflammatory/autoimmune diseases.

The severity of the symptoms is associated with the intensity and chronicity of the local immune response in the CNS, use of a mouse model is essential to perform kinetic of analysis of the entire infection process. Because *T. solium* is not infectious to mice, use of *Mesocestoides corti* (*M. corti*), a relative cestode, has allowed for a more controlled study of the entire infection process, and to better characterize the immune response in the CNS microenvironment. Importantly, analysis of the infected CNS in the murine NCC model paralleled results obtained with brain specimens from NCC patients showing release of glycan antigens that are uptaken by host immune cells in the parasite infected CNS of humans and mice. In this regard, multiples researchers had indicated that glycans from pathogens, including helminths, act as pathogen-associated molecular pattern molecules (PAMPs), which are knowns to be recognized by C-type lectin receptors (CLRs) through their carbohydrate recognition domains (CRDs) and function as important pattern recognition receptors (PRRs). We, therefore, hypothesized that *M. corti* larvae in the CNS release their antigens glycoconjugates molecules which induce the expression of specific

lectin receptors (LRs) such as galectins. The LRs may play important role in regulating the immune response and pathology during NCC.

Parasites, particularly helminths, tend to shift the balance of immunity to a Th2 type of response, and it is likely that Th2 immunity evolved in response to infections with these parasites. The Th2 response activates or expands alternatively activated macrophages (M2 macrophages) which are associated with suppressive activity. The development of M2 macrophages is thought to be crucial in establishment of chronic parasitic infections as they express various negative signaling accessory molecules to downregulate the proliferation of activated T cells, and promote healing and tissue remodeling (1-3). In this regard, previous studies involving the mouse model of NCC showed the presence of macrophages with M2 phenotype in the CNS microenvironment. Moreover, a reduction in CNS levels of M2 macrophages in STAT6$^{-/-}$ mice undergoing NCC is correlated with widespread tissue damage and greater mortality (4). This is exciting as M2 macrophage mediated anti-inflammatory/ neuro protective functions could be important in nervous tissue repair as well as containment of neuropathology in myriad neuro-inflammatory diseases. However, the specific role of these M2 cells during NCC infection still remind to be determined.

The tegument in helminths such as *T. solium* and *M. corti* is dynamically responsive to changing host environments or immune attack in the CNS. During CNS infection of both these parasites, glycan antigens from the rapidly released in the CNS. Interestingly the

glycan antigens containing terminal galactose and galactosamine (detected by the lectin IB4) is rapidly released (1-2d p.i.), and it is almost absent after 1 wk of infection. This was correlated with differential upregulation of the Galectins (Galectin-3, -7, and -9), a family of the galactose specific LRs, in the CNS during murine NCC. The aim of the studies is to study the specific roles of galectin 3, galectin 7, and galectin 9, in regulation of immune functions in the CNS microenvironment during murine CNS infection.

Here we report that M2 macrophages in the parasite-infected brain display abundant galectin-3 expression. Disease severity was increased in Galectin $3^{-/-}$ mice correlating with increased neurological defects, augmented cell death and, importantly, massive accumulation of neutrophils and M2 macrophages in the CNS of these mice. Because neutrophil clearance by efferocytosis is an important function of M2 macrophages, we investigated a possible role of galectin 3 in this process. Indeed, Galectin 3 deficient M2 macrophages exhibited a defect in efferocytic clearance of neutrophils in-vitro. Furthermore, adoptive transfer of M2 macrophages from Galectin 3 sufficient WT mice reduced neutrophilia in the CNS and ameliorated disease severity in parasite-infected Galectin $3^{-/-}$ mice. Together, these results demonstrate for the first time a novel role of galectin 3 in M2 macrophage function in neutrophil turnover and resolution of inflammatory pathology in the CNS. This likely will have implications in neurocysticercosis and neuroinflammatory diseases.

Analysis of helminth-infected mice revealed an abundant expression of Galectin 7 (Gal 7) that recognize galactose moieties in brain endothelial cells. The Galectin $7^{-/-}$ NCC

mice displayed a reduced accumulation of M2 macrophages in the parasite-infected brain. This reduction in M2 macrophage numbers in the CNS was correlated with an increased neuropathology and shortened survival. The use of adoptive transfer of fluorescently labeled cells demonstrated a decreased influx of M2 macophage into the CNS of helminth-infected NCC brains. The use of in-vitro transmigration assay conclusively demonstrated a key function of Galectin 7 expressed on endothelial cells in centrally regulating M2 macrophage entry into the CNS. The results are highly significant as the BBB restricts the flow of both cells into the brain, and the fact that galectin-7 regulates M2 macrophage extravasation into the CNS could be of great importance how to contain neuropathology in CNS infection.

Galectin 9 was the first to be upregulated as well as at a relatively high level in M2 macrophage. Parasite-infected Galectin $9^{-/-}$ mice displayed significantly increased susceptibility to parasite infection, despite a similar parasite burden in the CNS compared to infected WT mice. The accumulation of macrophages, γδ T cells, αβ T cells, or B cells in the brains of parasite-infected Galectin $9^{-/-}$ mice was similar to the WT mice. Moreover, AAMs which play a profound protective role in murine NCC were observed an increased level in infected WT and Galectin $9^{-/-}$ mice. Instead, the parasite-infected Galectin $9^{-/-}$ mice exhibited a massive neutrophilia in the CNS and an increase in neutrophil-associated neuroinflammation. These results suggest that galectin 9 may be involved in controlling and resolving neutrophilia and inflammation, however, not by efferocytosis. For the first, we showed in the possible correlation between the presence of Galectin 9 and neutrophilia

in NCC infection. Further experiments need to be done to determine the specific mechanism by which Galectin 9 is involved in this process.

In toto, our studies make an important contribution in establishing the novel protective mechanisms of M2 macrophages functions in the CNS pathological conditions. Our studies provide a better understanding of the immunopathogenesis of NCC which should lead to new therapeutic strategies in other CNS inflammatory disorders as well.

CHAPTER I

INTRODUCTION

Neurocysticercosis: Clinical Symptoms, Epidemiology, and Burden to Society

Neurocysticercosis (NCC), is the most common parasitic infection of the Central Nervous System (CNS) caused by the larvae stage metacestode of the zoonotic tapeworm *Taenia solium* (9-11). The most frequent clinical manifestation associated with NCC are seizures, hydrocephalus, strokes, and symptoms associated with increased intracranial pressure (12, 13). Particularly, strokes, seizures and late-onset epilepsy associated with NCC have devastating effects on the well-being of infected people and socio-economic development in affected communities (12). NCC accounts for 70-90% to seizures cases and 4%-12% of the total stroke case in endemic areas (12, 14, 15)

NCC is considered as a major public health problem because it contributes to more than 50,000 deaths worldwide every year (12, 14-17). The incidence of NCC is most common in developing countries of Asia, Africa, and Latin America, where this disease is considered to be endemic (17). NCC used to be rare in developed countries. However, the recent increase of travel to endemic areas as well as immigration from endemic places likely have contributed to the ever increasing incidences of NCC reported in developed countries (18, 19). In the United States, NCC is mostly found to be prevalent in a Hispanic population. More than 1000 new cases are diagnosed every year in the Southwest region

of the USA, where NCC account for more than 2% of neurological admission.

Life Cycle of Taenia Solium

The life cycle of *T. solium* is complex, which involves a larval stage (metacestode) and an intermediate host (pig) and human as the only known definitive host (Figure. I-1) (17-20). NCC is transmitted to humans through ingestion of contaminated food, mainly by eating raw/under-cooked larvae infected pork meat. Upon ingestion, metacestode reaches the small intestine and attach to the intestinal wall. The adult worm develops in the small intestine by forming segments called gravid proglottids. The eggs in these proglottids are self-fertilized and released into the environment, where they can survive for many months before entering another host. Pigs ingest *T. solium* eggs contaminated food or water in endemic areas. Once the eggs reached the intestine of the pigs, they transformed to an invasive form called oncospheres. Oncospheres can cross the intestinal wall and reach different tissues of the host through the body fluid. In the tissues, they transform into cysticerci in a period of 3 weeks to 2 months (12, 14, 16, 17). The infection cycle continues when the humans eat undercooked pork infected with *T. solium*.

In human's small intestine, embryo releases the oncospheres due to the action of gastric juices. The oncospheres hatch and cross the intestinal mucosa. They can migrate and established in muscles, subcutaneous connective tissues, CNS, eyes, liver and other organs, where they mature into cysticerci and cause pathogenesis. To migrate to striated muscles, liver, and other tissues, where they develop into cysticerci. In humans, when the parasite lodges in the CNS, the cysts can cause serious illness resulting in neurocysticercosis. (Figure. I-1).

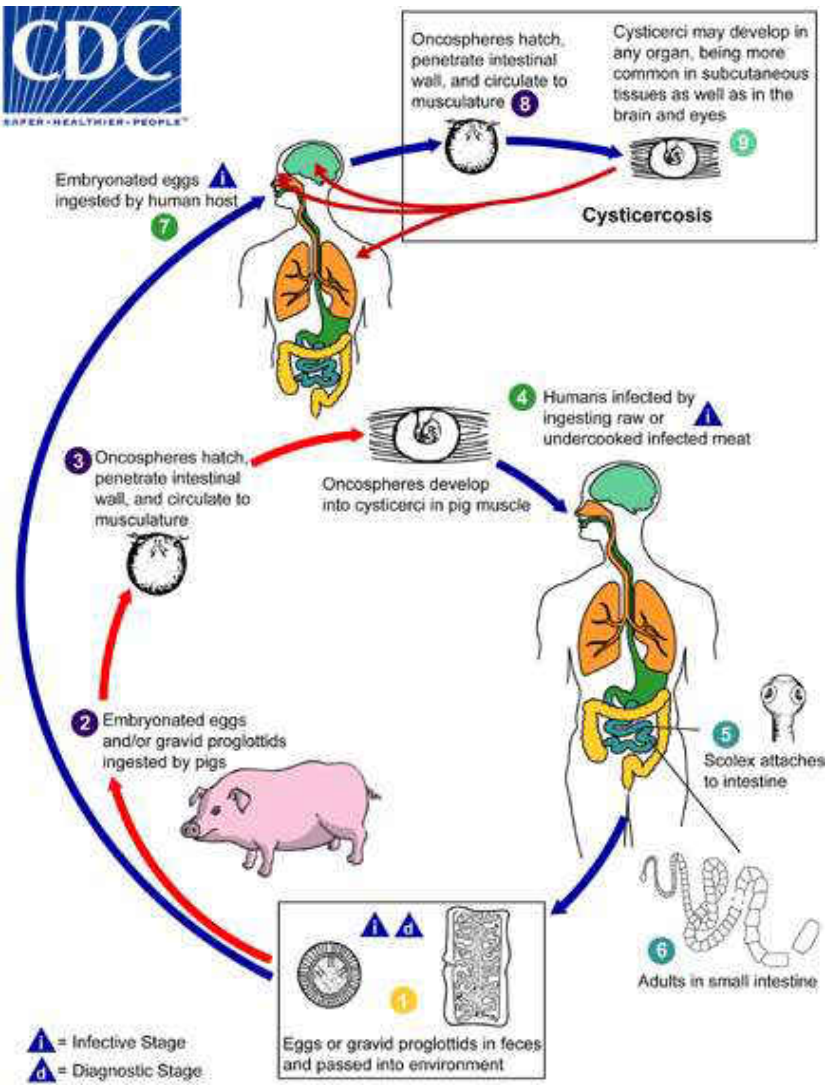

Figure I- 1. The life cycle of *Taenia solium*.

Neurocysticercosis is the result of the ingestion *Taenia solium*'s cyst. Human becomes infected either by ingesting contaminated food or autoinfection. In the cause of autoinfection, cysts are passed to the environment by an infected human and re-enter the infected body where they migrate to the central nervous system (CNS). Once in the brain, the eggs differentiate into oncosphere which matures into cysticerci which into neurocysticercosis. In the other hand, eggs can also contaminate vegetation in the environment where they last for months until, a pig which is the intermediate host, feed into it and become infected. Inside the animal, eggs migrate into the intestinal wall where they differentiate into oncosphere and migrate the muscle where they mature into cysticerci. Human becomes infected by eating contaminated meat where the cysticerci migrate to the brain and induce neurocysticercosis**.**

Type of Neurocysticercosis

Inactive Neurocysticercosis: Inactive NCC represents the cases in which only cysts or only trace residues of the cysts are visible by neuroimaging studies. Parenchymal calcifications are the most common form of inactive NCC. Some patients with inactive NCC reveal the presence of hydrocephalus (14).

Active intra-parenchymal Neurocysticercosis*:* Intra-parenchymal is the most common form of the disease, which involves the parenchyma of the cerebral hemispheres. Presence of lesion in the parenchyma commonly detected, particularly at the gray matter-white matter junction. Intraparenchymal NCC patients present seizures in most patients as well as headaches and hypertension (12, 20). Many patients with intraparenchymal NCC may resolve the infection in few years (12, 20). Whereas many others develop cysticercal encephalitis, another form of intra-parenchymal NCC present in young adolescents. Untreated patients are more likely to develop severe neurological disorders

Extra-parenchymal neurocysticercosis

Ventricular neurocysticercosis: About 10 to 20% of symptomatic patients will develop this type of NCC. The cysts are predominantly found in the ventricles and associated with the obstruction of the cerebrospinal fluid (CSF) flow (12, 20). Whereas minor percentage of patients with *subarachnoid cysticerci* display meningeal signs due to the presence of cyst(s) in the subarachnoid spaces (12, 20).

Pathogenesis of NCC

The intensity of the immune response is thought to play the most significant role in the pathogenesis of NCC. The disease severity also is depended on other factors, including

the number of parasites, the stage of the parasite, size, location in the CNS, and duration of infection (21).

Duration of infections:

Duration of parasite infection of the CNS is a critical determinant of NCC severity and disease outcome. The infection process in the CNS of NCC patient can be broadly defined in three different phases. In the first phase of infection, the parasite thought to enter the CNS and establish itself by evading the host immune responses. However, direct evidence regarding the initial clinical symptoms and immune responses in the CNS microenvironment is minimal. The second phase is the asymptomatic phase typically lasting from 3 to 5 years after infection of the CNS. During the asymptomatic phase, the host does not display any clinical symptoms thought to be due to viable cysticerci induce immune suppressive effects. This phase is characterized by little or no signs of inflammation detected around the live cysts (5, 7, 8, 14, 17). Because of technical limitations involved, the immunopathogenesis of asymptomatic phase is primarily established by analyzing the nervous tissue of patients who have died due to other co-morbidities other than the disease itself. The third phase is the symptomatic phase in which the parasite degenerates and releases parasitic molecules into the surrounding tissue causing a wide range of clinical symptoms caused by the resulting inflammatory responses of the host (14). Indeed, it is commonly believed that the viable cysticerci induce immune suppressive effects and loss of these effects when the parasite dies likely leads to activation of pathogen resulting in uncontrolled inflammatory response and neuropathology (5, 7, 8).

Stage of the Cysts

Previous studies using magnetic resonance imaging (MRI) and computed axial tomography (CT) had shown parasites go through different stages after entering the CNS (12, 14, 22). The first state is the vesicular stage, where the live parasites induce immune suppressive/ regulatory effects with little to none inflammatory response detected in the CNS microenvironment (18, 20). The second stage is the colloidal cysticerci, where there is a presence of gelatinous material in the cyst fluid. During this stage, the parasite is in the dying process and surrounded by a thick collagen capsule (21-24). The third stage is the granular nodular stage in which the cyst starts to contract, and the walls are transformed into coarse mineralized granules followed by necrosis (21-24). The last stage is the nodular calcified stage when the cyst has died and collagenous structures are formed by calcification of the cyst (21-24).

Immune response in patient undergoing NCC due to helminth infection of the CNS

General and immunological features to helminth infections

Helminths parasites are multicellular eukaryotes that often cause acute and chronic pathologies. The mechanisms of host defense that control microbial infections are broadly divided into two categories, innate and adaptive immune responses. Innate immune responses represent the first line of host defense and constitute a multifaceted and rapid response to pathogens. It is induced by the interaction of pattern recognition receptors (PRRs) on host cells with conserved pathogen-associated molecular patterns (PAMPs) (25). Such interactions result in the development of host Th1 type inflammatory responses that play an important role in the elimination of the organism (25, 26). However, helminth parasites have evolved to co-exist in the host and induce compensatory responses often

leading to the inability of the host to eliminate the parasite (25-27). Parasites, particularly helminths, tend to shift the balance of immunity to a Th2 type of response.

Immunological features in patients with neurocysticercosis:

In humans for a long period of time after the infection of the CNS, the host remain asymptomatic. The direct evidence on the kinetic of host-pathogen interaction in the CNS microenvironment involved in the sequential progression from asymptomatic to symptomatic stage remains to be clearly understood. However, correlative studies strongly suggest that the asymptomatic stage is associated with the presence of the live parasites in the CNS. As the absence of clinical symptoms in infected people coincides with a lack of detectable inflammatory response in the CNS, it has been assumed that the viable cysticerci induce immune suppressive effects to evade the immune response (4, 28). A number of immunomodulatory mechanisms the live parasites use to invade the host immune system include suppressing lymphocyte activation (27, 29). *T. solium* inhibits both the classical and alternative pathways of complement activation by the release of the molecules paramyosin (binds to C1q) and taeniaestatin (30, 31). Lymphocyte proliferation and inflammatory cytokine production are mitigated *ex vivo* (32). During this asymptomatic phase of infection, even the PBMCs stimulated with *T. solium* antigens produce T helper (Th2) 2 cytokines such as IL-4, IL-5, and IL-13 (33), suggesting an anti-inflammatory response prevails during the symptomatic phase. These live parasite-mediated immune suppressive/ anti-inflammatory mechanisms could be critical for parasite survival and to co-evolve with the host to establish chronic infections. In contrast, the analysis of CNS tissue specimens from NCC patients reveals that the immune response consists of an overt Th1 phenotype (32) or a mixed Th1, Th2, and Th3 phenotype depending upon the absence

or presence of granuloma formation (10, 23). Moreover, the Th1 cytokines are detected in higher concentration in the cerebral spinal fluid (CSF) of symptomatic NCC patients vs. asymptomatic patients (32). Although direct evidence that the Th1 inflammatory response contributes to the neuropathology is limited, treatment of NCC patients with corticosteroids help to control Th1-inflammatory response along-with neuropathology and disease severity (10, 32). However, the appropriate immune responses that are required to control the infection or the CNS inflammatory pathology in human patient are largely unknown. In this regard, the majority of studies in NCC are focused on T-cell mediated immune responses, possibly due to the presumed role of Th1 cells on the immunopathogenesis. Nevertheless, macrophages around the metacestode in the CNS of humans with symptomatic NCC display a low expression of MHC-II molecules, although it appears to be higher in cells located further away from the parasite (34). Indeed, Downregulation of MHC-II in macrophages by regulatory T cells (Tregs) and Th2 cells have been shown to play an important role in establishing chronic helminth infections with limited inflammatory pathology in their hosts (27). Owing to the critical roles of macrophages and microglia in both innate immune and adaptive immune effector pathways in the CNS, identifying their phenotypes and their associated functions in NCC is essential for understanding the disease process.

Murine Model of NCC

Examining brain specimens from patients that have undergone emergency craniotomies, have been critical to characterize the immune response in the CNS microenvironment in NCC and the associated pathology. As the severity of the symptoms is associated with the intensity and chronicity of the local immune response, use of a mouse

model is essential in order to allow for a kinetic analysis of the infection process in the CNS with a more controlled experimental setup. Attempts to develop animal models using *T. solium* to mimic host-pathogen interaction have failed in several laboratories as it does not infect the CNS naturally. Other Taenias such as *T. crassiceps* do not infect the CNS of mice through the fecal-oral route as occurs naturally during *T. solium* infection in human. Attempts to develop a CNS infection by intra-cranial injection of these parasites failed in many laboratories as the metacestodes displaced most of the nervous tissue due to their large size. Although *T. solium* infects the CNS naturally in pigs, the approach is not cost effective. Moreover, it is difficult to do a mechanistic study in infected pigs in laboratories setting to understand the kinetics of immunopathological events in the CNS during the entire course of infection (35). Many other laboratories had used the artificial infection caused by *Taenia crassiceps* cysticerci which is injected into the abdominal cavity of mice. This model has been useful to understand the impact of immune, sexual and endocrine factors on the disease, and to study the efficacy of anti-parasitic drugs for cysticercosis. However, it does not allow to study NCC (35, 36) as the intraperitoneal environment barely resemble human CNS condition (35).

We and Judy Teale's laboratory at the UTHSCSA/ UTSA have been using another closely related cestode, *Mecestoides corti* (*M. corti),* with a known life cycle similar to *T. solium*, to study the kinetic analysis of the events in CNS microenvironment in nice. The life-cycle of *M. corti* involves two hosts. The parasite is believed to ingested by terrestrial arthropods. An intermediate host such as mouse and lizard consume arthropod carrying *M. corti* (37, 38). Once inside the animal, the oncosphere develops into mature larvae. A definitive host such as dog, cat or skunk, consume the larvae, intermediate host. The larvae

develop into mature tapeworm releasing eggs in the definitive host for the life-cycle to continue (38). Owing to these remarkable similarities in life cycles of both *M. corti* and T. *solium*, murine model of NCC was developed by injecting *M. corti* intracranially. In laboratory, *M. corti* metacestodes are maintained by serial intraperitoneal inoculations of mice. Murine NCC is developed by injecting *M. corti* metacestodes i.c. in 3-5 wk old mice at the junction of the superior sagittal and the transverse sutures to avoid penetration of the brain tissue. During the first 24 hrs of infection, most of the metacestodes are found in the leptomeningeal space. Depending upon the duration of the infection and the type of host responses elicited, the distribution of *M. corti* metacestodes parallels that of *T. solium* as the parasite localizes in the subarachnoid space, ventricular areas and parenchyma (9, 38). In murine NCC, the first cells to infiltrate the brain are large numbers of macrophages, and T cells followed to a lesser extent by neutrophils, T cells and B cells. However, as observed in symptomatic NCC patients, the cytokine response of infiltrating cells in the CNS during *M. corti* infection is a mixed Th type response with higher IL-2, IL-12, IL-15, IFN-γ, and TNF-α indicative of a Th1 type of response (4, 9, 32, 39, 40), along with IL-4, IL-13 and IL-10 (Th2 type) detected in the CNS albeit at a relatively lower amount. Most importantly, similar to human NCC, the degree of inflammation in the CNS increases correlates with disease severity (4, 9, 32, 39, 40). Thus, murine NCC provides an important model for understanding the human disease which should aid in understanding the kinetics of the immunopathological events in the CNS microenvironment and the development of new therapeutic strategies.

Similar to human NCC, the degree of inflammation in the CNS correlates with the disease severity in murine NCC (10, 41). In these mice infected i.c. with *M. corti*, the Th1

cytokine response in the CNS microenvironment is mounted by γδ T cells (10, 42, 43). Use of γδ $T^{-/-}$ mice revealed that the inflammatory response by them contribute to infiltration of immune cells into the brain (30, 39) and enhance neuropathology and disease severity (10, 30). Thus, in the chronic stage of both human and murine neurocysticercosis, manifestation of the TH1 inflammatory response is the main cause of disease severity and mortality. Importantly, the murine NCC provides an important model for understanding the human disease which should aid in understanding the kinetics of the immunopathological events in the CNS microenvironment and the development of new therapeutic strategies.

Protective versus detrimental correlates in murine NCC

The last decade of research has demonstrated that Toll-like receptors of the innate immune system play an important role in eliciting host inflammatory responses (44, 45). In murine NCC, all the TLRs (TLRs1-13), except TLR5 are upregulated and differentially expressed among the various nervous tissue and infiltrating immune system cell types (46). Moreover, in the absence of MyD88, a central adaptor molecule associated with the signaling pathway induced by all the TLRs activation except for TLR3, infiltration of immune cells into the CNS was reduced. MyD88-deficient mice infected with M. corti, display an enhanced ability to survive the infection as compared to wild-type mice, correlated with a decreased Th1 inflammatory cytokine responses along with reduced neuropathology. Thus, inflammatory responses mediated by immune modules such as by TLR system and γδ T cells, play detrimental roles during murine NCC. In contrast, despite a reduced inflammatory response characterized by a decreased Th1 inflammatory responses observed in the CNS of $TLR2^{-/-}$ NCC mice, these mice displayed a higher

neuropathology correlated with an increased mortality. Interestingly, TLR2$^{-/-}$ NCC mice exhibited decreased numbers of M2 macrophages in the CNS, suggesting that maybe M2-macrophages play an important protective role in mice i.c. infected with *M. corti*. Indeed, after *M. corti* i.c. infection mice deficient in STAT6, which is a central regulator of M2 macrophage development and function (4), display a greater neuropathology and mortality correlated with a scarce presence of M2 macrophages in the CNS. Thus, the M2 macrophages mediated protective mediators could be important for controlling the neuropathology and overall disease severity in murine NCC.

Macrophages functional phenotypes in infections and injury

Macrophages are key effectors cells of the immune system (47). Macrophage plasticity is essential for innate immunity and tissue remodeling (47-49). The functional phenotypes of macrophage vary in response to external stimuli they receive through a wide variety of surface receptors (50). Macrophage with the M1, or classical activation phenotype, release multiple pro-inflammatory molecules (51). M1 or classically activated macrophages are activated by environmental signals such as IFN-γ or TLR ligands. IFN-γ binds to IFNGR-1 and IFNGR-2 chains which form INFGR-1 and recruit JAK1 and JAK2 adaptors proteins. Ultimately the activation of the signal transduction pathways involving phosphorylation of STAT1, IRF-1, and IRF-8, ultimately orchestrate the transcription of pro-inflammatory molecules (47). M1- macrophage activation associated production of inflammatory molecules, including the production of NOS2, IL6 and TNF-α, play critical role in protection from microbial pathogenic infection (47). In contrast, M2 macrophages or alternatively activated macrophages are involved in anti-inflammatory and tissue protective response (1, 49, 52-54). M2 macrophage activation results from exposure to

environmental cues such as cytokines IL-4 or IL-13 (M2a subtype), agonists of IL-1 receptor and TLR (M2b subtype), IL-10 and glucocorticoid hormones (M2c subtype), or activation of adenosine A2A receptors (M2d subtype) (17). The M2a macrophages are involved in allergies responses induced by th2 responses and killing of parasites (1, 47). The M2b phenotypes are involved in Th2 cells differentiation and humoral antibody production. The M2c macrophages play a role in immune regulation and tissues repair (1, 47). Whereas M2d macrophages believed to play a role in protecting from tissue injury (1). Thus, M2 macrophages are involved in many physiologic and pathological processes such as homeostasis, inflammation, metabolic function, allergy, and parasites infection.

M2 Macrophages Functions in Wood Healing

Wood macrophages are referred IL-4 induced macrophages due to the several tissues remodeling molecules produced by these macrophages including Arginase, and YM-1 (48). Arginase-1 an enzyme is a prototypic marker of AAM in mice. For a long time, Arginase-1 was believed to be the primary wound healing protein and it is induced transcriptionally by TGF-β, IL-4, and IL-13 proteins. Arginase-1 uses the amino acid L-arginine as substrate and break it down into L-ornithine and urea. L- Ornithine is believed to be involved in collagen biosynthesis which ultimately promotes fibrosis and tissue healing (55). YM-1 is a lectin with affinity to glycosaminoglycans like heparin. YM-1 is considered as M2 marker in a mouse model and it is upregulated by IL4 and IL-13 and its expression may be directly correlated IL-10 production. This protein has been associated with cellular recruitment and extracellular matrix deposition during tissue repair.

M2 Macrophages Functions in Helminth Infections

Immune response involving M2 macrophages are hallmarks of chronic type 2 pathological conditions, including in helminth parasitic infection (3, 56). Specifically, M2 macrophages have been suggested to involve in the expulsion of certain helminths (57). In this regard, M2 associated molecule YM-1, a chitinase, play an important role in cleaving the surface chitin on helminth parasites thereby making their plasma membrane accessible to host mediators. Importantly, M2 macrophages functions in wound healing (4, 43, 47, 58) thought to be crucial to repair tissues damages caused by the parasites or due to inflammation (47, 57).

As mentioned before, *M. corti* infection of the CNS of mice resulted in massive accumulation of M2 macrophages, correlated with a complete lack of any $iNOS^{+}$ M1 macrophages (4, 43). As $STAT6^{-/-}$ mice infected with *M. corti* displayed a decreased number of M2 macrophages correlated with severe disease sign (4), an understanding of the molecular mechanisms associated with the development, recruitment, and/ or functions of M2 macrophages in murine NCC could be important for understanding how NCC can be clinically controlled.

Glycan antigens in host-parasite immune responses during NCC

More than 95% of NCC patients exhibit humoral responses against glycan antigens (13, 24). Glycan antigens are released both in humans and mice NCC (9, 29) and are taken up by infiltrating immune cells around the metacestodes (9), suggesting their role in immunopathogenesis. It is commonly believed that in order establish a long-lasting infection in the host, parasites helminths release glycans present on their surface or through excretory /secretory products (59, 60). These helminth parasite-derived glycans are

grouped into two distinct sets: the host like and non-host like glycans (59). Various types of host-like glycans are found in different parasites (61-63). The terminal glycan structures shared by bost host parasites include LeX, LDN, and LDNF as well as the truncated-glycans known as the T (Galβ-3GaNAcα-O-Thr/Ser) and Tn (GalNAcα-O-Thr/Ser) antigens (61-63). Remarkably, the host-like glycans can also induce the production of anti-glycan responses resulting from the "foreign" presentation of the host-like glycan antigens in highly multivalent forms as well as their linkage to immunogenic helminth proteins. Interestingly, the synthesis of the non-host-like glycan moieties in the helminths appears to be strongly upregulated during infection of the mammalian host (59, 64). In contrast, non-host-like glycans contain unusual molecules including polyfucose, tyvelose, terminal GalNAc, phosphorylcholine, methyl groups, and sugars with unusual linkages (59). As can be expected, these non-host-like glycans are highly immunogenic in nature and induce the generation of dominant anti-glycan antibody responses in infected hosts (62). Interestingly, the synthesis of the nonhost-like glycan moieties in the helminths appears to be strongly upregulated during infection of the mammalian host (64) suggesting a biological relevance for their expression.

NCC patients exhibit humoral responses against N-glycan antigens, presumably released from the parasite in the CNS. Indeed, carbohydrate-based antigens are extensively used for immunodiagnosis of this disease. These glycan antigens are mostly expressed in the parasite's tegument (external surface) that remains in contact with the host as well as secreted molecules. Both in human and mouse NCC, the glycans in tegument of *T. solium* and *M. corti* are released into the CNS microenvironment (9). In murine NCC, the glycan antigens containing terminal galactose and galactosamine is rapidly released (1-2d p.i.),

whereas glycan antigens containing terminal glucosamines are constantly released during the CNS parasitic infection. We speculate that interaction of some of these parasite glycan antigens with PRRs e.g. lectin receptors dictate the immune regulatory mechanisms involved in NCC (9, 41).

Lectin Receptors, Galectins: Structure, and Functions

PRRs (Patterns recognizing Receptors) are accountable to detect the presence pathogens by recognizing their PAMPs (Pathogen Associated Molecule patterns) (25). PRRs are expressed by host cells mostly by APC (antigen presenting Cells) which include macrophages and dendritic cells but also by non-phagocytic cells. PRRs include the Toll-Like Receptors (TLRs), (RIG) - like receptors, the NOD-like receptors and the Lectin receptors (LRs) (65, 66).

Lectin Receptors

Lectin receptors (LR) are carbohydrates binding proteins found in most animals and plants (66, 67). Within multicellular organisms, protein–carbohydrate recognition is critical to intracellular processes, such as protein folding and transport (66, 67). Such interactions play various important homeostatic and protective functions in the hosts such as extracellular matrix in its function including cell differentiation, adhesion, and migration, and embryogenesis. Moreover, host lectins can also act like PRRs to sense pathogen released molecules (66). Thus, LRs represent a family of multifunctional host molecules that are involved in fine-tuning the host immune system in a variety of conditions. Owing to such flexibility of these LRs in modulating a variety of immune functions, it is necessary to study these and similar molecules in a microenvironment

specific manner. However, the role of targeted LRs in the regulation of immune functions during CNS helminth infection is limited, if any.

Galectins: Galectins are β- galactoside binding proteins which share the same carbohydrate recognition domain (CRD) (68, 69). Based on their structural features, mammalian Galectins had been classified into three different groups (26, 70). The proto-types galectins have one CRD per subunit and most of the time are associate as non-covalently bound homodimers through a hydrophobic interphase (26, 70). This group includes galectins 1, 2, 5, 7, 10, 11,12,13,14, and 15. The chimera-type galectins have C-terminal a CRD similar to the proto-type. In addition, they possess an N-terminal amino-terminal peptide that is rich in glycine, tyrosine, and proline which is involved in the formation of oligomers. The only member of that group is galectin 3. The last group is the tandem repeat (TR) galectins. They have two CRDs joined by a linker peptide. The members of this group include galectins 4, 6, 8, 9, and 12 (26, 70, 71).

Most galectins are non-glycosylated soluble proteins and are ubiquitously expressed and distributed in mammalian tissues, including innate cells such as dendritic cells, macrophages, mast cells, natural killer cells, γδ T cells, B1 cells and adaptive cells such as activated B and T cells of the immune system (26, 70). Although they lack signal sequence, they can be found in the extracellular, intracellular or nuclear milieu where they perform different functions which include cell migration, cell and protein interaction, and immune response regulation (26, 70). Their different functions are accomplished either by protein-protein interactions of protein-glycan interaction (69). Galectins have increase affinity to soluble extracellular matrix (ECM) glycoproteins such as fibronectins, lamins, and vitronectin (26, 68-75). For instance, Lysosome-associated membrane glycoprotein 1

(LAMP-1) is recognized by both galectin 3 and 1 (69). Moreover, galectin 3 binds to CD98 on macrophages and CD66 on neutrophils. Galectin 9 on the other hand has been known to interact with Tim 3 and CD44 on activated T cells (26, 69, 76, 77). These galectins – glycoproteins complexes can interact with other ECM proteins and lead to their regulation effects and signaling cascades (26, 69, 76, 77). In addition, galectins also bind to membrane-bound proteins. Their interactions modulate the immune responses and inflammation (26, 69, 76, 77). Even though the role of galectins has been intensively studied in tumors cells, recently, an increased attention has been given to explore their roles during chronic infections.

***Galectin 3, Immune Response, and the CNS*:** galectin 3 is unique in chimera group and has been extensively studied for the past few years. It may have pro-inflammatory or anti-inflammatory function depending on the pathogens as wells as it microenvironment. Studies had shown galectin 3 intervenes in multiples cellular processes in vitro. It has been shown to play a role in DC-T cells interaction as well as cell- matrix glycoprotein adhesion. Galectin 3 exerts chemotactic effects, control cells proliferation (78). Furthermore, extracellular galectin 3 promotes neutrophil functions such as survival, phagocytosis, and extravasation (78, 79).

The role of galectin 3 during inflammatory conditions in the CNS had been investigated. Even though galectin 3 is expressed at a low level by some brain cells including neurons astrocytes and microglia, its expression is increased during some CNS infection. Galectin 3 was considered as a pro-inflammatory molecule during murine EAE. Despite its involvement in the phagocytosis of myelin debris in CNS which is advantageous for neural regeneration, galectin 3 is also known to be involved in the aggravation of the

of EAE because it plays a critical role in preventing apoptosis, reduce the production of IL-10 and increase IL-17's level (6). In addition, galectin 3 was proving to play a role in mediating injury-induced microglia activation and proliferation in response to Ischemic injury (80).

Galectin 3 and Helminth Infections: Galectin 3 expressed by DCs has been shown to modulate immune and inflammatory response by controlling the magnitude of T-lymphocyte priming during helminthic infection (78). In addition, galectin 3 was found to be indispensable for the recognition and endocytosis of yeasts and is essential for TLR2-dependent cytokine production in response to *C. albicans* (81). However, its role modulating innate responses during NCC is yet to be determined.

Galectin 3 and phagocytosis and efferocytosis. Studies had explored the role played by galectin 3 during both phagocytosis and efferocytosis. Galectin 3 expressed on macrophages was found to be critical in their phagocytosis function (5, 6). However, a recent study had suggested that galectin 3 expressed on neutrophil may help with their clearance during acute infection (82). Moreover, galectin 3 has been proven to not only enhance alveolar Macrophages' differentiation into AAMs phenotypes, it also enhances the clearance of apoptotic cells, therefore improves COPD (65). This suggested that galectin 3 may play a role in the function of AAMs during NCC infection.

Galectin 7 and Immune Response: Most of the studies done on galectin 7 had been in cancers tumors. Galectin 7 is normally expressed by keratinocytes and restricted to stratified epithelia, nevertheless, its expression is considerably altered in tumors cells (83). It had been reported to up-regulate the growth and propagation of lymphoma cells in vivo (84) as well as helping to eliminate tumors cells with it pro-apoptotic function (84). It

typically non-cancerous infections, galectin 7 expression on epithelial cells was found to be associated with pro-apoptotic functions as well as cell-cell adhesion (26, 70, 85). However, the role of galectin 7 during helminth infections is yet to be determined.

Galectin 9 and Immune Response: Galectin 9 is another member of the galectins family which has an increase of interest and it's from the tandem repeat group. Studies had shown galectin 9-Tim-3 interaction pathway induces antibacterial activity in human macrophages which were infected with Mycobacterium tuberculosis (8). In addition, galectin 9 was shown to be highly expressed by iTreg cells and its interaction with its receptor CD44 increase iTregs cells stability and their functions (86). However, the specific role of galectin 9 in the innate immune response during parasitic infection, particularly NCC is yet to be determined.

Central Hypothesis

It has been assumed that viable cysticerci induce immune suppressive effects to evade the immune response. Whereas after the degeneration of larvae caused by either therapeutic treatment or normal attrition, the loss of parasite-mediated immune modulatory effects leads to uncontrolled hyper-inflammatory responses contributing to tissue pathology. In this regard, a considerable body of evidence indicates that the host inflammatory responses elicited in the CNS contributes to nervous tissue pathology and is responsible for most of the clinical symptoms observed with NCC patients. Thus, anti-inflammatory treatments with corticosteroids are often prescribed along with anti-parasitic drugs to kill the organism and control the host inflammatory response and neuropathology. However, long-term treatments with steroids lead to life-threatening side effects. Therefore, it is important to identify the pathophysiological basis of the inflammatory as well regulatory responses

elicited in parasite-infected brains to aid in developing safer therapeutic strategies. Although, we previously contributed in identifying the role of TLR signaling pathway in regulating CNS inflammatory response and pathology, but the presence of the myeloid cells with M2 activation phenotype seemed to be necessary for containment of tissue pathology and disease severity. In this regard, one of the most important similarities between human and murine NCC is the documented release of parasite glycans in the CNS microenvironment. In addition, the terminal galactose-containing glycan are first to be released from the parasite and to larger extent, which coincides with the presence of large numbers of myeloid cells with M2 activation phenotype in the parasite-infected CNS. Thus, our goal was to study the role of lectin receptors in regulating M2 cell functions in the parasite-infected CNS. We hypothesize that the differential expression of galectins that recognize galactose moieties, in endothelial cells play a critical role in immunopathology by modulating trafficking of M2 macrophages into the CNS, whereas galectins expressed in M2 cells controls the protective functions of these cell types.

1. **Characterization of the differential expression and distribution of galectins in endothelial cells and M2 cells during murine NCC.**
2. **Determine the function of galectin/s expressed in M2 cells, in regulating their protective functions in CNS immunopathogenesis of murine NCC**

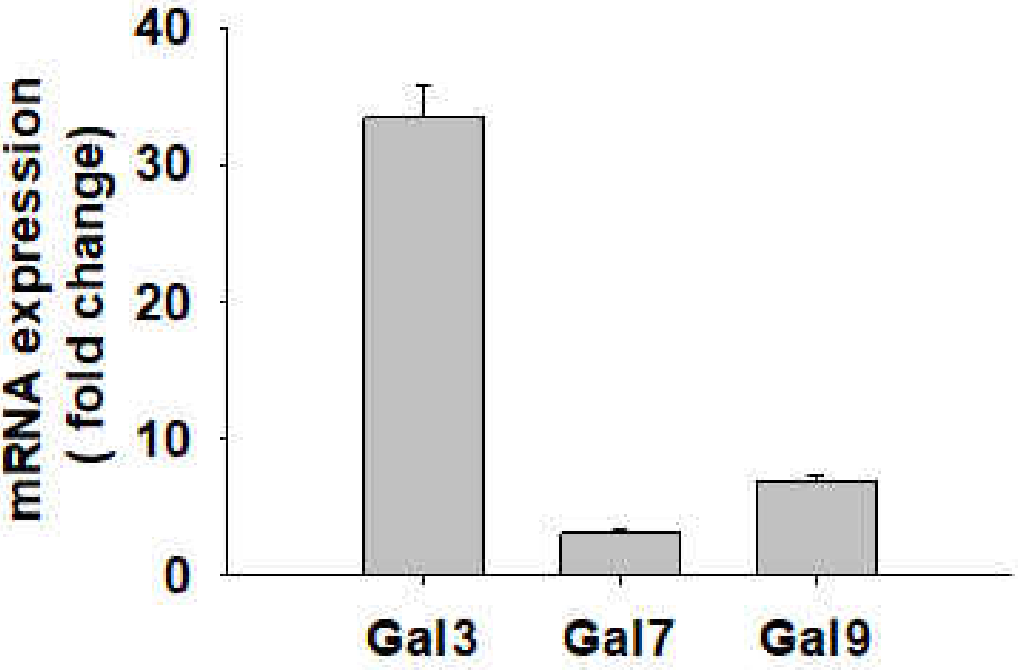

Figure I- 2. Galectins are upregulated during NCC.
WT mice were intracranially infected with *M. corti*. At 2 weeks post infected, brains were isolated and galectins mRNA were measured. Galectins were found to be up regulated at mRNA level.

CHAPTER II

GALECTIN-3 IN M2 MACROPHAGES PLAYS A PROTECTIVE ROLE IN RESOLUTION OF NEUROPATHOLOGY IN BRAIN PARASITIC INFECTION BY REGULATING NEUTROPHIL TURNOVER

Fredice O. Quenum Zangbede, Jyotika Sharma, Bibhuti B Mishra*.

Department of Biomedical Sciences

University of North Dakota School of Medicine and Health Sciences

Grand Forks, ND 58201.

Manuscript in preparation

Abstract

Macrophages/ microglia with M2- activation phenotype are thought to play an important anti-inflammatory and tissue reparative functions in the brain, yet the molecular basis of their functions in the central nervous system (CNS) remain to be clearly defined. In a preclinical model of neurocysticercosis using brain infection with a parasite *Mesocestoides corti*, we previously reported the presence of large numbers of M2 cells in the CNS. Here we report that M2 macrophages in the parasite-infected brain display abundant galectin-3 expression. Disease severity was increased in Galectin $3^{-/-}$ mice correlating with increased neurological defects, augmented cell death and, importantly, massive accumulation of neutrophils and M2 macrophages in the CNS of these mice. Because neutrophil clearance by efferocytosis is an important function of M2 macrophages, we investigated a possible role of galectin 3 in this process. Indeed, Galectin 3 deficient M2 macrophages exhibited a defect in efferocytic clearance of neutrophils in-vitro. Furthermore, adoptive transfer of M2 macrophages from Galectin 3 sufficient WT mice reduced neutrophilia in the CNS and ameliorated disease severity in parasite-infected Galectin $3^{-/-}$ mice. Together, these results demonstrate for the first time a novel role of galectin 3 in M2 macrophage function in neutrophil turnover and resolution of inflammatory pathology in the CNS. This likely will have implications in neurocysticercosis and neuroinflammatory diseases.

Key words: M2 Macrophage, Galectin 3, CNS, brain, neurocysticercosis, neutrophil turnover, neuroinflammation

INTRODUCTION

Inflammation in the central nervous system (CNS) is often associated with catastrophic neurological disabilities (87). A major component of this inflammatory process is a robust innate immune response by microglia and their counterparts, the peripherally derived macrophages that infiltrate into the brain during pathological insult (31, 88, 89). Indeed, microglia/macrophages are potent modulators of CNS repair/regeneration in several neuropathological conditions, including brain infections, stroke, traumatic brain injury, and spinal cord injury (1, 90).

Macrophages and microglia respond to environmental cues by adopting polarized functional phenotypes between activated M1-pro-inflammatory or M2-anti-inflammatory and wound healing/ tissue reparative phenotypes (49, 50, 54, 91, 92). In this regard, exposure to environmental factors such as pathogen-associated molecular patterns (PAMPs) or host molecules such as IFN-γ and TNF-α, leads to activation of STAT1 and NF-kB signaling cascades culminating in M1-inflammatory molecules production (51, 53, 57). These M1 inflammatory effector cells generally promote the destruction of pathogens but cause widespread tissue damage when unchecked (93). On the other hand, M2 macrophage/ microglia activation results from exposure to environmental cues such as cytokines IL-4 or IL-13 (M2a subtype), agonists of IL-1 receptor and TLR (M2b subtype), IL-10 and glucocorticoid hormones (M2c subtype), or activation of adenosine A2A receptors (M2d subtype recently identified in macrophages, but not in microglia) (94). In general, the M2 cells dampen excessive host inflammatory responses and promote tissue repair, including in the CNS (95, 96). Emerging evidence suggests that M2 cells are involved in clearance of cellular debris by phagocytosis and release tissue

protective/trophic factors (97, 98). Although functions of M2 subpopulations are not well characterized in neuropathological conditions, M2 cells promote these restorative processes in nervous tissue such as axonal remodeling and remyelination, oligodendrogenesis and neurogenesis (99). In contrast, M1-macrophage/microglia are found to impair these CNS repair process (90). These insights, although informative, have primarily been derived from in vitro studies or in vivo by selective depletion of these cells using chemicals. Direct mechanistic evidence showing the protective function of M2-macrophages/ microglia in the CNS microenvironment thus, is lacking.

M2-macrophage associated response is a signature of helminth infections. Therefore, experimental models involving helminth infections in the CNS can be important to identify critical molecules and mechanisms in M2- macrophage/ microglia functions to control brain pathology. In this regard, neurocysticercosis (NCC) is a common central nervous system (CNS) parasitic disease caused by the presence of *Taenia solium* larvae in the CNS (14, 100). The degree of immunological responses in CNS microenvironment plays a critical role in the pathogenesis of this disease (12, 17, 101). A mouse model for NCC, in which mice are intra-cranially (i.c.) inoculated with a related helminth *Mesocestoides corti* (*M. corti*), has been used to systematically characterize various aspects of the immune responses in the CNS microenvironment (17, 30, 43). Indeed, an absence of M2 activation phenotype in macrophages/ microglia in the brain of parasite-infected mice, as shown by us, causes severe neurological manifestations and mortality (4, 13) Although the factors regulating M2-microglia/macrophages functions in murine NCC brains are not completely understood, of particular importance are glycan antigens containing terminal galactose and galactosamine that are released from the parasite in the

CNS (17). These glycans released in the CNS microenvironment during murine as well as in human neurocysticercosis interact with the infiltrating macrophages (9, 102), suggesting a possible role for the host lectin receptors in M2- cell associated functions in the CNS, a highly understudied area.

Galectins are host lectins that can recognize pathogen- or host-derived β-galactosides (74, 75). They interact with carbohydrates through conserved carbohydrate-recognition domains (CRDs) and contribute to homeostatic as well as immune modulatory functions in several pathological conditions (103). Galectins are broadly divided into three groups: those containing one CRD (Galectin-1, 2, 5, 7, 10, 11, 13, 14 and 15); those containing two distinct CRDs (galectin-4, 6, 8, 9 and 12); and galectin-3 (73, 104). Galectin 3 is a unique member of the galectin family of molecules characterized by the presence of unusual tandem repeats of proline- and glycine-rich regions. Although there is evidence for the importance of galectin 3 to influence M2 macrophages development and trafficking (105), virtually nothing is known about its role in M2-macrophage/microglia functional phenotype in chronic CNS pathological conditions.

The goal of this study was to identify the expression and distribution of Galectin 3 in M2-macrophage and microglia in murine model of NCC. We compared the susceptibility and immunopathology, including the dynamics of leukocytes infiltration in the CNS, between the Galectin $3^{-/-}$ and WT NCC mice. As galectin-3 was primarily detected in infiltrating M2-macrophages, M2-macrophages from WT mice (Galectin 3 sufficient) were adoptive transferred into the Galectin $3^{-/-}$ NCC mice to analyze resolution of the CNS inflammation and disease severity. Based on our results from these studies, we report a protective role of M2-macrophage specific galectin 3 in mitigating CNS pathology

and disease severity, by regulating efferocytosis mediated neutrophil turnover during brain parasitic infection. To our knowledge, this is the first report showing a protective function of M2-macrophages critically regulated by galectin 3 in the CNS, in a neuropathological condition.

Materials and Methods

Mice and Parasite Inoculation

Female mice age 4-6 weeks were used in this study. Galectin $3^{-/-}$ mice on C57BL/6 were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and used as wild-type (WT) controls. Both WT and Galectin $3^{-/-}$ mice were bred in the animal facility of the University of North Dakota. The animals were used according to institutional and federal guidelines

Murine model of neurocysticercosis

M. corti metacestodes used in this study were maintained in the peritoneal cavity of BALB/c mice by serial intra-peritoneal (i.p.) inoculation as described by us before (40, 42). Mouse model of neurocysticercosis was developed as described by us (93, 106). Briefly, anesthetized 4-6 wk old mice were intracranially (i.c.) injected with 50 µl of HBSS containing about 40 parasites (103, 104). Mock control mice were i.c. injected with 50 µl sterile HBSS using the same protocol. Death was recorded as infection-induced mortality. Mice displaying severe signs of distress (non-responsiveness to cage tapping) were humanely sacrificed and also recorded as infection-induced mortality. To analyze various immunopathological parameters at indicated times post-inoculation, mice were

anesthetized with 100 µl of the above mixture, perfused through the left ventricle with 10 ml cold PBS, before the brains were aseptically collected.

Parasite burden, Histopathological and Immunofluorescence analysis

From the perfused control and parasite-infected mice, the brains were quickly dissected out, embedded in O.C.T. (optimal cutting temperature), and snap frozen. Serial horizontal cryosections of 10µm in thickness were placed on silane prep slides (Sigma-Aldrich, St. Louis MO). The slides were air-dried overnight and fixed in fresh acetone for 10s at room temperature (RT). Acetone-fixed sections were processed immediately for hematoxylin and eosin (H&E) or were wrapped in aluminum foil and stored at -80°C to perform immunofluorescence (IF) staining in future.

For H&E staining, after fixation in 4% formalin for 10 min at room temperature, slides were washed twice in deionized water, dehydrated for 30s in 100% ethanol, stained 30s in hematoxylin, and washed in distilled water for 2 min (40, 42). Tissue sections were stained with eosin for 15s followed by 2 min treatment with 95 and 100% ethanol each. Slides were allowed to air dry, then submerged in xylene for 3 min, and mounted using cytoseal mounting medium (Stephens Scientific, Riverdale, NJ). The number and location of parasites were determined by microscopic examination of the stained tissues. Tissues were also analyzed for the presence or absence of infiltrating leukocytes.

For IF microscopy, the frozen brain tissue sections were stained with relevant antibodies to detect immune parameters such as galectin 3, and immune cells (107). Briefly, brain tissue sections were incubated with specific primary antibodies in staining buffer (PBS buffer with 3% host serum) to prevent non-specific binding. After 45 min of incubations, sections were washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with

0.1% Tween-20 and incubated with appropriate secondary antibodies in staining buffer for 30 min. Sections were then washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20. For double IF staining, the above-mentioned procedures were sequentially repeated for each additional staining. The sections were mounted using FluorSave reagent (Calbiochem, La Jolla, CA) containing 0.3 μM 4', 6'-diamidino-2-phenylindole (DAPI)-diacetate (Molecular Probes). Additional control staining was performed to rule out any nonspecific staining. In each case, sections were blocked with saturating concentrations of appropriate host serum antibodies to eliminate false positive staining due to FcR-mediated nonspecific binding. Staining in the absence of primary antibodies provided additional negative controls. Primary and secondary antibodies used for IF staining are: Rabbit anti-galectin 3 (Abcam, San Diego, CA) and Alexa Fluor® 546 or Alexa Fluor® 488 goat anti-rabbit (Molecular Probes, OR), Rat anti-mouse 7/4 (Cedarlane, Burlington, NC) and Alexa Fluor® 546 or Alexa Fluor® 488 Goat anti-rat (Molecular Probes, OR), Goat anti-mouse MGL1/2 (R&D system, Minneapolis, MN) and Alexa Fluor® 546 or Alexa Fluor® 488 Donkey anti-goat Alexa 488/Alexa546 (Molecular Probes, OR). We evaluated tissue damage on frozen brain tissue sections from control and parasite-infected brains by performing the terminal deoxyribonucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining as per manufacturer's instructions (Chemicon International, CA). In all the cases, the images were acquired using a Nikon eclipse 80i upright microscope (Nikon Corporation, Tokyo, Japan) with an attached cooled RTke Spot 7.3 three spot color camera (Diagnostic Instruments Inc., Sterling Heights, MI). The images were processed and analyzed using Adobe Photoshop 7.0 software (Adobe, Mountain View, CA).

Brain mononuclear cells isolation and flow cytometric analysis

Leukocytes from mouse brains were isolated as described by (108). In order to prepare single cells suspension, each perfused brain was gently minced using Dounce homogenizer in 3 ml of HBSS (w/o Ca2+, Mg2+) containing HEPES (10mM) (Invitrogen, CA). The mixture was suspended in 30% percoll and then slowly overlaid on 70% percoll (GE Healthcare, CA), centrifuged at 500 x g for 30 min at RT. The interface was collected, pelleted at 200 x g for 5 min and re-suspended in HBSS, washed three times in 1 ml of HBSS with 0.1% BSA (Sigma-Aldrich), and quantified by trypan blue staining before performing flow cytometric analysis. Following antibodies were used for flow cytometry: FITC anti-mouse CD11b (clone M1/70), PE-Cy7 anti-mouse F4/80 (clone BM8), PE anti-mouse TCR γδ (clone GL-3), PE-Cy7 anti-mouse Siglec F (clone FC50-2440), APC anti-mouse CD206 (clone C068C2), PE anti-mouse PD-L2 (clone T425), Pacific blue anti-mouse CD45 (clone 30-F11) and APC anti-mouse Ly6G (Clone 1A8) antibodies (All from Biolegend, San Diego, CA), and PE-anti-mouse galectin 3 (clone eBioM3/38) from eBioscience.

Flow cytometric analyses for cell surface expression of relevant molecules were performed on a BD LSR II flow cytometer (BD Biosciences) as we have previously described (28). For intracellular galectin 3 staining in macrophages/ microglia, cells were rinsed, stained for relevant surface markers at 4°C. Cells were fixed and permeabilized using fixation and permeabilization buffer (eBiosciences) before intracellular galectin 3 was detected by staining in intracellular FACS buffer (eBiosciences) containing PE anti-mouse galectin 3 (eBiosciences) and analyzed on a BD LSRII flow cytometer.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) analysis

The qRT-PCR analysis was performed as described by us (34, 109). Total RNA from mock control and parasite-infected brains were isolated using Trizol reagent following manufacturers' instructions (Invitrogen) and as described by us before (40, 108). One microgram of total RNA from each sample was reverse transcribed into cDNA by using a high capacity cDNA reverse transcription kit according to the manufacturers' instructions (Applied Biosystems, CA, and the USA). Transcript levels of galectin 3 or the housekeeping ribosomal 18 S RNA were analyzed by RT-PCR using specific primers: 18S (forward) 5'-CATGTGGTGTTGAGGAAAGCA-3' and (reverse) 5'GTCGTGGGTTCTGCATGATG-3', galectin 3 (forward) 5′-CAGTGCAGAGGCGTCGGGAAA-3′ and (reverse) 5′-CTGCCCCAGCAGGCTGGTTT-3′. Expression levels of galectin 3 were normalized to the mRNA level of the housekeeping 18 S RNA gene in the same sample. The fold change was calculated by dividing the normalized value of galectin 3 in parasite-infected brain samples with the corresponding normalized value in mock-infected brain samples.

Macrophage Adoptive Transfer

For adoptive transfer of M1 or M2 macrophages in vivo, bone marrow cells were isolated from WT mice and differentiated to macrophages as previously described (34, 110). Bone marrow-derived naïve macrophages on day 6 of differentiation were exposed to IFNγ (20ng/ml, Peprotech) or to IL-4 (20ng/ml) to generate the M1-inflammatory or M2-antiinflammatory phenotype respectively in vitro (92, 111). M1 macrophages were labeled with Cell-tracker orange CMTMR (Life technologies) and M2 macrophages were labeled with Carboxyfluorescein succinimidyl ester (CFSE cell proliferation kit Life technologies) CellTracker at 37°C for 15 min as described by us before (112). $5x10^6$

labeled cells were then injected intravenously via tail vein (112) into WT and Galectin $3^{-/-}$ mice i.c. infected with M. corti for 6d prior to transfer. As control, WT and Galectin $3^{-/-}$ mice undergoing NCC in same experimental set up were injected with vehicle only. Twenty-four hours after transfer, brains were harvested and processed for various immunopathological analysis on parameters described in results section by following above mentioned experimental protocols.

Ex-vivo Efferocytosis

For these studies, peritoneal neutrophils were isolated from WT mice 12-16hr after intraperitoneal injection of sterile 4% thioglycollate (BD Biosciences, San Jose, CA) (46). For macrophages, WT and Galectin $3^{-/-}$ mice were injected i.p. with 4% thioglycollate on day-0. On day-0 and day-2 mice were injected i.p. IL-4c mixture containing 5 µg of recombinant murine IL-4 (Peprotech) and 25 µg of anti-IL-4 mAB (11b11, BioXcell), whereas control animals received the vehicle, PBS. On day-4 peritoneal exudate cells (PECs) were harvested and macrophages were analyzed for expression of M1/ M2 mediators by flow cytometry and RT-PCR before they were used for efferocytosis analysis ex vivo. To compare efferocytosis by M2 macrophages induced by parasite infection, WT and Galectin $3^{-/-}$ mice were infected i.p. with M. corti (60-100 in numbers) and PECs were harvested after 4-5wk p.i. to collect M2 cells. Mock control mice instead received vehicle (PBS). On day-4 PECs were harvested to purify naïve macrophages for their role in this process. To obtain neutrophils, WT mice were injected i.p. with 4% thioglycollate for 16-18 hours.

The purity of the cells was ascertained by flow cytometry analysis (Ly6G+ neutrophils 80-85%; F4/80+ macrophages 85-90%). Isolated neutrophils were labeled with

Carboxyfluorescein succinimidyl ester (CFSE; Cell Trace™ CFSE Cell Proliferation Kit from Invitrogen). Macrophages seeded on 6 well plates ($1x10^6$ cell/ml) were incubated with CFSE-labeled neutrophils at a ratio of 5:1(neutrophils: macrophages). After 2 hrs of efferocytosis, non-internalized neutrophils were removed by washing thoroughly. Macrophages were scraped and stained with F4/80 and Ly6G antibodies for flow cytometry. Gating scheme to quantitate Ly6G-F4/80+ CFSE+ efferocytic macrophages that had internalized labeled neutrophils is shown in Figure.II-8. For individual experiments, efferocytic index was calculated as percentage of Ly6G-F4/80+CFSE+ macrophages from WT and Galectin $3^{-/-}$ mice that engulfed neutrophils (112).

Statistical Analysis

The survival of the infected WT and Galectin $3^{-/-}$ mice was compared using Log-rank (Mantel-Cox) Test. The statistical comparison between levels of host mediators was done with Student's t test using Sigma Plot 8.0

H&E and Immunofluorescence Staining

Parasite and HBSS infected brains were immediately removed from perfused WT and Galectin $3^{-/-}$ mice, embedded in OCT resin and snap frozen. Serial horizontal cryo-sections with 10um in thickness were positioned on silane prep slides (from Sigma-Aldrich, St. Louis, MO). Every 5^{th} slides were sectioned and stain following the H&E staining protocol previously described

Quantitative Real-time PCR

Brain from infected and mock control mice 1 WK and 3 WKS post infection were directly perfused and the total RNA was extracted using trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis of the samples was

done using SYBRO green (Applied Biosystems, CA (USA). Transcript levels of the housekeeping ribosomal 18S ,Galectin 9 gene Galectin 3 genes were measured in each sample by PCR amplification using specific primers: removed 18S (forward) 5'-CATGTGGTGTTGAGGAAAGCA-3' and (reverse) 5'GTCGTGGGTTCTGCATGATG-3'; Galectin 9 (forward) 5'- TCAAGGTGATGGTGAACAAGAAA-3' and (reverse) 5'-GATGGTGTCCACGAGGTGGTA -3';Gal3 Galectin 3 (sense) *5'CAGTGCAGAGGCGTCGGGAAA-3'* and (anti-sense) *5'CTGCCCCAGCAGGCTGGTTT-3'*

Statistical Analysis

The survival of the infected WT and Galectin $3^{-/-}$ mice was compared using Log-rank (Mantel-Cox) Test. The statistical comparison between levels of host mediators was done with student's test using Sigma Plot 8.0.

Results

Galectin 3 is abundantly expressed in M2 macrophage recruited into the CNS during murine NCC

To examine the role of galectin 3 in parasite-infected brains, its transcript-level of expression was determined by qRT-PCR using RNA from mock and M. corti i.c. infected brains. Increased mRNA expression of galectin 3 was observed at both 1wk and 3wk p.i. over mock control (~ 27-fold increase at 1wk p.i. and ~14-fold increase at 3wk p.i) (Figure.II-1A). To identify the cell-specific expression of galectin 3 protein, in-situ co-immunostaining was performed on brain cryosections of mock control and parasite-infected mice. In mock-infected mice brains, galectin 3 was scarcely detected (Figure.II-1B). In contrast, parasite-infected brains exhibit abundant galectin 3 protein expression at both 1wk and 3wk p.i. (Figure.II-1B & C). Double IF staining on the parasite-infected brain sections showed an accumulation of large numbers of round monocyte/ macrophage-like CD11b+ or F4/80+ cells in meninges and ventricles that displayed positive staining for galectin 3 (Figure.II- 1C). We have previously reported that the infiltrating macrophage in the CNS of mice undergoing NCC display M2-, but not M1- activation phenotype (40, 42, 43, 58). Indeed, the MGL1/2+ infiltrating macrophages were stained positive for galectin 3 (Figure.II- 1D), suggesting that the M2- macrophages accumulating in the CNS during parasite infection abundantly express galectin 3. In addition, some microglia cells as determined by CD11b+ staining combined with typical cell morphology and brain anatomical location exhibited galectin 3 expression (data not shown). Galectin 3 protein expression was largely undetected in other nervous tissue or immune cell types e.g. CD11c (dendritic cells), αβT cells, γδT cells, neutrophils (7/4), B-cells (CD19) (data not shown).

To further characterize the cell-specific expression of galectin 3 in infiltrating macrophage and/ or microglia as well as to identify its cellular localization, the flow-cytometric analysis was performed. It is important to note that $CD45^{hi}F4/80+$ cells in the CNS are known to be infiltrating macrophages, whereas $CD45^{int}F4/80+$ cells represent as microglia. Consistent with IF microscopy analysis, galectin 3 was primarily detected in $CD45^{hi}F4/80+$ macrophages in the CNS at both 1wk and 3wk p.i. (Figure.II-2B & 2C). In contrast, only 7% of $CD45^{int}F4/80+$ that are presumably microglia, displayed galectin 3 positive staining (Figure.II-2B & 2C). Interestingly, the majority of the CD45hiF4/80+ cells exhibited intracellular staining of galectin 3 (Figure.II-2B & C). In both 1wk and 3wk p.i. mice brains, 80–90% of the $CD45^{hi}F4/80+$ cells were MGL1/2+ or PD-L2+ (data not shown), indicating that the galectin 3 expression is largely localized to the M2 macrophages in the CNS of *M. corti* i.c. infected mice undergoing NCC.

Galectin $3^{-/-}$ mice exhibit increased disease severity during murine NCC

In light of abundant expression of galectin 3 in M2 macrophages, we sought to examine the role of galectin 3 in overall disease severity during parasite infection of the brain. For this, the development of neurological signs and survival of WT and Galectin $3^{-/-}$ was compared. Neurological signs related to i.c. *M. corti* infection including abnormal vestibular function, tilted head, and cerebral abnormalities were observed between 2-3wk p.i. in WT mice. However, infected Galectin $3^{-/-}$ mice displayed these signs much earlier and were more accentuated between 2-3wk p.i. Importantly, M. corti-infected Galectin $3^{-/-}$ mice exhibited decreased survival as compared to the WT (Figure.II- 3A). At 3wk p.i., only 40% (4 of 10) of the M. corti i.c. infected Galectin $3^{-/-}$ survived the CNS infection as opposed to 100% (9 of 9) of the WT mice (Figure.II- 3A, $p<0.005$). Together these data

suggest a protective role of galectin 3 in the pathogenesis of CNS parasite infection, possibly relating to its expression in the infiltrating M2 macrophages in the CNS.

Galectin $3^{-/-}$ mice exhibit no defect in parasite clearance or their segregation

Microscopic analysis of the H&E stained serial brain cryosections was performed to determine the clearance of parasites and/ or their extravasation into specific CNS areas in the absence of galectin 3. No statistically significant difference in the parasite loads was observed in the CNS of WT and Galectin $3^{-/-}$ NCC mice at 1wk and 3wk p.i. (Figure.II-3B). Moreover, brain parenchyma or extra-parenchyma of WT and Galectin $3^{-/-}$ mice at 1wk and 3wk p.i. exhibited no statistically significant difference in parasite count (Figure.II-3B), although the Galectin $3^{-/-}$ mice tended to exhibit a relatively lower number of organisms in extra-parenchymal regions (Figure.II- 3B2). Thus, the protective function of galectin 3 did not appear to relate to parasite clearance and/ or their segregation to specific CNS sites of infected mice.

Galectin $3^{-/-}$ mice exhibit severe neuropathology

We evaluated the gross CNS immunopathological changes in mock and i.c. *M. corti*-infected WT and Galectin $3^{-/-}$ mice. H&E stained brain cryosections of the control animals of both strains that were i.c. inoculated with HBSS, displayed similar normal brain tissue morphology with scarce presence of immune cells (Figure.II- 3C). In the parasite-infected WT mice brains, presence of mixed leukocyte subsets were detected. A majority of these infiltrating cells appeared to be monocyte/ macrophages at both 1wk and 3wk p.i. (Figure.II- 3C, inset). In contrast, infected Galectin $3^{-/-}$ mice brains appeared to exhibit an increased CNS inflammation with transiently higher numbers of infiltrating immune cells

compared to WT mice. In addition to the high numbers of immune cells with morphology consistent with monocytes/ macrophages, polymorphonuclear cells (PMNs) were abundantly detected between 2-3wk p.i. in Galectin $3^{-/-}$ mice compared to their WT counterparts (Figure.II- 3C, insert, shown at 3wk p.i.). To examine the impact of Galectin-3 deficiency on the tissue pathology, TUNEL staining was performed on the brain tissue sections of WT and Galectin $3^{-/-}$ mice. Very little to no TUNEL positive cells were detected in the CNS of mock and at 1wk p.i. in WT and Galectin $3^{-/-}$ mice (data not shown). At 3wk p.i. the TUNEL staining remained low in the WT brains, which was in contrast to the Galectin $3^{-/-}$ brains that displayed increased TUNEL detected staining at that time p.i. (Figure.II- 3D). Interestingly, this increased TUNEL staining was detected in immune cells present in the extra-parenchymal regions and surrounding nervous tissue cells in the CNS of Galectin $3^{-/-}$ mice. These data suggest that absence of galectin 3 dependent responses leads to greater CNS inflammatory pathology during NCC.

To further examine the impact of galectin 3 deficiency on inflammatory response, levels of multiple inflammatory cytokines and chemokines in brain homogenates were compared between WT and Galectin $3^{-/-}$ mice i.c. inoculated with HBSS or M. corti. (Figure.II- 4). CBA analysis for key inflammatory molecules TNF-α, IL-1β, and IL-12p70 showed low basal levels of these cytokines in WT and Galectin $3^{-/-}$ mice brains (Figure.II-4). In contrast, *M. corti*-infected WT and Galectin $3^{-/-}$ mice brains exhibited increased levels of these mediators at 1wk and 3wk p.i., albeit there were no statistically significant differences in IL-1β, and IL-12p70 levels between infected Galectin $3^{-/-}$ and WT mice (Figure.II- 4). TNF-α was detected at a lower level in Galectin $3^{-/-}$ mice at 3wk p.i., compared to the WT counterparts (Figure.II- 4). On the other hand, significantly higher

levels of neutrophil chemo-attractants (CXCL1, CXCL2), and monocyte/ macrophage chemo-attractants (CCL2, CCL4) were detected in the brain homogenates of parasite-inoculated Galectin $3^{-/-}$ mice at 1wk p.i. compared to the WT. Intriguingly, no significant differences in the levels of these chemokines were detected in the CNS of Galectin $3^{-/-}$ mice at 3wk p.i. (Figure.II- 4), a time of peak disease severity displayed by them. Our results suggest a possibility of higher accumulation of PMNs together with increased cell death in the CNS to be likely the cause of enhanced disease severity observed in Galectin $3^{-/-}$ mice after brain parasite infection.

Galectin $3^{-/-}$ mice display increased neutrophil and M2 macrophage accumulation in the CNS after parasite infection

Majority of the immune cells recruited to the brain after parasite infection are macrophages (2-3d p.i.), γδ T cells (3-5 days p.i), dendritic cells (1wk p.i.), αβ T cells (1wk p.i) and B-cells (3wk p.i.) (40, 42, 43, 58). In the *M. corti* infected brains, macrophages are the most predominant leukocyte populations, whereas neutrophils are detected in relatively in low numbers throughout the infection process (27). Within this context, H & E analysis of the brain cryosections of *M. corti* parasite infected Galectin $3^{-/-}$ mice appeared to display a massive increase in numbers of infiltrating cells with morphology consistent with neutrophils (Figure.II-3C). To determine whether a deficiency of galectin 3 alters neutrophil numbers in the CNS, the expression of neutrophil marker 7/4 was analyzed by in situ IF microscopy. In mock-infected brains of both WT and Galectin $3^{-/-}$ mice, 7/4+ cells were scarcely detected (Figure.II- 5A). In the WT brain at 1wk p.i. little change in the numbers of 7/4+ cells was detected as compared to the mock control mice, which increased by 3 wk p.i.. Galectin $3^{-/-}$ brains, on the other hand, exhibited a massive increase in 7/4+

cells at 3wk p.i. as compared to WT mice (Figure.II- 5A). This suggested that absence of galectin 3 cause increased of neutrophils accumulation in the infected brain.

Next, flow cytometry analysis was performed to enumerate immune cells infiltrating the CNS of Galectin $3^{-/-}$ and WT infected mice. Mononuclear cells from the whole brain were isolated at an early stage of infection (1wk p.i.) and at the peak of inflammation (3wk p.i.) (Figure.II- 5 and Figure.II- 6). The numbers of neutrophils in the CNS of Galectin $3^{-/-}$ and WT infected mice were determined by quantifying Ly6G+CD11b+ cells. Consistent with the results from IF microscopy analysis, we found no significant differences in the percent as well as absolute numbers of CD11b+Ly6G+ neutrophils in the CNS of parasite-infected Galectin $3^{-/-}$ mice compared to the WT at 1wk p.i. (Figure.II-5B). However, at 3wk p.i. the numbers of Ly6G+CD11b+ neutrophils were significantly increased in the CNS of parasite-infected Galectin $3^{-/-}$ mice as compared to the WT mice (Figure.II-5B), confirming that galectin 3 deficiency leads to increased accumulation of neutrophils in the CNS microenvironment during chronic infection. We found no significant differences in the numbers of dendritic cells (CD11c+), γδ T cells (TCRδ+), αβ T cells (TCRβ+) or B cells (CD19+) at 1wk or 3wk p.i. CNS of Galectin $3^{-/-}$ and WT mice (data not shown). To determine if the infiltration of M2 macrophages is affected in the absence of galectin 3, the numbers of MR1+ or PD-L2+ was enumerated as we previously have shown MR1 is upregulated on M2 macrophages in parasite-infected brains whereas PD-L2 is a well-known marker of M2 cells (29, 43). Indeed, $CD45^{hi}$F4/80+MR1+, as well as $CD45^{hi}$F4/80+PD-L2+ M2 macrophages, were detected at both higher percentage and numbers in the CNS of infected Galectin $3^{-/-}$ mice at 1wk and 3 wk p.i. as compared to their WT counterparts (Figure.II- 6A& 6B). This increased

accumulation of M2-macrophages in the CNS of parasite-infected Galectin $3^{-/-}$ mice was statistically significant. Together our data suggested that galectin 3 in M2 macrophages likely plays a role in containment of neutrophilia in the CNS.

Adoptive transfer of M2-macrophage from WT mice reduces neutrophilia in the CNS and improves disease severity in recipient Galectin $3^{-/-}$ mice during brain parasitic infection

Galectin 3 was found to be expressed primarily in infiltrating M2 macrophage in the CNS of *M. corti* i.c. infected mice. Because Galectin $3^{-/-}$ NCC mice exhibited neutrophilia and severe disease signs, we sought to examine if the adoptive transfer of galectin 3 sufficient M2-macrophages improves the disease severity. The effect on neutrophil turnover in the CNS and disease severity was compared WT and Galectin $3^{-/-}$ mice after adoptive transfer of WT M2 macrophages. In initial experiments, macrophages were isolated from the brains of i.c. infected WT mice to use as donor cells. However, even though macrophages are the most predominant immune infiltrating cells found in the parasite-infected WT mouse brains, it was difficult to isolate sufficient numbers of these cells from the brain to perform adoptive transfer experiments in different groups. Thus, naïve bone marrow-derived macrophages were polarization to the M2- phenotype using well-characterized stimulus IL-4 for adoptive transfer experiments. M2- phenotype of these cells was verified by analyzing M2-markers Arginase-1 and CCL24, but not NOS2 expression in these cells (data not shown). The *M. corti* i.c. infected WT and Galectin $3^{-/-}$ NCC mice received vehicle or WT-M2 cells i.v. at 1wk, and 2wk p.i. followed by analysis of neutrophils numbers in the CNS of recipients. Flow cytometry analysis revealed that the i.v. injected WT-M2 cells migrated into the CNS microenvironment of the recipient

Galectin $3^{-/-}$ or WT mice with similar relative efficiency (Supplementary Figure II. S1). Indeed, more than 50% of the CFSE labeled WT-M2 cells (F4/80+CFSE+) injected i.v. migrated into the CNS of the infected recipient mice at 24-h post adoptive transfer (Figure. S1). In-situ IF microscopy with anti-7/4 was performed on brain cryosections from recipient mice at 3wk of NCC to detect the accumulation of neutrophils. Transfer of WT-M2 (experimental Group: WT NCC-WT M2) or vehicle (experimental Group: WT NCC-Vehicle) into the i.c. infected WT mice caused no apparent change in the number of 7/4+ neutrophils accumulated in the CNS (Figure.II- 7B). On the other hand, infected Galectin $3^{-/-}$ mice i.v. injected with vehicle (experimental Group: Galectin $3^{-/-}$NCC-Vehicle) at 1wk, and 2wks p.i., revealed the abundant presence of 7/4+ neutrophils in the CNS which was substantially more compared to the experimental Group: WT NCC-WT M2 (Figure.II- 7B). Importantly, transfer of WT M2 cells into i.c. infected Galectin $3^{-/-}$ mice (experimental Group: Galectin $3^{-/-}$NCC- WT M2) resulted in a significant decrease in the number of 7/4+ neutrophils accumulated in the CNS as compared to the Galectin $3^{-/-}$NCC mice that received vehicle only (Figure.II- 7B). These results confirmed that Galectin 3 expressing M2-macrophages regulate neutrophilia in the CNS during brain parasitic infection.

To determine whether adoptive transfer of galectin 3 sufficient WT-M2 cells would prevent disease severity in Galectin $3^{-/-}$ NCC mice, WT and Galectin $3^{-/-}$ mice inoculated i.c. with M. corti were i.v. injected with vehicle or WT M2 cells at 1wk, and 2wks p.i. as discussed above. WT NCC-Vehicle displayed typical neurological signs (abnormal vestibular function, tilted head, and cerebral abnormalities) by 3wk p.i.. These infection induced disease signs appeared earlier and were more pronounced in Galectin $3^{-/-}$NCC-vehicle group of mice. In addition, these Galectin $3^{-/-}$NCC that received vehicle exhibited

decreased survival (Figure.II-7D). By 26 days p.i, % of M. corti-infected WT mice Galectin 3 was found to be expressed primarily in infiltrating M2 macrophage in the CNS of *M. corti* i.c. infected mice. Because Galectin $3^{-/-}$ NCC mice exhibited neutrophilia and severe disease signs, we sought to examine if adoptive transfer of galectin 3 sufficient M2-macrophages improves the disease severity. The effect on neutrophil turnover in the CNS and disease severity was compared WT and Galectin $3^{-/-}$ mice after adoptive transfer of WT M2 macrophages. In initial experiments, macrophages were isolated from the brains of i.c. infected WT mice to use as donor cells. However, even though macrophages are the most predominant immune infiltrating cells found in the parasite-infected WT mouse brains, it was difficult to isolate sufficient numbers of these cells from the brain to perform adoptive transfer experiments infected Galectin $3^{-/-}$ mice (experimental Group: Galectin $3^{-/-}$NCC- WT M2) resulted in a significant decrease in the number of 7/4+ neutrophils accumulated in the CNS as compared to the Galectin $3^{-/-}$ NCC mice that received vehicle only (Figure.II-7B). These results confirmed that galectin 3 expressing M2-macrophages regulate neutrophilia in the CNS during brain parasitic infection. By 26 days p.i, %25 of M. corti-infected WT mice succumbed to the infection as compared to 80% of Galectin $3^{-/-}$ mice (4of 5) (Figure.II- 1, $p<0.005$). In contrast, none of the Galectin $3^{-/-}$NCC-WT M2 mice exhibited severe neurological signs during the 3wk p.i. and all survived the infection. This data strongly showed that adoptive transfer of Galectin 3-sufficient M2-MΦ ameliorated disease severity and improved survival during CNS parasite infection in Galectin $3^{-/-}$ mice.

Galectin $3^{-/-}$ M2-MΦ exhibit impaired efferocytosis of neutrophils

A prominent anti-inflammatory and tissue reparative function of M2-macrophages is efferocytic clearance of infiltrating immune cells (97, 98, 113, 114). In order to gain mechanistic insights into the role of Galectin 3 expressed in M2-MΦ in regulating neutrophil accumulation in helminth-infected brains, we compared ex-vivo efferocytosis of CFSE- labeled neutrophils by M2-macrophages from WT and Galectin $3^{-/-}$ mice by flow-cytometry (Figure.II-8). For this, Galectin $3^{-/-}$ and WT peritoneal M2-macrophages isolated from the M. corti i.p. infected (Figure.II-8B) or thioglycollate + IL-4 complex i.p. injected mice (Figure.II-8C) were incubated with CFSE labeled neutrophils. M2-phenotype of macrophages was ensured by flow cytometry analysis of differential expression of M1/M2 markers PD-L2, MR-1, ARG-1, and NOS2, as described above. As shown in (Figure.II-8B and 8B'), after 3 hrs of incubation, efferocytic uptake of CFSE-labelled neutrophils was significantly less in Galectin $3^{-/-}$ M-macrophages as compared to their WT counterparts. Similar results were obtained with WT and Galectin $3^{-/-}$ M2 macrophages differentiated with peritoneal injection of IL-4 complex (Figure.II- 8C and 8C'). These data strongly suggested that galectin 3 expression in M2-macrophages is required for efferocytic uptake of neutrophils by these cells.

DISCUSSION:

Increasing evidence from several experimental neuropathological models such as in traumatic brain injury suggests that M2-macrophage are the first to infiltrate into CNS tissues (97, 115). As the injury persists, a shift to increased accumulation of M1 phenotypic macrophages in the CNS coincides with the inflammatory pathologic phase (94). Whereas regulatory immune mediators such as IL-10, TGF-β have been implicated in M2 cells mediated immune suppression/ anti-inflammatory response, important central regulatory mechanisms involved in M2-macrophage functions to contain CNS inflammation and pathology need to be identified. The data presented here show that during brain helminth infection of the CNS, cytosolic galectin 3 is increased specifically in M2 macrophages, but not substantially in microglia. Concomitantly, absence of galectin 3 resulted in a massive increase in the accumulation of neutrophils correlating with heightened disease severity in M. corti infected Galectin $3^{-/-}$ mice as compared with WT mice. Galectin 3 deficiency in M2-macrophages decreased efferocytic clearance of neutrophils by these cells. Importantly, pathological sequelae, including severe neutrophilia in CNS parasite infected Galectin $3^{-/-}$ mice, were ameliorated by adoptive transfer of Galectin 3 sufficient M2 macrophages. Our study shows, for the first time, a protective role of M2-macrophage associated galectin 3 in brain parasitic infection by controlling neutrophil turnover in the CNS. There has been a growing appreciation for a role of macrophage/ microglia with M2 functional phenotype having a profound role in different aspects of CNS repair and containment of immunopathology in the brain (90). Indeed, results from our previous studies with $STAT6^{-/-}$ or $TLR2^{-/-}$ mice provide direct evidence for the critical protective role of the myeloid cells with M2 functional phenotype in the brain microenvironment, during CNS parasite infection (13, 43). Here we observed that the parasite-infected

Galectin $3^{-/-}$ mice, despite showing the presence of an increased number of M2-macrophages in the CNS compared to their WT counterparts, exhibit severe neurological signs and are highly susceptible to the infection. In the CNS of Galectin $3^{-/-}$ mice, perhaps the observed elevated amount of the chemokines including, CCL2 that are known to involve in trafficking of monocytes/ macrophages (116, 117), facilitates the increased influx of M2 cells. This indicated to us that the protective function, but not the development, of M2-cells is dependent on galectin 3. Indeed, in the parasite-infected brains, galectin 3 was primarily detected in infiltrating M2 macrophages and the transfer of Galectin 3 sufficient M2 macrophage from WT mice reversed the infection-induced CNS pathology and improved the survival of parasite-infected Galectin $3^{-/-}$ mice. These results highlight the importance of galectin 3 in M2 macrophages mediated protective functions to contain CNS pathological process. However, it remains to be determined the consequence as well as the mechanisms involved in the increased accumulation of M2-macrophages in the CNS of parasite-infected Galectin $3^{-/-}$ mice. In this regard, it has been suggested that galectins, including galectin 3, can play a role in immune cell trafficking (118-120). However, our results showed that adoptively transferred galectin 3 sufficient as well as -deficient M2-macrophages accumulated in the CNS of both WT and Galectin $3^{-/-}$ mice with similar frequency. This strongly supports the idea that the enhanced disease severity in Galectin $3^{-/-}$ NCC mice was a direct consequence of a lack in the galectin 3 mediated response from M2 cells, but not due to a defect in M2 macrophage development or trafficking (105, 120). In M. corti- infected WT mice brains neutrophils were detected as a minor proportion of the immune cells present in the brain of M. corti- infected WT mice. Earlier studies have also identified similar low numbers of neutrophils in the CNS

microenvironment during murine NCC (30). However, infected brains in Galectin $3^{-/-}$ mice display a massive increase in the number of neutrophils as compared to the WT mice, suggesting a role for galectin 3 in regulating neutrophil turnover. The transfer of M2-macrophage from WT could reverse this increased of neutrophil accumulation in the CNS microenvironment of *M. corti* –infected Galectin $3^{-/-}$ mice, thus providing additional support for a role of galectin 3 in M2 macrophage-mediated containment of neutrophilia. This observation is exciting, as the absence of M2-macrophages has been shown to associate with increased neutrophilia and reduced eosinophilia during infection with a nematode parasite, *Brugia malayi,* but the mechanism remained unknown (3). Indeed, in M. corti i.p. infected Galectin $3^{-/-}$ mice, we observed a reduced eosinophilia coinciding with the increased neutrophilia (data not shown) further supporting a fundamental role of M2 macrophage-associated functions of galectin 3 in controlling neutrophilia. Although the molecular pathway of galectin 3 mediated containment of neutrophilia in the CNS microenvironment remains to be determined, our data showing a defect in efferocytosis of neutrophils by Galectin $3^{-/-}$ M2 macrophages in vitro suggests its regulatory role in efferocytic ability of these cells. In this context, a recent report has suggested that galectin 3 expressed and secreted by neutrophils auto-regulate their turnover during a self-resolving model of peritonitis with zymosan injection (82). It has further been suggested that galectin 3 secreted by neutrophils facilitates efferocytosis of these cells by interacting with its ligand on macrophage surface (65). However, the data from our studies clearly suggested a cytosolic, but not extracellular presence of galectin 3 in M2 macrophages in parasite-infected brains. Moreover, if secreted galectin 3 was involved in containing neutrophilia in parasite-infected brain, we would expect to detect galectin 3 on the surface of macrophages

and/ or neutrophils, which was not the case. Thus, galectin 3 in M2 macrophages regulates neutrophil turnover by efferocytosis in the CNS microenvironment. The findings that several signature inflammatory mediators are present at a similar level in the CNS Galectin $3^{-/-}$ mice despite exhibiting low survival raised the fundamental question of whether M2 subsets are sufficient to contain NCC and other CNS pathological conditions. Indeed, in human NCC patients the hyper-inflammatory responses in the CNS thought to contribute to tissue pathology and mortality (121). Thus, along with anti-parasitic drugs, treatment of NCC patients with immunosuppressive/anti-inflammatory factors such as corticosteroids is frequently used to control the associated neuropathology, despite the life-threatening side effects associated with long-term treatments with steroids (82). Within that context, we and others have shown a decreased expression of the inflammatory mediators in M. corti infected $\gamma\delta T^{-/-}$ or $MyD88^{-/-}$ mice is associated with reduced pathology and longer survival times. But, despite presence of lower brain levels of these inflammatory mediators, M. corti infected $TLR2^{-/-}$ mice presented an increased CNS pathology and disease signs (13). We have also shown that the reduced numbers of M2 macrophages observed in $TLR2^{-/-}$ mice, and more importantly in the CNS of $STAT6^{-/-}$ mice, coincided with severe disease signs in murine NCC (42). Thus, the findings from these studies that galectin 3 can profoundly influence M2- macrophage cell functions in the CNS may be directly relevant to human disease notably involving CNS pathological conditions.

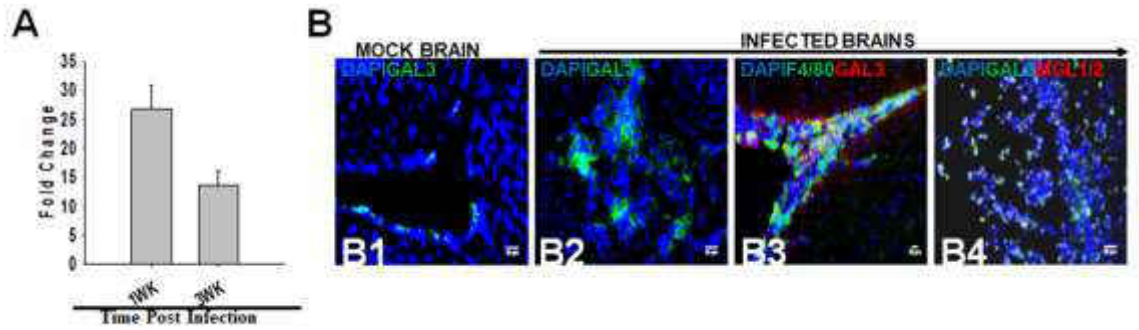

Figure II- 1. Expression and distribution of galectin 3 in the parasite-infected brains.

Quantitative RT-PCR analysis and IF microscopy were performed on parasite-infected and mock control mice i.c. injected with helminth parasite *M. corti*. (A) The total RNA was extracted from the brains at 1wk and 3wk post inoculation and mRNA expression of galectin 3 was measured by qRT-PCR as described in Methods. The fold changes in parasite-infected brains were calculated over the levels in mock control mice using the formula $2^{-(\Delta\Delta Ct)}$. Data shown are the mean ± SEM of 3–4 mice per time point in two independent experiments. (B1& B2) Double immunofluorescence staining was performed on brain tissue cryosections from mock or parasite- infected C57/BL6 mice. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Galectin 3 was stained using an affinity purified anti-mouse galectin 3 goat IgG followed by Alexa Fluor® 488 labeled (Green) goat anti-rabbit IgG. (B3) galectin 3 expression was co-visualized with macrophages using an affinity purified anti-mouse F4/80 Alexa Fluor® 488 labeled (Green) or (B4) MGL1/2+ cells using an affinity purified anti-mouse MGL1/2 goat IgG followed by Alexa Fluor® 546 labeled (red) donkey anti-goat IgG. Images shown are representative of 3 independent experiments with 3–4 mice each. Magnification of 20X.

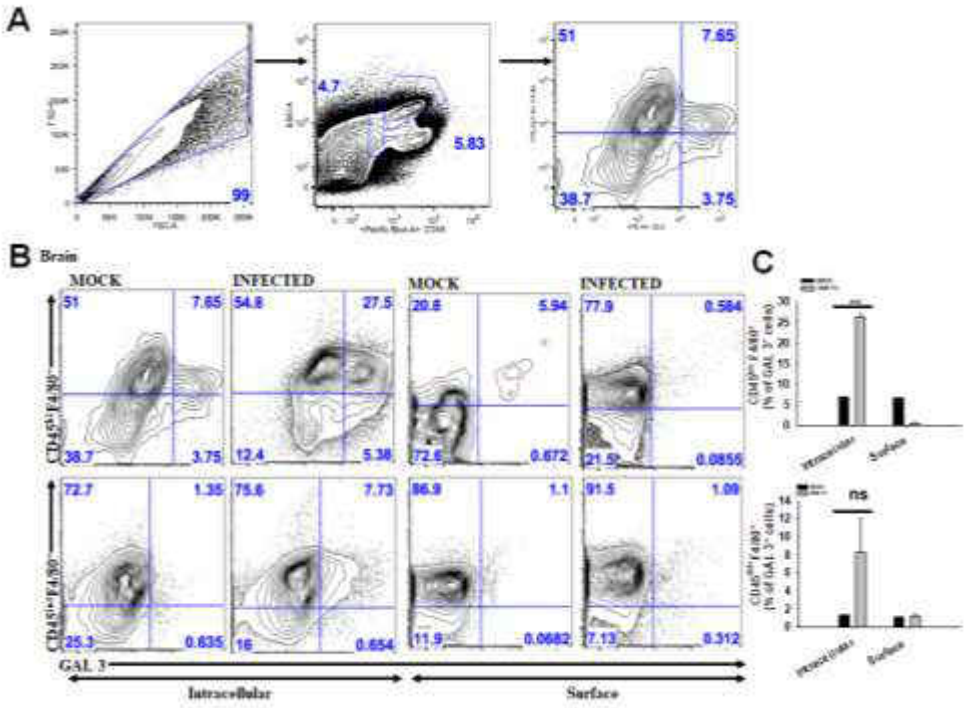

Figure II- 2. Infiltrating M2 macrophages in the parasite-infected brains express galectin 3 in the cytosol, but not on cell surface.

Mice were injected i.c. with HBSS (mock) or 60 helminth parasite (infected), and brain mononuclear cells were isolated at 3wk post inoculation. (A) Gating scheme to select $C45^{hi}$F4/80+Galectin 3 positive cells. Collected cells with gated with FSC and SCC Singlet cells. The cells were then gating on CD45 and F4/80 and galectin 3 positive cells. (B) Representative FACS plots gated on $CD45^{hi}$ and F4/80+ (infiltrating macrophages) or gated on $CD45^{int}$ and F4/80+ (microglia) cells from individual mouse brain showing intracellular or cell surface expression of galectin 3. (C) Quantification of galectin 3 expression in $CD45^{hi}$F4/80+ macrophages or $CD45^{int}$F4/80+ microglia. Results are representative of 3 independent experiments. **P <0.005 as determined by t test.

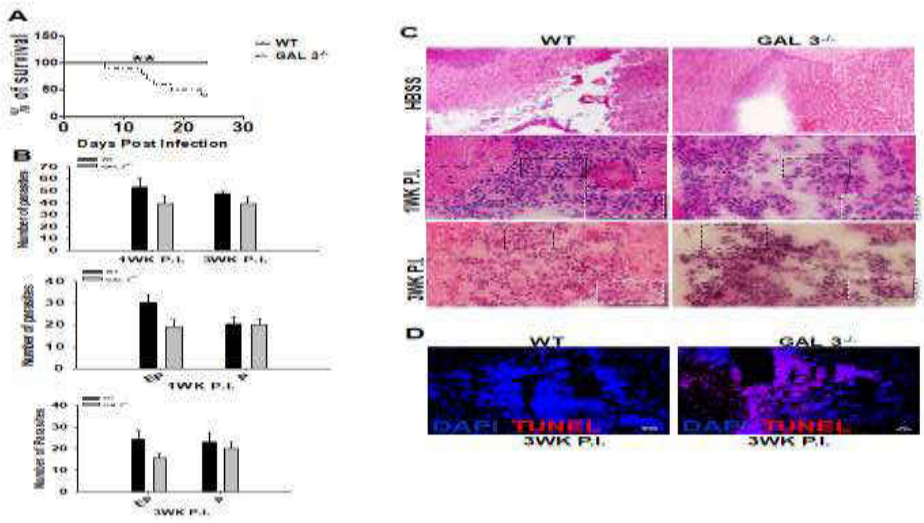

Figure II- 3. Galectin $3^{-/-}$ mice exhibit reduced survival and increased brain pathology despite exhibiting similar parasite burden as the WT mice.

(A) WT and Galectin $3^{-/-}$ mice were i.c. infected with 60 *M. corti* and were assessed daily for disease severity. The survival was monitored for 3 weeks post inoculation. Statistical comparison of susceptibility was done by Log-rank (Mantel-Cox) Test, p= 0.0047 **, n=9-10. (B) Number of parasites at 1wk and 3wk p.i. in parenchymal (P) and extra parenchymal (EP) regions in individual Galectin $3^{-/-}$ and WT brains were calculated by microscopic examination of serial H&E-stained brain sections. The bars show average +/- SEM parasites from 5-6 mice per group. (C) Representative microscopic evaluation of H&E stained brain cryosections from WT and Galectin $3^{-/-}$ mice i.c. injected with HBSS (mock) or with 40 parasites at indicated times p.i.. Magnification 200X. (D). IF microscopy analysis was performed on brain cryosections from WT and Galectin $3^{-/-}$ mice i.c. infected with 60 *M. corti* parasites to detect TUNEL positive staining (Red). Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Images are representative of 5-6 mice in each group from three independent experiments.

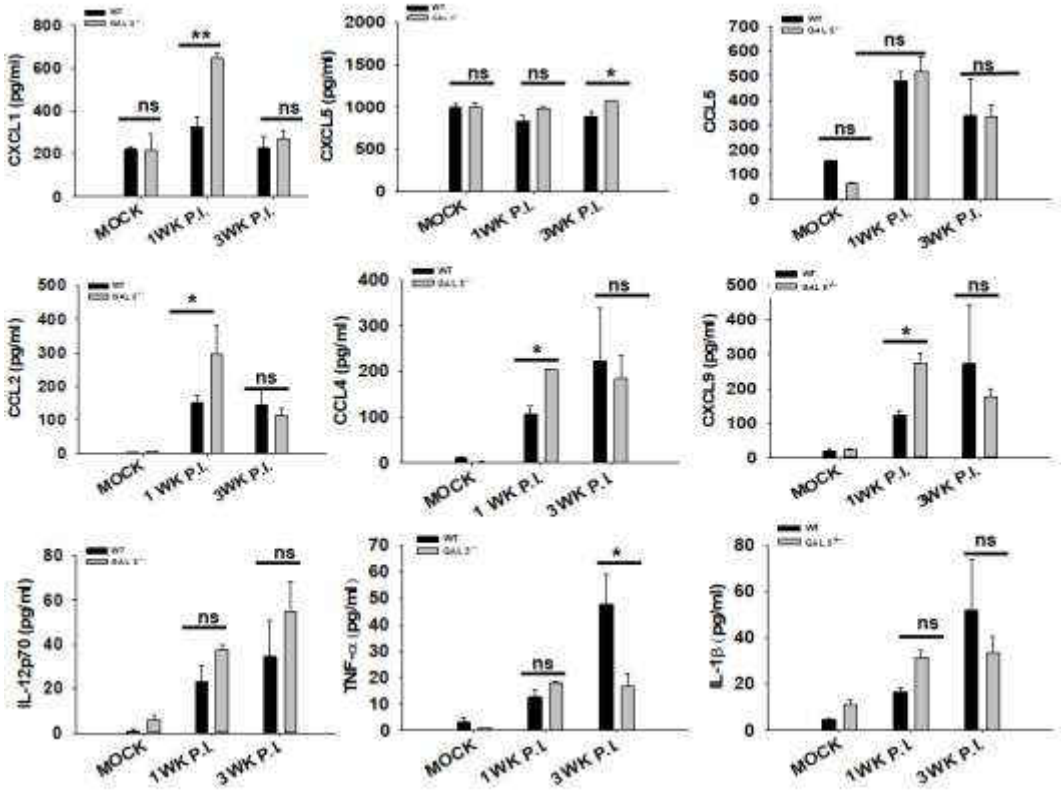

Figure II- 4. Inflammatory mediators in the CNS of parasite-infected Galectin 3$^{-/-}$ and WT mice.

The brains from mock control and *M. corti* infected WT and Galectin 3$^{-/-}$ mice were harvested at 1wk and 3wk i.c., homogenized, and the protein level of host immune mediators measured by CBA using flow cytometric analysis (BD Biosciences). Results shown are mean ± SEM of 3–4 each infected and mock control mice from 2–3 independent experiments. Statistical significances are denoted by asterisks (*, $p<0.05$; **, $p<0.005$).

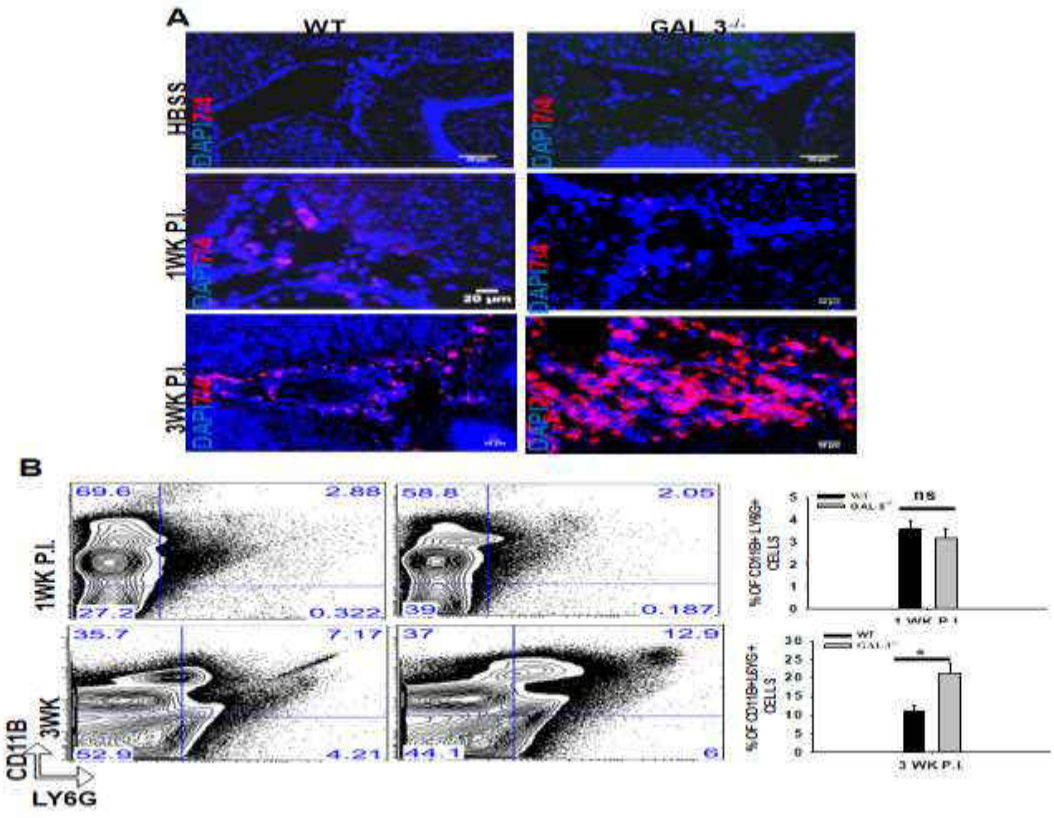

Figure II- 1. Parasite-infected Galectin $3^{-/-}$ mice exhibit increased neutrophil accumulation in brain.

(A)IF microscopy on brain cryosections from WT and Galectin $3^{-/-}$ mice i.c. injected with HBSS (mock) or with 60 parasites at 1wk and 3wk i.c. Neutrophils (7/4+) were detected by using an affinity purified anti-mouse 7/4 goat IgG followed by Alexa Fluor® 546 labeled (red). Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Images shown are representative of 3 independent experiments with 3–4 mice each group.(B) Flow cytometry analysis of CD11b and Ly6G on brain mononuclear cells harvested From mock control and *M. corti* infected WT and Galectin $3^{-/-}$ mice at 1wk and 3wk i.c. The cells were double-stained with anti-Ly6G-APC and anti-CD11b-Pacific Blue Antibodies as markers for neutrophils. Representative contour plots show CD11b+Ly6G+ neutrophils in brains of parasite-infected WT and Galectin $3^{-/-}$ mice. The bar graph on the right shows mean ± SEM of percent of CD11b+Ly6G+ neutrophils in brains of *M. corti* infected WT and Galectin $3^{-/-}$ mice each from 3 independent experiments. Statistical significances are denoted by asterisks (* $p<0.05$).

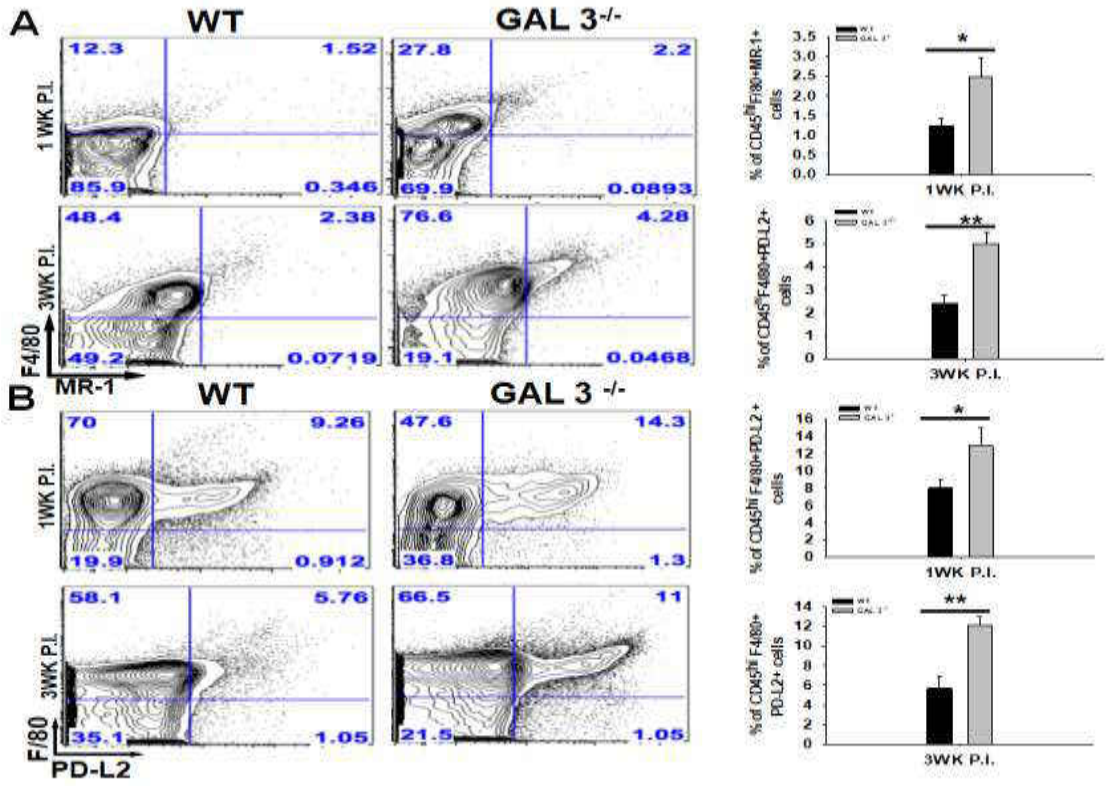

Figure II- 6. Parasite-infected Galectin 3$^{-/-}$ mice exhibit increased M2 macrophages accumulation in brain

(A) Flow cytometry analysis of CD45, F4/80 and Mr-1 or (B) CD45, F4/80 and PD-L2 on brain mononuclear cells harvested from mock control and *M. corti* infected WT and Galectin 3$^{-/-}$ mice at 1wk and 3wk i.c. The cells were with anti-CD45-Pacific Blue, anti-F4/80-PE-cy7, anti-MR-1-APC and PD-L2-PE antibodies as markers for infiltrating M2 macrophages in brains of *M. corti* infected WT and Galectin 3$^{-/-}$ mice each from 3 independent experiments. Statistical significances are denoted by asterisks (* $p<0.05$, ** $p<0.005$)

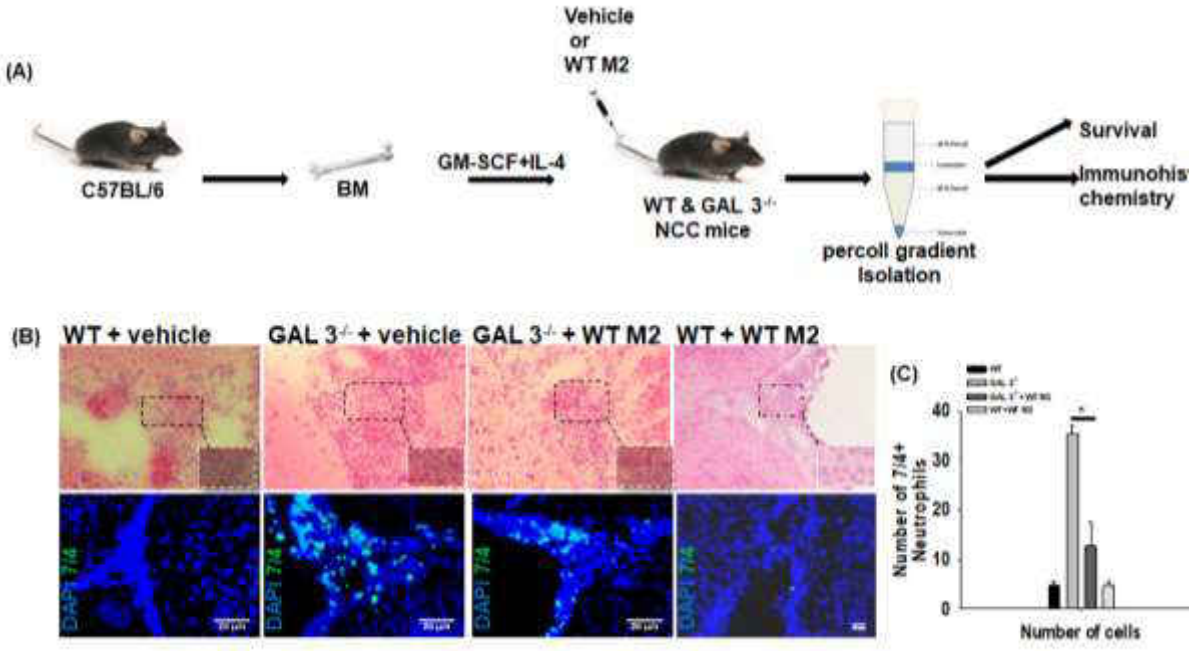

Figure II- 7. Galectin 3 expressing M2 macrophages exhibit protective role during NCC infection.

(A)Schematic representation of the adoptive transfer experiment as described in the Methods. Purified bone marrow macrophages from WT mice were differentiated to M2 phenotype by exposure to IL-4 *in vitro*. These M2-macrophages or vehicle alone were injected i.v. into WT or Galectin $3^{-/-}$ recipient mice at 1wk and 2wk of i.c. infection with *M. corti*. Survival of mice, brain tissue pathology and neutrophil accumulation in the CNS were compared to 3wk i.c. (B) Top panel shows representative images of H&E stained brain cryosections. Bottom panel show IF microscopy images of brain cryosections to compare accumulation of 7/4+ (green) neutrophils. (C) Bar graph showing 7/4+ neutrophils manually counted from images shown in (B). (D) The survival of NCC-WT or NCC-Galectin $3^{-/-}$ mice adoptively transferred with WT-M2 macrophages or vehicle was monitored for three weeks. Statistical significance was determined by student t test (** $p<0.005$).

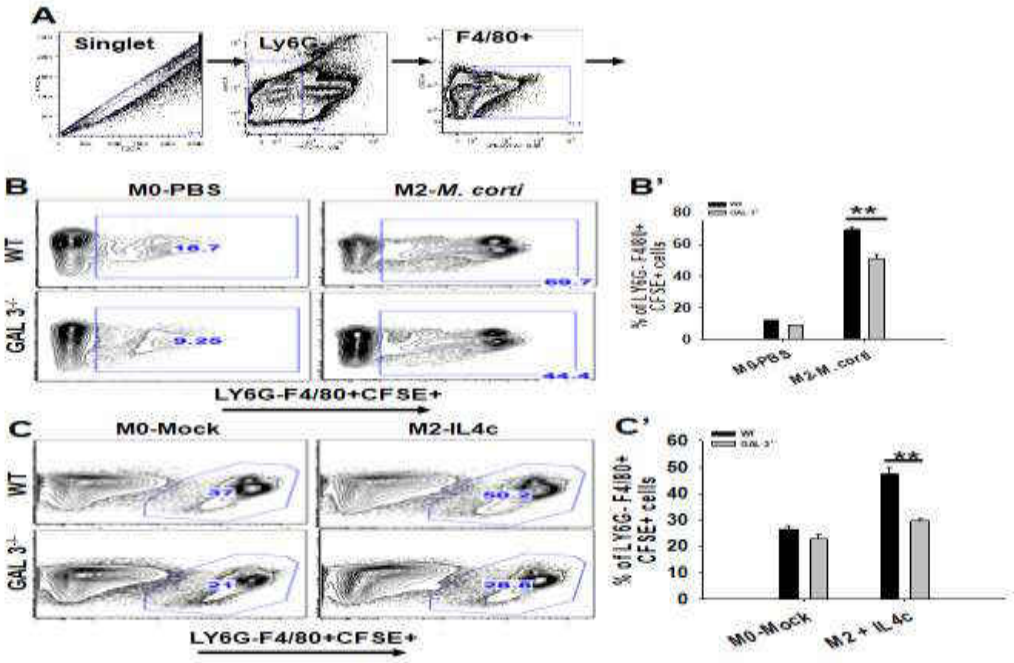

Figure II- 8. Galectin $3^{-/-}$ M2-macrophage exhibit reduced efferocytosis of neutrophils *ex-vivo*

(A). Gating scheme to select efferocytic cells. singlet cells gated on FSC-H and FSC-A and the Ly6G negative to eliminate untaken neutrophils that may be on the surface of macrophages and CFSE positive to quantify the macrophages that internalized the neutrophils (Ly6G-F4/80+ CFSE+ cells) (B). Efferocytosis of CFSE+ neutrophils by macrophages from WT and Galectin $3^{-/-}$ mock control mice that received vehicle (M0 PBS) or *M. corti* parasite (M2- *M. corti*) intraperitoneally. The bar graph (B') indicates is the percent of Ly6G-F4/80+CFSE+ macrophages that have internalized CFSE labeled neutrophils calculated from 3 independent experiments that are shown in the representative density plots (B). (C). Macrophages from WT and Galectin $3^{-/-}$ mock mice that received vehicle (M0-Mock) or IL4complex (M2- IL4c) by i.p. were analyzed for efferocytosis of CFSE labeled neutrophils. The bar graph (C') indicates is the percent of Ly6G-F4/80+CFSE+ macrophages that have internalized CFSE labeled neutrophils calculated from 3 independent experiments that are shown in the representative density plots (C).

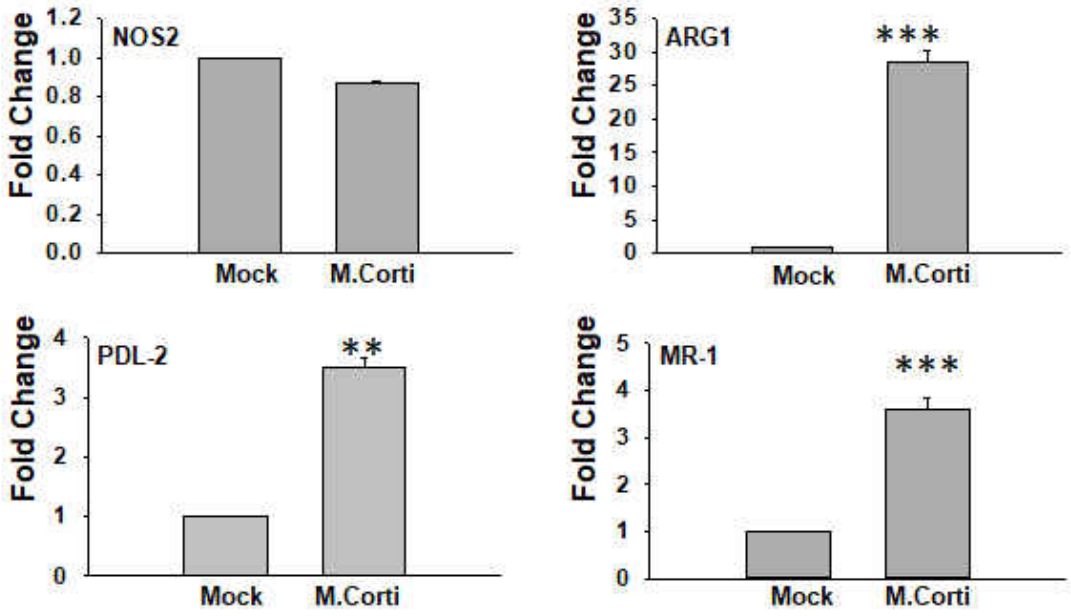

Figure II- S1. Peritoneal macrophage from WT mice exhibit M2-activation phenotype during *M. corti* i.p. infection.

Peritoneal macrophages from mock control and *M. corti* i.p. infected mice were harvested, and the relative mRNA expression of M2 mediators, Arg1, PDL-2, MR-1, and M1 inflammatory mediators, NOS2 was measured by qRT-PCR.
** $p \leq 0.01$ and *** $p \leq 0.001$ (Student's t-test).

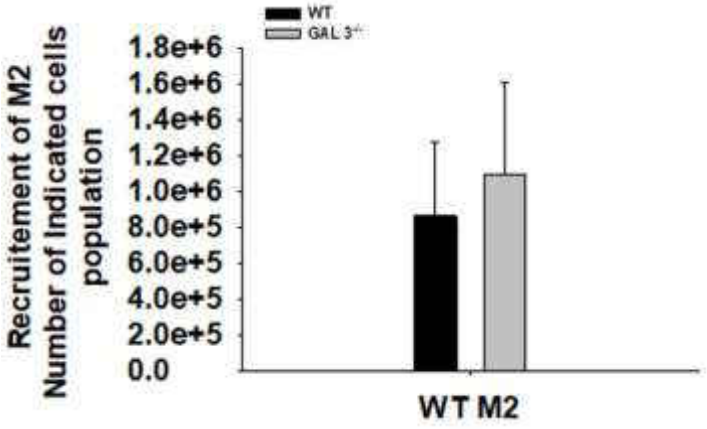

Figure S2. M2 macrophages extravasate into the CNS of WT and Galectin $3^{-/-}$ mice at similar rate

Purified bone marrow macrophages from WT mice differentiated to M2 activation by exposure to IL-4 *in vitro*, labeled with Carboxyfluorescein succinimidyl ester (CFSE) CellTracker Green, i.v. injected into WT or Galectin $3^{-/-}$ recipient mice at 1wk i.c. infection with *M. corti* prior to injection. Twenty four hour post adoptive transfer brains were harvested and processed for flow cytometry to measure the accumulation of CFSE positive cells in the CNS.

CHAPTER III

GALECTIN 7 ON ENDOTHELIAL CELLS IS INVOLVED IN ALTERNATELY ACTIVATED MACROPHAGE RECRUITMENT INTO THE CENTRAL NERVOUS SYSTEM

Fredice Quenum Zangbede, Arun Chauhan, Jyotika Sharma, Bibhuti B Mishra*

Department of Biomedical Sciences
University of North Dakota School of Medicine and Health Sciences
Grand Forks, ND 58201

Manuscript in preparation

Abstract

M2 macrophages function to dampen host inflammatory responses and promote wound healing and tissue repair. As the blood-brain barrier (BBB) restricts the flow of both cells into the brain, understanding how M2 macrophages extravasate into the central nervous system (CNS) is of great interest. In a mouse model of neurocysticercosis (NCC) we previously demonstrated that M2 macrophages are essential in containing neuropathology in helminth-infected CNS. Analysis of helminth-infected mice revealed an abundant expression of Galectin 7 (Gal 7) that recognize galactose moieties in brain endothelial cells. The Galectin $7^{-/-}$ NCC mice displayed a reduced accumulation of M2 macrophage in the parasite-infected brain. This reduction in M2 macrophage numbers in the CNS was correlated with an increased neuropathology and shortened survival. The use of adoptive transfer of fluorescently labeled cells demonstrated a decreased influx of M2 macrophage into the CNS of helminth-infected NCC brains. The use of in-vitro transmigration assay conclusively demonstrated a key function of Galectin 7 expressed on endothelial cells in centrally regulating M2 macrophage entry into the CNS.

INTRODUCTION

The central nervous systems (CNS) is regarded as an immunologically privileged organ due to the absence of a classically defined lymphatic drainage system and the presence of the blood-brain barrier (BBB) that restricts the influx of blood-borne molecules and immune cells. However, when brain homeostasis is compromised as a result of challenges such as during infection, injury, or trauma, innate immune activation can lead to infiltration of various leukocytes to mount stimuli specific responses in the CNS microenvironment (116). In this regard, microglia and infiltrating macrophages function as the primary regulators of the innate immune-mediated development of inflammatory responses in the CNS (122-125). The functional phenotypes of macrophage/ microglia vary in response to external stimuli they receive through a wide variety of surface receptors (50). These cells with the M1, or classical activation phenotype, release multiple pro-inflammatory molecules (51). M1-macrophages are critical effectors of inflammation and innate immunity as well as orchestrators of adaptive immunity to microbial infections (126, 127). Whereas, macrophage/ microglia with M2, or alternatively activated phenotype, produces factors involved in anti-inflammatory and tissue remodeling functions (49, 53, 54, 128). Interestingly, persistent M1 microglia/macrophages mediated pro-inflammatory mediators often have tissue destructive effects eventuating into severe neuropathological outcome in diseases affecting the CNS. In contrast, M2 phenotypes of these cells release numerous protective/trophic factors in vitro settings (129, 130). Because of the presumed ability of M2 cells to promote tissue repair/regeneration, transplantation of ex vivo activated M2 macrophages was used in clinical trial as a possible cell-based regenerative

strategy in spinal cord injury (131). Unfortunately, the phase II trial was not a complete success, and necessitating the requirement of further understanding of different aspect of M2 macrophage functions in the CNS. Use of pre-clinical models, where M2 macrophages play an important contributor to the CNS immunopathogenesis, could be vital to identify critical molecules responsible for their functions in brain.

The immune response during helminth parasitic infections predominantly involves M2 macrophages (132). Indeed, in a murine model of neurocysticercosis, due to intra-cranial infection with a helminth *Mesocestoides corti*, the newly recruited macrophages display an M2 phenotype (40, 43, 58). Importantly, a lack of the M2 phenotype in macrophages was correlated with profound nervous tissue pathology and severe disease in STAT6-/- NCC mice (42). Interestingly, the M2 macrophages influx coincided with the release of glycan containing antigens from the tegument of the parasite in the CNS microenvironment (40). Furthermore, glycan antigens with terminal galactose/galactosamine are released from the parasite early and to larger extent. This raised the possibility of these antigens may induce glycan (with-terminal galactose)-recognizing receptors to shape M2 macrophages trafficking into the CNS and the overall pathogenesis during brain parasitic infection. In this regard, the egress of leukocytes from the blood into tissues is tightly regulated and is mediated by a multistep process involving; leukocyte rolling, rapid activation of leukocyte, adhesion to endothelium, and diapedesis (117, 133). The interaction of leukocytes to the endothelium and successful transmigration involves different selectins that bind to specific carbohydrates, such as sialyl Lewis X (sLeX) on the endothelium or vice versa (117). Furthermore, studies show that C-type lectin receptors (CLRs) that bind to specific carbohydrates influence the outcome of

leukocyte accumulation in tissues by positively or negatively influencing leukocyte trafficking into the affected site (14). For example, CLRs such as MR1 and DC-SIGN have been observed to mediate cell trafficking across endothelium (134-138) whereas MGL and galectin 1 can inhibit migration of leukocytes through endothelium (137, 139). An important unanswered question, therefore, is whether specific lectin receptor/s are induced on brain endothelium during helminth infection to control M2 macrophage extravasation, which in turn play an essential role in the containment of this disease.

In this study, we identified that intracranial infection of mice with *M. corti* metacestodes results in upregulated expression of galectin 7 on brain endothelial cells. As galectin 7 expression was also upregulated in brain-derived endothelial cells in vitro after exposure to helminth factors or IL-4, the best-documented cytokines produced by the host in helminth infection. The susceptibility and immunopathology was compared between Galectin $7^{-/-}$ and wild-type (WT) mice infected intra-cranially with *M. corti*. The contribution of galectin 7 to CNS inflammation was assessed by measuring infiltration of various immune cells into the brain, as well as pro-and anti-inflammatory mediators response in CNS of Galectin $7^{-/-}$ and WT mice. By studying Galectin $7^{-/-}$ and WT mice in vivo or brain-derived endothelial cells ex-vivo, for the first time, we demonstrate that galectin 7 on brain endothelial cells regulate trafficking of M2 macrophage.

Materials and Methods

Mice and parasite inoculation.

Female mice age 4.5 to 6 weeks were used in this study. The C57BL/6 WT mice were purchased from Charles River Laboratories, Wilmington, MA. The Galectin $7^{-/-}$ mice were kindly provided to us by Francoise Portier (Universite Paris Diderot, France). Mesocestoides corti (*M. corti*) was kindly provided by Dr. Debrosky Herbert (Cincinnati Children's Research Foundation, Cincinnati, OH 45229, USA). *M. corti* metacestodes were maintained in the peritoneal cavity of BALB/c mice by serial intraperitoneal (i.P.) infection for propagation.

For intracranial infections, *M. corti* larvae were aseptically collected from the intraperitoneal cavity of the infected BALB/c mice and were extensively washed in HBSS. The metacestodes (about 60 parasites) were suspended in 50 µl of sterile HBSS and injected intracranially into 4.5 to 6 weeks old female mice using 1 ml syringes and a 25-gauge needle. Control mice were given 50 µl of sterile HBSS. Priority to the inoculation, the mice were anesthetized with a mixture of xyline (120 µl /ml), ketamine (220 µl /ml) and PBS. 50 µl of the mixture was given to each mice.

At 1 week and 3 weeks, mice were anesthetized using isoflurane and perfused through the left ventricle with 20 ml of cold PBS.

Brain mononuclear cell isolation and Flow cytometry

The brain was instantly removed from perfused mice and leukocytes were processed for antibodies staining for flow cytometry analysis. Briefly, leukocytes single cells suspensions were isolated from parasite-infected brains of WT and Galectin $7^{-/-}$ using percoll density gradient from GE healthcare (CAT# 17-0891-01). The cells were counted using trypan blue. $2x10^6$ cells were pre-incubated with Fc blocker for 10min and then incubated with 50 µl of antibodies cocktail for 30 min. after incubation, the cells were washed 2 times with 500 µl FACS buffer (10% FBS in PBS) and the pellets were suspended in 350 µl of FACS buffer and analyzed on a BD LSRII flow cytometer (BD Biosciences).

H&E Staining.

Parasite and HBSS infected brains were immediately removed from perfused WT and Galectin $7^{-/-}$ mice, embedded in OCT resin and snap frozen. Serial horizontal cryo-sections with 10 µm in thickness were positioned on slides (from Sigma-Aldrich, St. Louis, MO). Every 5^{th} slides were sectioned and stain following the H&E staining protocol previously described.

Adoptive transfer experiment

WT and Galectin $7^{-/-}$ mice were intracranially infected with M. corti for 6 days. They were then adoptively transferred with labeled BMDM which were previously cultured with IL-4 (M2) or IFN-γ (M1). The M2 macrophages were labeled with cell trace CFSE (CAT# C34554, Thermo scientific Waltham, MA) and the M1 was labeled with Cell Trace Far-Red orange dye (CAT# C34564, Thermo scientific Waltham, MA).The cells labeled cells were a mix at 1:1 ratio and intravenously injected to the infected mice. via tail

vein injection. Brains were harvested 24 hrs after the tail vein injections, the infiltrating cells were collected and processed for flow cytometry as described above. Adoptively transferred macrophages recruited to the brains were enumerated using an LSR II flow cytometer and analyzed using FlowJo software (BD Biosciences, San Jose, CA).

Endothelial cell's isolation

Mouse brain endothelial cells were isolated like it was described (140) and with the help of Dr. Abhay Sagare and Dr. Zlokovic V. Berislav with little modifications. Briefly, Brains were aseptically isolated for 8 weeks of WT and Galectin $7^{-/-}$ mice using the MCDB31 medium (GIBCO BRL, New York) supplemented with 2% FBS, 100U/ml penicillin, and 100 µl/ml streptomycin. Meninges and big vessels were removed. The cortices were then cut into small pieces and homogenized using tissues Dounce homogenizer. The mixture was suspended in 15% dextran and centrifuged. The pellet was re-suspended in 0.1% collagenase/dispase made with 2% FBS medium. The mixture was incubated for 6hr at 37°C with sporadic agitation. After the incubation, the mixture was centrifuged at 1000Xg for 5 and the endothelial cells and microvessels found in the top layer were collected, centrifuged and plated in tissues culture plate coated with rat tail collagen I (Roche Diagnostics, Mannheim, Germany) and incubated at 37 with 5% $C0_2$. Every 2 days, the media was changed to enhance recovery.

After few days of culture, the cells were collected using enzyme-free dissociation buffer and a magnetic purification step was used to reduce contamination. Briefly, the cells were incubated with rat CD31 primary antibody (1:50, BD Pharmagen, Lexington, KY) for 30 min at 4°C with gentle mixing. After incubation, cells were centrifuged and incubated with Dynabeads M-450 Sheep anti-rat for 20 min with shaking. The cells were

then washed and collated using magnet and plate on collagen I coated plate for father incubation. In addition, 70,000 cells were seeded in the insert with 8 µm pore (CAT# Corning) with media in the low chamber of the transwell.

Trans-endothelial migration essay

Once the cells had grown to appropriate confluency in the insert, FITC labeled dextran was used is a tie junction between the endothelial cells, 5µg/ml of dextran (3000MW, CAT#D3305 Thermo Fischer Scientific) was diluted in PBS and added to the endothelial cells. the concentration of dextran was measured in the low media using colorimeter. The cells were stimulated with Parasite derived molecules for 12 hrs. The lower media was changed with fresh media. Then the bone marrow-derived macrophages that were previously cultured with IL-4 to induce M2 phenotype were added to the upper chamber of the insert $5x10^5$ macrophages were added with 500 µl of media. In order to mediate migration of the macrophages to the lower chamber, macrophages MCP-1 at 100ng/ml was added to the media in the low chamber. After 24 hr of incubation, cells in the low chamber were counted.

Immunofluorescence

Immunofluorescence microscopy was used to determine the presence of immune mediators and/or specific cells types in brains of parasites infected and non-infected mice at various times post-infection 1wk. Brains from mock (HBSS infected) mice were used as controls and IF staining. All steps were carried out at room temperature. Briefly, the sections were incubated 10% host serum for 30 min, then with specific antibodies in PBS buffer with 3% host serum to prevent non-specific binding. After 40 min, the sections were washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20 washing

buffer. The sections were then incubated with appropriate secondary antibodies for 30 min and were washed seven times with the above washing buffer. For double immunofluorescence staining, the above-mentioned procedures were sequentially repeated for each additional staining. The sections were mounted using Fluorsave reagent (Calbiochem, La Jolla, CA) containing 0.3 μM 4', 6'-diamidino-2-phenylindole (DAPI)-diacetate (Molecular Probes). Additional control staining was performed to rule out any nonspecific staining. In each case, sections were blocked with saturating concentrations of appropriate host serum antibodies to eliminate false positive staining due to FcR-mediated nonspecific binding. Staining in the absence of primary antibodies provided additional negative controls.

In addition, IF microscopy in vitro settings using primary brain-derived endothelial cells (isolation described above) and bEnd.3 [BEND3] (ATCC, USA), a brain capillary endothelial cell line. Cells were pulsed with medium alone or exposed to helminth secreted/ soluble factors (HSF) or with IL-4. Bend.3 cells line ([Bend.3] (ATCCR CRL-2299TM). with specific antibodies in PBS buffer with 3% host serum to prevent non-specific binding. After 40 min, the sections were washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20 washing buffer. The sections were then incubated with appropriate secondary antibodies for 30 min and were washed seven times with the above washing buffer. For double immunofluorescence staining, the above-mentioned procedures were sequentially repeated for each additional staining.

Antibodies and reagents

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. For detection of Galectin 7 by immunofluorescence (IF) staining, a purified rat anti-mouse

galectin 7 antibody clone 212923 (R&D system, Minneapolis, MN) followed by Alexa 546 conjugated goat anti-rabbit antibody (Molecular Probes, OR) was used. A rat anti-mouse CD31 antibody conjugated to Alexa 488 (BD Pharmingen) were used for IF staining. A purified goat anti-mouse MGL1/2 (CAT# AF4297, R&D system, Minneapolis, MN) followed by donkey anti-goat Alexa 488/Alexa546 (Molecular Probes, OR). A purified goat anti-mouse MR-1 (CAT# AF2535, R&D system, Minneapolis, MN) followed by donkey anti-goat Alexa 488/Alexa546 (Molecular Probes, OR). For flow cytometry FITC anti-mouse CD11b (clone M1/70), PE-cy7 anti-mouse F4/80 (clone BM8), PE anti-mouse TCR γδ (clone GL-3), PE-cy7 anti-mouse siglec-f (clone FC50-2440), APC anti-mouse CD206 (clone C068C2), PE anti-mouse PD-L2 (T425), pacific blue anti-mouse CD45 (clone 30-F11), and APC anti-mouse Ly6G (Clone 1A8) antibodies (Biolegend, San Diego, CA) were used. The terminal deoxyribonucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining kit was purchased from Chemicon International, CA. The endotoxin level was <1.0 EU per μg of protein. Ultrapure E. coli endotoxin was purchased from Invivogen and lactose was purchased from Sigma.

Quantitative Real-Time PCR

Brain from infected and mock control WT and Galectin $7^{-/-}$ mice 1 week and 3 weeks post infection were directly perfused and the total RNA was extracted using trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis of the samples was done using SYBRO green (Applied Biosystems, CA (USA). Transcript levels of the housekeeping ribosomal 18S and Galectin 7 genes were measured in each sample by PCR amplification using specific primers: 18S (sense) *5′*- CAT GTG GTG TTG AGG AAA GCA-3' and (antisense) 5′- GTC GTG GGT TCT GCA TGA TG-3′; galectin

7 (sense) 5'-GGC CAC TTT-GTT-GTA-TTG-3' and (antisense) 5'-GAC CAT GCC TCG AAT TCT CAT-3'

Statistical Analysis

The survival of the infected WT and Galectin $7^{-/-}$mice was compared using Log-rank (Mantel-Cox) Test. The statistical comparison between levels of host mediators was done with student's t-test using Sigma Plot 8.

Results

Galectin 7 is abundantly expressed in brain endothelial cells during murine NCC

To examine the role of galectin 7 in parasite-infected brains, its transcript-level of expression was determined by qRT-PCR using RNA from mock and *M. corti* i.c. infected brains. Increased mRNA expression (by ~ 2.5-fold) of galectin 7 was observed at 1wk p.i. over mock control at 1wk p.i. (Figure.III-1A). To identify the cell-specific expression of galectin 7 protein, in-situ co-immunostaining was performed on brain cryosections of mock control and parasite-infected mice. In mock-infected mice brains, galectin 7 was undetected (Figure.III-1B1). In contrast, parasite-infected brains exhibit an increased expression of galectin 7, which was primarily evident in periventricular and leptomeningeal areas of the NCC brain (Figure.III-1B1-B2). A unique extracellular matrix (ECM)-like fibrillar staining pattern for galectin 7 was observed (Figure.III-1B1-B2). Double IF staining on the parasite-infected brain sections showed large numbers of round monocyte/ macrophage-like CD11B+ or F4/80+ cells extravasating through the pial vessels in meninges and detected in proximity of galectin 7 (Figure.III-1B3).

The expression of galectin 7 on brain endothelial cells is unknown. To test the physiological relevance during helminth infections, the specificity of galectin 7 expression on brain endothelial cells was evaluated by IF microscopy in *vitro* settings using primary brain-derived endothelial cells as well as bEnd.3 [BEND3], a brain capillary endothelial cell line. Cells were pulsed with medium alone or exposed to helminth secreted/ soluble factors (HSF) or with IL-4. In this regard, IL-4 has been shown to be the critical cytokine

shaping many of the host response to helminth infections, including the signature M2 phenotypic response by macrophage. Both bEnd.3 [BEND3], cells and primary brain endothelial cells exposed to HSF exhibited increased galectin 7 expression (Figure. III-1C). On the other hand, galectin 7 was undetected in the naïve/ unstimulated control cells cultured in presence of medium only (Figure. III-1C). Moreover, galectin 7 expression at protein level was detected at a massively higher level in IL-4 exposed bEND3 cells compared to the corresponding unstimulated naïve/ control cells (figure.III-1C). Together, the results confirmed that HSF and IL-4 stimulation induces an increased galectin 7 expression, specifically in endothelial cells (CD31+) *in vitro*.

Galectin 7$^{-/-}$ mice exhibit increased disease severity during murine NCC

To investigate whether Galectin 7 influences host immunity to parasite infection of the brain, disease severity was compared between WT and Galectin 7$^{-/-}$ mice were infected i.c. with *M. corti*. As reported previously (141, 142), infected WT mice displayed typical neurological signs including abnormal vestibular function, tilted head, cerebral abnormalities, morbidity, and weight loss between 1-3wk p.i. In general, these overt signs appeared earlier and were more accentuated in Galectin 7$^{-/-}$ mice. In addition, Galectin 7$^{-/-}$ mice exhibited decreased survival. By 5wk p.i., only ~10% % of *M. corti*-infected WT mice succumbed to the infection as opposed to ~70% of Galectin 7$^{-/-}$ mice (Figure.III- 2A, $p<0.0005$).

To determine the parasite burden, serial horizontal sections of parasite-infected WT and Galectin 7$^{-/-}$ brains of mice were stained with H&E, evaluated by microscopic analysis.

The brains of Galectin $7^{-/-}$ mice exhibited increased numbers of parasites as compared to the infected brains of WT mice (Figure.III- 2B). Additionally, an increased numbers of *M. corti* was observed in brain parenchyma in Galectin 7-deficient mice, however, it was not statistically significant (Figure.III- 2B). Together, the results suggest that in the absence of galectin 7 dependent responses lead to an increased parasite growth in the CNS.

Next, H&E staining was performed to determine immunopathological changes in WT and Galectin $7^{-/-}$ mice (Figure.III- 2C). Both the WT and Galectin $7^{-/-}$ mice displayed normal brain tissue morphology. In contrast, a large number of infiltrating immune cells was detected in the CNS of both WT and Galectin $7^{-/-}$ (Figure.III- 2C), albeit a somewhat lower number of infiltrating cells with morphology consistent with monocytes/ macrophages (round cells) was observed in the extra-parenchyma of parasite-infected Galectin $7^{-/-}$ mice as compared to the WT (Figure.III-. 2C).

To examine the impact of galectin 7 deficiency on the tissue pathology, TUNEL staining was performed on the brain tissue sections of WT and Galectin $7^{-/-}$ mice. Very little to no TUNEL positive cells were detected in the CNS of mock of both WT and Galectin $7^{-/-}$ mice (data not shown). Although the TUNEL staining remained low in the parasite-infected WT brains, the Galectin $7^{-/-}$ mice displayed an increased TUNEL staining in the CNS (Figure.III- 2C). These data suggest that absence of galectin7 dependent responses leads to greater CNS inflammatory pathology during NCC.

Galectin $7^{-/-}$ mice display decreased M2 macrophage accumulation in the CNS after parasite infection

To determine the physiological relevance of galectin 7 on the infiltration of immune cells into the CNS during NCC, flow cytometry analysis was performed. Mononuclear cells from the whole brain of Galectin $7^{-/-}$ and WT infected mice at 1wk p.i. The percent of macrophages (CD11b+F4/80+), neutrophils (CD11b+Ly6G+), eosinophils (Siglec-F+), γδ T cells (TCRδ+), αβ T cells (TCRβ+) or B cells (CD19+) were measured. No significant differences in the numbers of neutrophils, eosinophils, γδ T cells, αβ T cells or B cells at 1wk p.i. in the CNS of parasite-infected Galectin $7^{-/-}$ compared to the WT mice (Figure.III-3). However, infected Galectin $7^{-/-}$ mice displayed significantly lower numbers of infiltrating macrophages in the CNS at 1wk p.i. Thus, these data demonstrate that absence of galectin 7 leads to a significant reduction in macrophage recruitment into the brain during NCC.

Our previous studies have demonstrated that mice deficient in STAT6, an adaptor molecule contribute to the development of M2 macrophage phenotype (4), exhibit a lack of macrophages with M2 phenotype in the CNS correlated with increased pathological signs and severe mortality in NCC. To determine the effect of galectin 7 deficiency on the level of macrophages with M2 phenotype in the CNS during NCC, the expression of MGL1/2 (a cell surface marker specific for M2 cells) was analyzed in Galectin $7^{-/-}$ mice by IF microscopy (Figure.III- 4A). Consistent with our previous findings, parasite-infected WT mice displayed an accumulation of large numbers of macrophages (CD11b+ cells)

with MGL1/2 (M2 activation phenotype) in the CNS at 1wk p.i. (Figure.III- 4A). In contrast, the parasite-infected Galectin $7^{-/-}$ mice exhibited reduced accumulation of CD11b+MGL1/2+ M2 macrophages in the CNS (Figure.III- 4A). Taken together, the results here suggested that deficiency of galectin 7 is associated with a reduced influx of M2 macrophages in the CNS during NCC.

Next, to determine if the infiltration of M2 macrophages is affected in the absence of galectin 7 flow-cytometric analysis was performed by staining the cells with anti-CD45, anti-F4/80, and PD-L2. It is important to note that $CD45^{hi}F4/80^{+}$ cells in the CNS are known to be infiltrating macrophages, whereas PD-L2 is a well-known marker of M2 activation phenotype in macrophages (29, 45). Indeed, CD45hiF4/80+PD-L2+ M2 macrophages were detected at significantly reduced numbers in the CNS of infected Galectin $7^{-/-}$ mice at 1wk p.i. as compared to their WT counterparts (Figure.III- 4B). Together our data suggested that galectin 7 likely plays a role in the accumulation of M2 macrophages in the CNS.

Galectin 7 deficiency causes decreased M2 macrophages extravasation *in vivo* and *in vitro*

In order to confirm the role of galectin 7 in M2 macrophage recruitment into the CNS of NCC mice, we performed adoptive transfer of WT (Galectin $7^{+/+}$) M2- and M1-macrophage into WT and Galectin $7^{-/-}$ mice (Figure.III- 5A, B). For this, naïve bone marrow-derived macrophages were polarized to M2- phenotype after exposure to IL-4,

whereas cells were stimulated with IFN-γ to drive their polarization to M1-functional phenotype (verified by expression of M1 activation marker NOS2) (data not shown). For adoptive transfer experiments, M1 and M2 macrophages were labeled with two different intracellular dyes (M1 cells with CFSE and M2 cells with CellTrace^{TM} far-red dye). Injecting a mix of M1- and M2- types of macrophages and in equal numbers allowed us to compare the relative migration efficiency of these cells in the exact same microenvironment. The *M. corti* i.c. infected WT and Galectin $7^{-/-}$ mice at 1wk p.i. received M1 or M2- macrophages by i.v. followed by flow cytometry analysis to measure CFSE+ cells and CellTrace^{TM}+ cells after in the whole brain mononuclear cells. We observed that M2 macrophages were recruited in lesser numbers in parasite-infected brains of recipient Galectin $7^{-/-}$ mice (Figure.III- 5B). However, no difference in the recruitment of M1-macrophages was observed in recipient WT and Galectin $7^{-/-}$ mice. (Figure.III-5B). Notably, i.v. injected M1 cells migrated with a lower relative efficiency compared to the M2 cells in the CNS microenvironment of the recipient parasite-infected WT and Galectin $7^{-/-}$ or WT mice. This is likely due to a CNS microenvironment, which is conducive for M2 macrophage recruitment. Nonetheless, these results further solidified the fact that WT (Galectin $7^{+/+}$) M2- macrophages, but not the M1 macrophages, infiltration into the brain of Galectin $7^{-/-}$ NCC mice is reduced. Next, transwell migration assay was performed to measures the capacity of M2 macrophages to transmigrate across a monolayer of brain-derived endothelial cells from WT and Galectin $7^{-/-}$ mice. Brain endothelial cells were isolated from both WT and Galectin $7^{-/-}$ mice. Cells were cultured in a transwell insert to

form a monolayer as described in methods and by Dr. Zlokovic group (140). Cells were stimulated with helminth soluble/ secreted factors for 24h to induce galectin 7 expression. Bone marrow-derived macrophages that were polarized to M2- phenotype (described before), CFSE labeled, and $5x10^5$ number of cells added to the upper chamber. The lower chamber contained media with 100ng/ml of MCP-1 chemoattractant for macrophages. Deficiency of galectin 7 on endothelial cells in upper chamber appear to affect M2-macrophage trans-endothelial migration as evidenced by lower numbers of CFSE+ cells accumulated in the corresponding lower chamber (Figure.III- 5C). Together, the findings suggested a novel role of galectin 7 in specifically controlling M2 macrophages extravasation through brain en Galectin $7^{-/-}$ NCC mice display reduced M2-macrophage associated anti-inflammatory/ immune mediators in the CNS

Galectin $7^{-/-}$ mice display decreased M2- macrophage-associated anti-inflammatory/ tissue protective mediators in the CNS after parasite infection

To determine a functional relevance of galectin 7 in the anti-inflammatory/ tissue protective response in parasite-infected CNS microenvironment, levels of multiple immune mediators e.g. cytokines, chemokines, and enzymes with tissue repair function, were compared in brain homogenates by CBA analysis and/ or RT-PCR analysis (Figure.III- 6). As compared to the mock animals the M. corti-infected WT and Galectin $7^{-/-}$ mice brains exhibited increased levels of several inflammatory molecules, including key factors e.g. TNF-α, IL-1β, IL-12p70, IL-6, GM-CSF, IFN-γ, CXCL-9, -10, and NOS2, however there

were no statistically significant differences in their levels observed between infected Galectin $7^{-/-}$ and WT mice (Figure.III- 6). On the other hand, significantly lower levels of anti-inflammatory factors (IL-13, IL-9, and especially the IL-10) were detected in the brain homogenates of parasite-inoculated Galectin $7^{-/-}$ mice at 1wk p.i. compared to the WT. Moreover, the significance of galectin 7 was further corroborated with the RT-PCR analysis to measure the mRNA level of the factors directly involved in tissue regeneration/ repair such as YM1, Fizz1, and ARG-1. M. corti-infected brains of WT mice displayed a massive upregulated expression of these molecules (25 to several hundred-fold) over those in mock animals at both 1wk p.i. (Figure.III- 6B). In contrast, there was only a moderate increase in the mRNA level of these anti-inflammatory/ tissue protective factors in the CNS of parasite-infected Galectin $7^{-/-}$ mice (Figure.III- 6B). Our results strongly suggest a decreased anti-inflammatory/ tissue protective response in the CNS of Galectin $7^{-/-}$ mice after brain parasite infection.

Discussion

Increasing evidence supports an important role for M1 and M2 phenotype-specific roles of macrophages in multiple steps of CNS repair/regeneration (130). A switch from M1 to M2- phenotypic macrophages/ microglia facilitate diverse protective functions such as remyelination, oligodendrocyte renewal and white matter integrity, axonal regeneration, promote neurogenesis in models of multiple sclerosis to CNS injuries (143-145). On the other hand, a phenotype shift from M2-dominant to M1-dominant of macrophages at the site of injury impairs CNS repair (146-150). As most of the macrophages are recruited from the blood circulation to the CNS site in pathological settings, a better understanding of the mechanism of trafficking of these cells to the CNS is needed. It is also critical to identify how the macrophage trafficking regulator/s affect the anti-inflammatory/ tissue protective effect of these cells once inside the brain. Together, these could lead to the identification of novel therapeutic strategies to modulate macrophage function to promote CNS repair and/ or contain CNS pathology. Here we report that galectin 7, a host lectin receptor is upregulated specifically on the brain endothelial cells, and impacts the outcome of brain helminth infection as evidenced by a reduced survival, enhanced CNS pathology, because of decreased accumulation of M2- macrophages and associated immune mediators in the CNS of Galectin $7^{-/-}$ mice. The use of *in vitro* transmigration assay as well as *in vivo* adoptive transfer experiments provided mechanistic basis of a key regulatory function of galectin 7 on endothelial cells in centrally regulating M2 macrophage entry into the CNS.

Our study shows, for the first time, a CNS protective role of galectin 7 by controlling M2 macrophage influx into the helminth-infected brain.

After the infection of the CNS by *M. corti* during murine NCC, both parasites and immune cells are initially accumulated in extra parenchymal areas of the brain, which include ventricles, meninges and subarachnoid spaces (10, 30, 32, 39, 40). As the infection progresses, parasites and immune cells extravagate into the brain parenchyma typically depending upon the duration of infection (10, 30, 32, 39, 40). Macrophages represent the first type of immune cells to infiltrate into the brain (within first 3d p.i.) and are the predominant leukocyte populations throughout the infection process (4, 30). It was recently demonstrated that the initial release of galactose/ galactosamine containing glycan antigens by 1-2d p.i. precedes the accumulation of infiltrating macrophages with M2 activation phenotype. The early upregulated expression of galectin 7 on endothelial cells of the extra-parenchymal CNS vasculature in the *M. corti*-infected WT brain, and in brain-derived endothelial cells after activation with helminth soluble/ secreted factors or IL-4 *in vitro*, suggest that galectin 7 may regulate the recruitment of immune cells into the brain. The severe reduction in M2 macrophages accumulation in the CNS of *M. corti*-infected Galectin $7^{-/-}$ mice supports this idea. However, the early accumulation of all other immune cells were unaffected in Galectin $7^{-/-}$ mice compared to their WT counterparts, indicating that Galectin 7 may be specifically involved in M2-macrophages influx only. Indeed, the adoptive transfer of both M1 and M2-macrophages of the WT origin demonstrated that

Galectin 7 was required only for the accumulation of M2-macrophages, but not M1-macrophages, in the parasite-infected brains.

Our findings raised the fundamental question of whether the direct interaction galectin 7 on brain endothelial cells have with M2 macrophage facilitates regulates the M2 cells trafficking. Alternatively, perhaps galectin 7 has an indirect role in this process by functioning as a PRR, whose activation leads to the induction of chemokines modulating M2 macrophages trafficking into the CNS. However, the expression of various key macrophage recruiting chemokines were unaffected in the CNS of Galectin 7-deficient mice following parasite infection. In case galectin 7 functioning as a PRR, we would expect to observe a decrease in macrophage chemoattractants in the CNS of Galectin $7^{-/-}$ mice. Furthermore, we would expect the adoptively transferred M1 and M2 macrophages to reach the CNS of both WT and Galectin $7^{-/-}$ NCC mice with similar frequency, which was not the case. On the other hand, *in vitro* transmigration assay settings, the extravasation of M2-macrophages, but not M1-macrophages, was impaired through the monolayer brain-derived endothelial cell from Galectin $7^{-/-}$ mice compared to their WT counter parts. These results further highlight the likely critical function of the galectin 7 on brain endothelial cells directly interacting with the M2 macrophages and mediating their transmigration into the CNS during brain parasitic infection.

Galectin 7 deficiency resulted in increased parasite burden both in the extra-parenchyma and parenchyma of the brain. It is likely that reduced numbers of M2 macrophages play a role in limiting parasite growth in the brain of Galectin $7^{-/-}$ mice. This

is consistent with the results from our previous studies (4, 10) in murine NCC as well as from studies involving other parasite infections, a defect in Th2 dependent M2-macrophages associated responses contribute to parasite growth correlated with increased disease severity in the host (151-153). Although a direct role of Galectin 7- mediated M2-macrophage accumulation and associated responses, contributing to disease severity was not established in the present study, the enhanced disease severity of Galectin $7^{-/-}$ NCC mice was correlated with decreased IL-10 and IL-13 in the CNS as compared to WT mice. This indirectly indicates a possible protective role of galectin 7 through influencing M2-macrophages trafficking in NCC. We are currently performing relevant adoptive transfer experiments to identify whether the transfer of WT M2 macrophages directly into the CNS of Galectin $7^{-/-}$ NCC mice by i.c. injection with WT M2-macrophages would ameliorate the disease severity. The findings that galectin 7 on brain endothelial cells function as a central regulator in M2-macrophages influx into the CNS, and in its absence the reduced accumulation of M2 cells contribute to the lethality of Galectin $7^{-/-}$ mice, may offer important mechanisms to target in CNS inflammatory disease conditions.

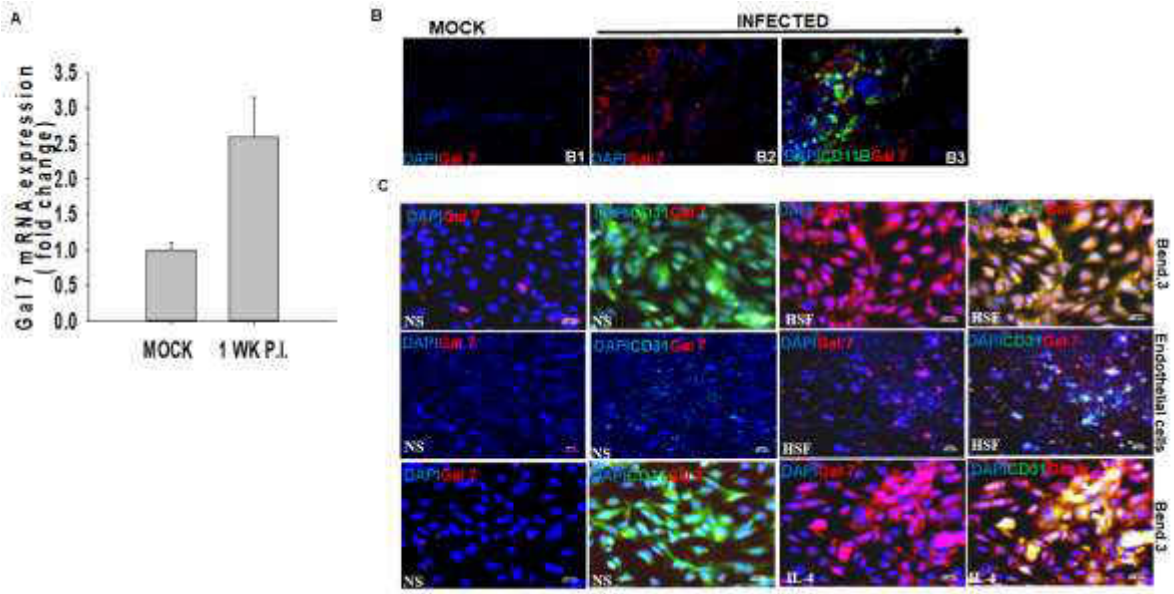

Figure III- 1. Expression and distribution of galectin 7 in the parasite-infected brains and endothelial cells.

Quantitative RT-PCR analysis and IF microscopy were performed on parasite-infected and mock control mice i.c. injected with helminth parasite *M. corti* and endothelial cells. (A) The total RNA was extracted from the brains at 1wk post inoculation and mRNA expression of galectin 7 was measured by qRT-PCR as described in Methods. The fold changes in parasite-infected brains were calculated over the levels in mock control mice using the formula $2^{-(\Delta\Delta Ct)}$. Data shown are the mean ± SEM of 3–4 mice per time point in two independent experiments. (B1-B2) Immunofluorescence staining was performed on brain tissue cryosections from mock or parasite- infected C57/BL6 mice. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Galectin-7 was stained using an affinity purified anti-mouse galectin 7 goat IgG followed by Alexa Fluor® 546 labeled (red) goat anti-rabbit IgG. (B3) Immunofluorescence staining was performed using purified anti-mouse galectin 7 and purified anti-mouse CD11B followed by Alexa Fluor® 488 labeled (Green). Images shown are representative of 3 independent experiments with 3–4 mice each. Magnification of 20X. (C) Immunofluorescence staining was performed brain Bend3 endothelial cells lines and primary endothelial cells pulsed with medium alone or exposed to helminth secreted/ soluble factors (HSF) or with IL-4. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Galectin 7 was stained using an affinity purified anti-mouse galectin 7 rat IgG followed by Alexa Fluor® 546 labeled (red) goat anti-rat IgG and CD31 was stained using an affinity purified anti-mouse CD31 rat IgG followed by Alexa Fluor 488 labeled (green). Images shown are representative of 3 independent experiments with 3–4 mice each. Magnification of 20X.

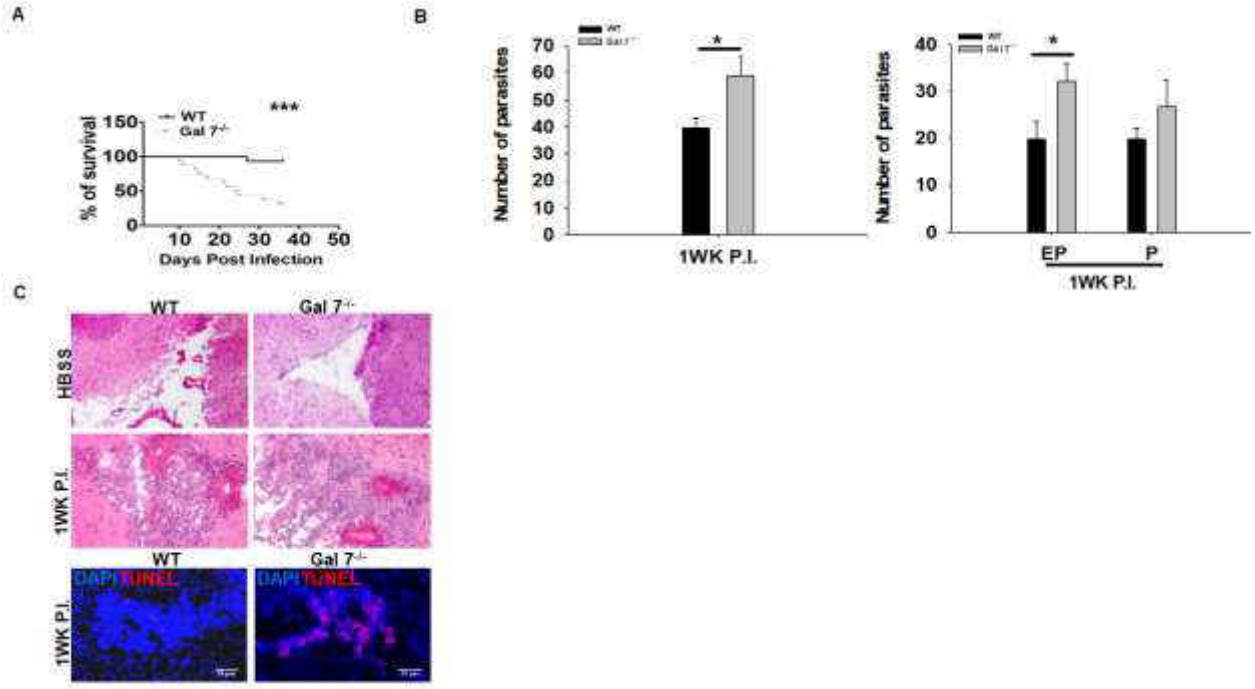

Figure III- 2. Galectin $7^{-/-}$ mice exhibit reduced survival and increased brain pathology and parasite burden as the WT mice.

(A) WT and Galectin $7^{-/-}$ mice were i.c. infected with 60 *M. corti* and were assessed daily for disease severity. The survival was monitored for 3 weeks post inoculation. Statistical comparison of susceptibility was done by Log-rank (Mantel-Cox) Test, P<0.0005***, n=9-10. (B) Number of parasites at 1wk and 3wk p.i. in parenchymal (P) and extra parenchymal (EP) regions in individual Galectin $7^{-/-}$ and WTbrains were calculated by microscopic examination of serial H&E-stained brain sections. The bars show average +/- SEM parasites from 5-6 mice per group. (C) Representative microscopic evaluation of H&E stained brain cryosections from WT and Galectin $7^{-/-}$ mice i.c. injected with HBSS (mock) or with 60 parasites at indicated times p.i.. Magnification 200X. IF microscopy analysis was performed on brain cryosections from WT and Galectin $7^{-/-}$ mice i.c. infected with 60 *M. corti* parasites to detect TUNEL positive staining (Red). Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Images are representative of 5-6 mice in each group from three independent experiments.

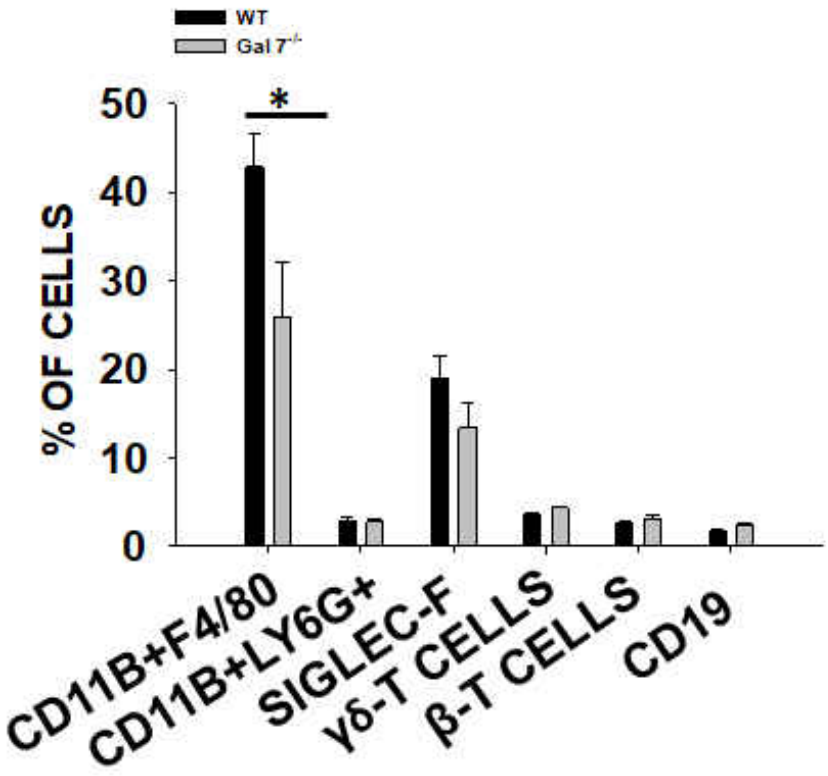

Figure III-3. Galectin $7^{-/-}$ mice display decreased M2 macrophage accumulation in the CNS after parasite infection

Brains were isolated from infected WT and Galectin $7^{-/-}$ and infiltrating cells were isolated, stained and analyzed using flow cytometry. The cells were stained for different infiltrating cells. Each bar represents percent cells positive for individual markers in brains of indicated experimental mice. Data were analyzed using the flowjo software.

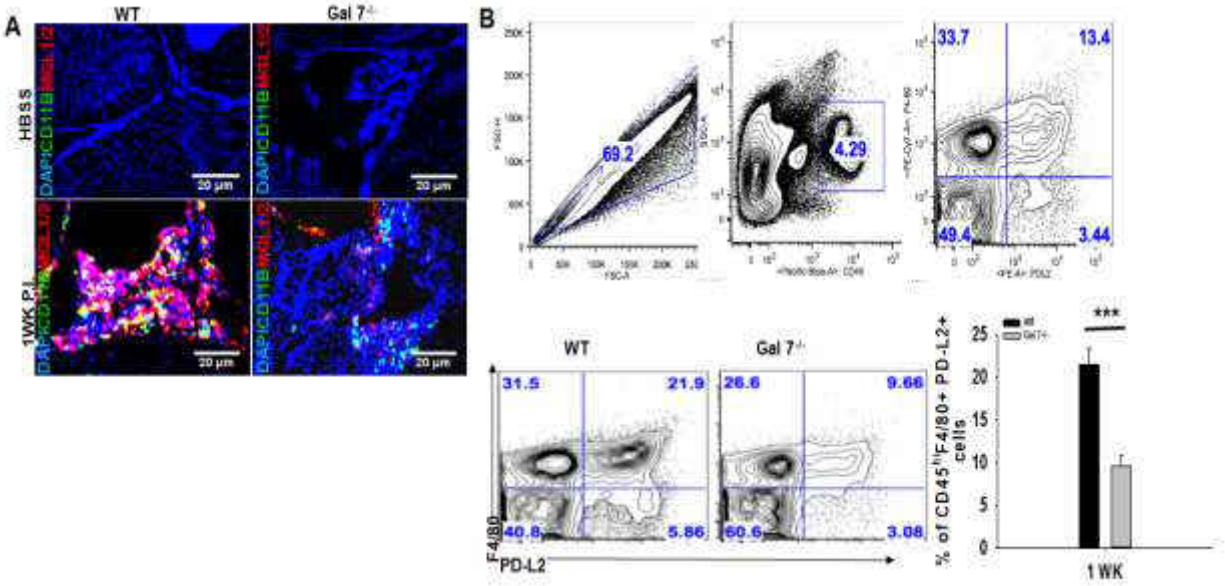

Figure III- 5. Parasite-infected Galectin 7$^{-/-}$ mice exhibit decreased M2 macrophages accumulation in brain.

(A) Double immunofluorescence staining was performed on brain cryosections from WT and Galectin 7$^{-/-}$ mice i.c. injected with HBSS (mock) or with 60 parasites at 1wk .i.c. Macrophages (MGL-1^{+}) were detected by using an affinity purified anti-mouse MGL-1 goat IgG followed by Alexa Fluor® 546 labeled (red). CD11B was stained using purified CD11B goat IgG followed by Alexa Fluor® 488 labeled (green). Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Images shown are representative of 3 independent experiments with 3–4 mice each group. (B) Gating scheme to select C45^{hi}F4/80+PD-L2 positive cells. Collected cells with gated with FSC and SCC singlet cells. The cells were then gating on CD45 and F4/80 and PD-L2 positive cells. Representative FACS plots gated on CD45^{hi}, F4/80+ and PD-L2+ (infiltrating M2 macrophages) from individual mouse brain showing cell surface expression of PD-L2. Results are representative of 3 independent experiments. ***P <0.005 as determined by t test

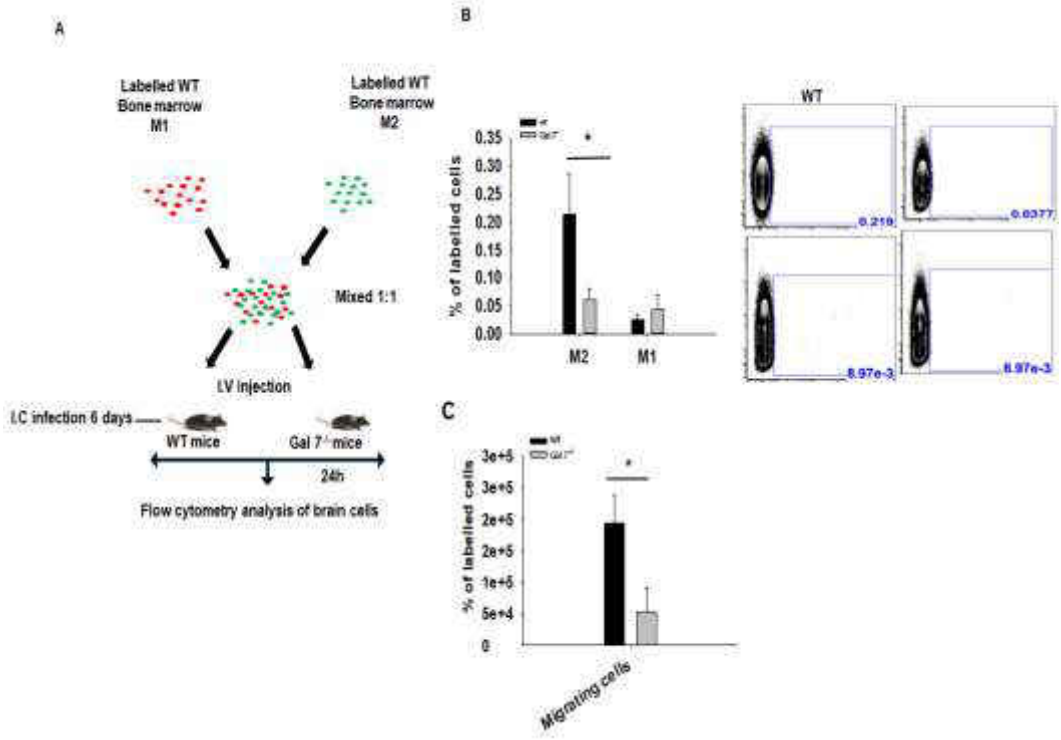

Figure III- 5. Galectin 7 expressing brain endothelial cells regulates M2 trafficking during NCC infection.

(A) Schema of experimental procedure on adoptive transfer experiment as described in methods. (B) Purified bone marrow macrophages from WT mice were cultured with IFN-γ (M1) or Il-4 (M2). The M1 were labeled with CellTracker far-red and M2 with CellTracker Green CFSE. They mixed in 1:1 ratio and injected intravenously (i.v.) into WT or Galectin $7^{-/-}$ recipient mice infected intracranially with *M. corti* 6 days prior to injection. Brains were harvested 24 hrs after the adoptive transfer and processed for flow cytometry. Data shown are mean ± SEM form 9 mice per group in 3 independent. Statistical significance are denoted by asterisks (*, $p<0.05$). (B). WT and Galectin 7 brains endothelial cells were isolated and cultured in the upper layer invasion insert chamber. Upon, differentiation into endothelial cells, the cells were stimulated in the presence of parasite-derived molecules for 24hrs. BMDM were then incubated into the top of the chamber. CCL2 is added to the low chamber with media. After 24hrs, the number of cells that migrated into the lower compartment was counted using trypan blue. Results are representative of 2 independent experiments Statistical significance was determined by student t test (* $p<0.05$).

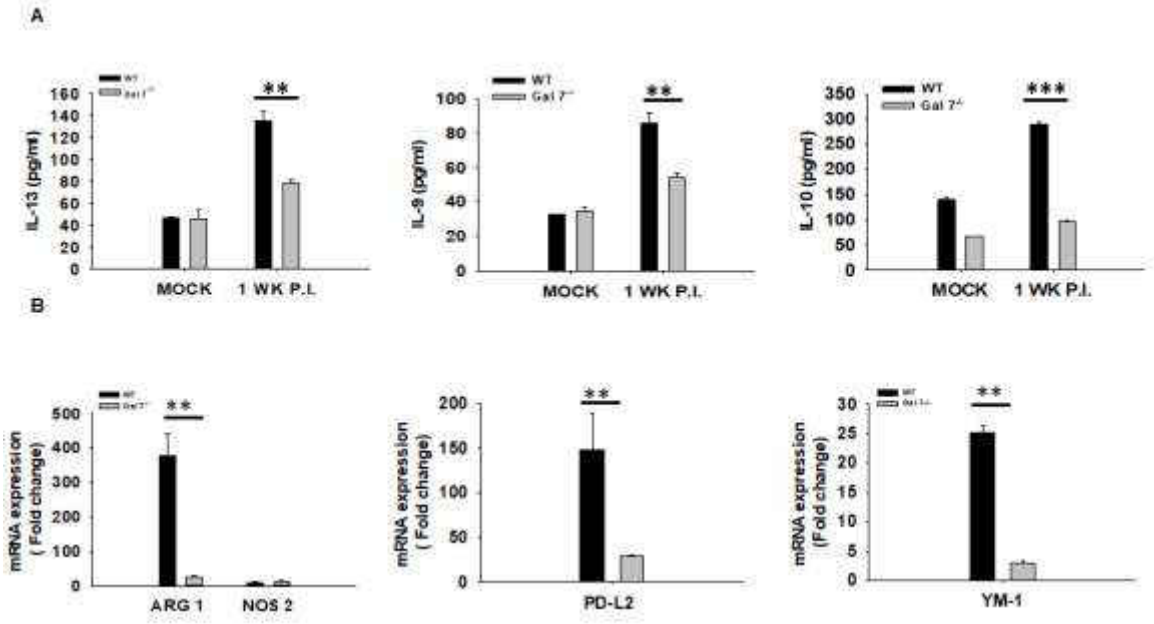

Figure III- 6. Inflammatory mediators in the CNS of parasite-infected Galectin 7$^{-/-}$.

(A) The brains from mock control and *M. corti* infected WT and Galectin 7$^{-/-}$ mice were harvested at 1wk .i.c., homogenized, and the protein level of host immune mediators measured by CBA using flow cytometric analysis (BD Biosciences). Results shown are mean ± SEM of 3–4 each infected and mock control mice from 2–3 independent experiments. Statistical significances are denoted by asterisks (* $p<0.05$; ** $p<0.005$). (B) The total RNA was extracted from the brains at 1wk post inoculation and mRNA expressions of Arginase 1 (ARG 1), PD-L2, and YM-1 were measured by qRT-PCR as described in Methods. The fold changes in parasite-infected brains were calculated over the levels in mock control mice using the formula $2^{-(\Delta\Delta Ct)}$. Data shown are the mean ± SEM of 3–4 mice per time point in two independent experiments. Significant differences were measured by Student's t test ($p < 0.005$)

CHAPTER IV

GALECTIN 9 IS REQUIRED FOR CONTAINMENT OF NEUTROPHIL RECRUITMENT INTO THE CNS: IMPLICATIONS IN INFLAMMATION AND BRAIN PATHOLOGY DURING MURINE NEUROCYSTICERCOSIS

Fredice Quenum Zangbede, Jyotika Sharma, Bibhuti B Mishra*

Department of Biomedical Sciences
University of North Dakota School of Medicine and Health Sciences
Grand Forks, ND 58201

Manuscript in preparation

Abstract

Helminth associated with parasitic infections are believed to release glycol-proteins which modulate the infections process. However, the specific receptors involved and their function during the parasitic infection, are yet to be completely determined. We examined the function of galectins, mammalian beta galactose-binding lectins, in a murine model for neurocysticercosis (NCC) induced by intracranial inoculation of the helminth parasite *Mesocestoides corti*. Parasite infection induced multiple galectins, among which Galectin 9 was the first as to be upregulated as well as at a relatively high level in M2. Parasite-infected Galectin $9^{-/-}$ mice displayed significantly increased susceptibility to parasite infection, despite a similar parasite burden in the central nervous system (CNS) compared to infected wild-type (WT) mice. The accumulation of macrophages, γδ T cells, αβ T cells, or B cells in the brains of parasite-infected Galectin $9^{-/-}$ mice was similar to the WT mice. Moreover, AAMs which play a profound protective role in murine NCC were observed an increased level in infected WT and Galectin $9^{-/-}$ mice. Instead, the parasite-infected Galectin $9^{-/-}$ mice exhibited a massive neutrophilia in the CNS and an increase in neutrophil-associated neuroinflammation. These results suggest that galectin 9 may be involved in controlling and resolving neutrophilia and inflammation, however, not by efferocytosis. For the first, we showed in the possible correlation between the presence of Galectin 9 and neutrophilia in NCC infection. Further experiments need to be done to determine the specific mechanism by which Galectin 9 is involved in this process.

Key words: Galectin 9, neurocysticercosis, neuroinflammation, helminth, CNS infection

INTRODUCTION

Neurocysticercosis is a common central nervous system (CNS) parasitic disease caused by the presence of *Taenia solium* (9, 38). In humans, the infection manifests in a long asymptomatic phase where the parasite stays in the CNS as a cyst with no detectable inflammatory response detected surrounding the parasite (4, 11, 33, 40, 42, 43, 58). In contrast, virtually all the patients with symptomatic disease are characterized by prominent immunological responses in the brain (4, 58). Indeed, the severity of the symptoms is associated with the intensity of the immunological response in the CNS microenvironment (4, 40, 43, 58).

A mouse model of neurocysticercosis by infection with a related cestode *Mesocestoides corti* (*M. corti*), has been used to systematically characterize various aspects of the host-pathogen response during the entire infection process. In mice, the infection was found to associate with severe neurological manifestations. A predominant influx of immune cells consisting of macrophages and γδ T cells (3-5 days p.i), followed by αβ T cells (1wk p.i) and B-cells (3wk p.i) (141, 154), are differentially recruited into the brain after parasite inoculation. Furthermore, macrophages with M2-activation (alternative activation) phenotype of cells are thought to play an important immunological response to *M. corti* infection of the CNS as illustrated by the finding that mice deficient in STAT6 exhibit severe CNS pathology with shorter time to death. Importantly, the degree of leukocyte accumulation in the CNS playing a critical role on neuropathology in the murine model parallels the results obtained with brain specimens from human patients (40), suggesting a key role of immune cells in the pathogenesis of the neurocysticercosis.

Parasitic helminths (worms) in their pursuit to establish long-term infections and survival in the host use glycans which are abundant on their surface and in their excretory/secretory products (155, 156) to regulate and suppress host immune responses. In fact, more than 95% of NCC patients exhibit humoral responses against carbohydrate-based antigens which are extensively used for immunodiagnosis of this disease. Indeed, glycan antigens from the tegument of *T. solium* and *M. corti* are found to be released in the CNS of humans and mice respectively (9, 157). Importantly, glycan antigens with terminal galactose are released from the parasites and are taken up by the host cells in the CNS environment in parasite-infected brains (60, 118, 158). Thus lectin receptors that recognize carbohydrates are likely to be critical in the subsequent immunological events and overall immunopathogenesis of NCC.

Galectins are important receptors involved in recognition of glycan structures, particularly containing terminal galactose, on a wide range of molecules in the host or from pathogens (26, 69-71, 74, 159). Indeed, galectins have been identified as key molecules that play important roles in cell proliferation, including neural progenitor cells, and contribute to tissue remodeling during experimental CNS injury (26, 69-71, 74, 160, 161). Members of galectins have been shown to play diverse roles such as in regulation of inflammation by sensing necrotic cells (162) or by exhibiting chemoattractant properties for leukocyte infiltration when secreted extracellularly (163, 164). Given the significant immunoregulatory role of galectins, the present study is focused on determining the role of galectin 9 on immunopathogenesis in mouse model of neurocysticercosis. The results indicate that both galectin 9 play a major role in controlling the initial influx of neutrophils into the CNS, and providing protection from brain parasite infection.

Materials and Methods

Mice and parasite inoculation.

Female mice age 4.5 to 6 weeks were used in this study. The C57BL/6 mice were purchased by Charles River Laboratories, Wilmington, MA. Galectin $9^{-/-}$ mice were purchased from Jackson Laboratories. *Mesocestoides corti* (*M. corti*) was kindly provided by Dr. Debrosky Herbert (Cincinnati Children's Research Foundation, Cincinnati, OH 45229, USA). *M. corti* metacestodes were maintained in the peritoneal cavity of BALB/c mice by serial intraperitoneal (i.P.) infection for propagation.

For intracranial infections, M. corti larvae were aseptically collected from the i.p. the cavity of the infected BALB/c mice and were extensively washed in HBSS. The metacestodes (about 60 parasites) were suspended in 50 µl of sterile HBSS and injected intracranially into 4.5 to 6 weeks old female mice using 1 ml syringes and a 25-gauge needle (10). Control mice were given 50 µl of sterile HBSS. Priority to the inoculation, the mice were anesthetized with a mixture of xylene (120 µl/ml), ketamine (220 µl/ml) and PBS. 50 µl of the mixture was given to each mice.

At 1 week and 3 weeks, mice were anesthetized using isoflurane and perfused through the left ventricle with 20 ml of cold PBS.

Brain mononuclear cell isolation and Flow cytometry

The brain was instantly removed from perfused mice and leukocytes were processed for antibodies staining for flow cytometry analysis. Briefly, leukocytes single cells suspensions were isolated from parasite-infected brains of WT and Galectin $9^{-/-}$ using percoll density gradient from GE healthcare (CAT# 17-0891-01). The cells were counted

using trypan blue. $2x10^6$ cells were pre-incubated with Fc blocker for 10min and then incubated with 50 µl of antibodies cocktail for 30 min. after incubation, the cells were washed 2 times with 500 µl FACS buffer (10% FBS in PBS) and the pellets were suspended in 350 µl of FACS buffer and analyzed by BD LSRII flow cytometer (BD Biosciences).

H&E and Immunofluorescence Staining.

Parasite and HBSS infected brains were immediately removed from perfused WT and Galectin $9^{-/-}$ mice, embedded in OCT resin and snap frozen. Serial horizontal cryo-sections with 10um in thickness were positioned on slides (from Sigma-Aldrich, St. Louis, MO). Every 5^{th} slides were sectioned and stain following the H&E staining protocol previously described (42). Briefly, the slides were washed twice in deionized water, dehydrated for 30s in 100% ethanol, stained 30s in hematoxylin, and washed in distilled water for 2 min (42, 108). Tissue sections were stained with eosin for 15s followed by 2 min treatment with 95 and 100% ethanol each. Slides were allowed to air dry, then submerged in xylene for 3 min, and mounted using the cytoseal mounting medium (Stephens Scientific, Riverdale, NJ). The number and location of parasites were determined by microscopic examination of the stained tissues. Tissues were also analyzed for the presence or absence of infiltrating leukocytes.

For IF microscopy, the frozen brain tissue sections were stained with relevant antibodies to detect immune parameters such as galectin 9, and immune cells (107). Briefly, brain tissue sections were incubated with specific primary antibodies in staining buffer (PBS buffer with 3% host serum) to prevent non-specific binding. After 45 min of incubations, sections were washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20 and incubated with appropriate secondary antibodies in staining buffer for

30 min. Sections were then washed seven times 3 min each in 50 mM Tris-HCl, pH 7.6 with 0.1% Tween-20. For double IF staining, the above-mentioned procedures were sequentially repeated for each additional staining. The sections were mounted using FluorSave reagent (Calbiochem, La Jolla, CA) containing 0.3 μM 4', 6'-diamidino-2-phenylindole (DAPI)-diacetate (Molecular Probes). Additional control staining was performed to rule out any nonspecific staining. In each case, sections were blocked with saturating concentrations of appropriate host serum antibodies to eliminate false positive staining due to FcR-mediated nonspecific binding. Staining in the absence of primary antibodies provided additional negative controls. Primary and secondary antibodies used for IF staining are: Rabbit anti-galectin 9 (Abcam, San Diego, CA) and Alexa Fluor® 546 or Alexa Fluor® 488 goat anti-rabbit (Molecular Probes, OR), Rat anti-mouse 7/4 (Cedarlane, Burlington, NC) and Alexa Fluor® 546 or Alexa Fluor® 488 Goat anti-rat (Molecular Probes, OR), and Alexa Fluor® 546 or Alexa Fluor® 488 (Molecular Probes, OR). We evaluated tissue damage on frozen brain tissue sections from control and parasite-infected brains by performing the terminal deoxyribonucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining as per manufacturer's instructions (Chemicon International, CA). In all the cases, the images were acquired using a Nikon eclipse 80i upright microscope (Nikon Corporation, Tokyo, Japan) with an attached cooled RTke Spot 7.3 three spot color camera (Diagnostic Instruments Inc., Sterling Heights, MI). The images were processed and analyzed using Adobe Photoshop 7.0 software (Adobe, Mountain View, CA).

Ex-vivo Efferocytosis

For these studies, peritoneal neutrophils were isolated from WT mice 12-16hr after intraperitoneal injection of sterile 4% thioglycollate (BD Biosciences, San Jose, CA) (46). For macrophages, WT and Galectin $9^{-/-}$ mice were infected i.p. with M. corti (60-100 in numbers) and PECs were harvested after 4-5wk p.i. to collect M2 cells. Mock control mice instead received vehicle (PBS). On day-4 PECs were harvested to purify naïve macrophages for their role in this process. To obtain neutrophils, WT mice were injected i.p. with 4% thioglycollate for 16-18 hours.

The purity of the cells was ascertained by flow cytometry analysis (Ly6G+ neutrophils 80-85%; F4/80+ macrophages 85-90%). Isolated neutrophils were labeled with Carboxyfluorescein succinimidyl ester (CFSE; Cell Trace™ CFSE Cell Proliferation Kit from Invitrogen). Macrophages seeded on 6 well plates (1x106 cell/ml) were incubated with CFSE-labeled neutrophils at a ratio of 5:1(neutrophils: macrophages). After 2 hrs of efferocytosis, non-internalized neutrophils were removed by washing thoroughly. Macrophages were scraped and stained with F4/80 and Ly6G antibodies for flow cytometry. Gating scheme to quantitate Ly6G-F4/80+ CFSE+ efferocytic macrophages that had internalized labeled neutrophils is shown in Figure 8. For individual experiments, efferocytic index was calculated as percentage of Ly6G-F4/80+CFSE+ macrophages from WT and Galectin $9^{-/-}$ mice that engulfed neutrophils (112).

Quantitative Real-Time PCR

Brain from infected and mock control mice 1 week and 3 weeks post infection were directly perfused and the total RNA was extracted using trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis of the samples was done using SYBRO green (Applied Biosystems, CA (USA). Transcript levels of the

housekeeping ribosomal 18S, Galectin 9.genes were measured in each sample by PCR amplification using specific primers.: removed 18S (forward) 5'-CATGTGGTGTTGAGGAAAGCA-3' and (reverse) 5'-GTCGTGGGTTCTGCATGATG-3'; Gal-9 (forward) 5'- TCAAGGTGATGGTGAACAAGAAA-3' and (reverse) 5'-GATGGTGTCCACGAGGTGGTA -3'

Statistical Analysis

The survival of the infected WT and Galectin $9^{-/-}$mice was compared using Log-rank (Mantel-Cox) Test. The statistical comparison between levels of host mediators was done with Student's t test using Sigma Plot 8.

Results

Predominant expression of galectin 9 in macrophages and astrocytes in the CNS after *M. corti* infection.

Quantitative real-time PCR analysis was performed to determine the expression profile of galectins in mock-infected and parasite-infected brain. Upon infection, gene expression of several galectins was significantly upregulated. Among them, galectin 9 mRNA expression was significantly upregulated at 1wk p.i. (~7-fold) and at 3wk p.i. (~3-fold) (Figure.IV-1A). Next, *in situ* IF microscopy analyses were performed to determine the expression and distribution of galectin 9 at the protein level in brain tissues from mock-infected control mice and in mice undergoing neurocysticercosis. In the uninfected animals, galectin 9 expression was largely undetected (Figure.IV-1B). At both 1wk and 3wk p.i. approximately half of the immune cells present in meninges and ventricles were positive for galectin 9 protein (Figure.IV-1B). Double immunofluorescence analysis with anti-galectin 9 and antibodies to cell surface markers specific for various immune cells

indicated that the majority of cells detected positive for galectin 9 were CD11b positive myeloid cells detected in the intraparenchymal areas of CNS (Figure.IV-1B). Morphological, anatomical correlation, and colocalization staining strongly suggested that the upregulated galectin 9 at protein level was mainly detected in infiltrating myeloid cells. However, with the progression of infection time, some the astrocytes and their foot processes proximal to the pial vessels showed galectin 9 positive staining (data not shown).

Galectin $9^{-/-}$ mice are more susceptible to parasite infection in murine NCC

To investigate whether galectin 9 deficiency influences the overall immunity during the CNS infection with M. corti, disease severity was compared between WT and Galectin $9^{-/-}$ mice. The WT mice injected i.c. with M. corti displayed typical neurological signs including abnormal vestibular function, tilted head, cerebral abnormalities, morbidity, and weight loss between 1-3wk p.i. In general, these overt signs appeared earlier and were more accentuated in Galectin $9^{-/-}$ mice. In addition, infected Galectin $9^{-/-}$ mice exhibited decreased survival. By 7wk post-*M. corti* infection, 25% of M. corti-infected WT mice succumbed to the infection compared to 70% of Galectin $9^{-/-}$ mice (Figure.IV- 2A, $p<0.05$).

Galectin 9 deficiency has no effect on parasite load in infected

To examine whether increased disease severity in galectin 9- deficient mice was associated with less effective control of parasite growth, serial horizontal sections of infected WT and Galectin $9^{-/-}$ brains of mice were stained with H&E, and the number of *M. corti* metacestodes was determined by microscopic analysis. Both WT and Galectin $9^{-/-}$ mice exhibited similar numbers of parasites as compared to the infected brains of WT mice both early (1wk) and later (3wk) (Figure.IV- 2B) post-*M. corti* infection, suggesting

that galectin 9 dependenting responses have no influence on overall parasite growth in the CNS.

Galectin $9^{-/-}$ mice exhibit increased accumulation of neutrophils in parasite-infected brains

Galectin $9^{-/-}$ mice exhibited increased susceptibility to murine NCC. To investigate whether galectin 9 deficiency had any effect on immunopathological events, H&E staining was performed to determine immunopathological changes in WT and Galectin $9^{-/-}$ mice at 3wk p.i. (Figure.IV- 3A) depicts normal brain tissue morphology in WT and Galectin $9^{-/-}$ control animals i.c. inoculated with HBSS. In contrast, a large number of infiltrating immune cells was observed in both parasite infected WT (Figure.IV-3A) and Galectin $9^{-/-}$ mice (Figure.IV-3A). In parasite infected WT mice, a high proportion of infiltrating cells appeared to be myeloid (Figure.IV- 3B, inset), whereas in Galectin $9^{-/-}$ mice the predominant cell type was polymorphonuclear cells (PMNs) (Figure.IV- 3A, inset). Next, using IF microscopy, the presence of neutrophils was analyzed at 3 wks after parasite inoculation. In wild-type mice, neutrophils (7/4+ cells) were scarcely detected in the brain at 3wk of parasite inoculation (Figure.IV- 3A). In contrast, the immune response revealed the presence of large numbers of 7/4+ neutrophils (Figure.IV- 3B) associated with the infiltrates in extra-parenchymal regions (ventricles, meninges, and subarachnoid spaces) at 3wk p.i (Figure.IV- 3B).

The flow-cytometry analysis was performed to quantify the differences in the levels of neutrophils recruited into the CNS of Galectin $9^{-/-}$ and WT infected mice. The differences in recruitment of neutrophils in Galectin $9^{-/-}$ mice were analyzed at both an early stage of infection (1 wk p.i.) and at the peak of inflammation (3 wk p.i.) (Figure.IV- 3C). *M. corti*

infection in both WT and Galectin $9^{-/-}$ mice resulted in infiltration of similar numbers of CD11b+ly6G+ activated neutrophils at 1wk p.i. (Figure.IV- 3C). However, at 3wk p.i. infected Galectin $9^{-/-}$ mice displayed significantly increased numbers of CD11b+Ly6G+ neutrophil cells as compared to the WT infected mice (Figure.IV- 3C). Thus, taken together, the histopathological and flow cytometric analyses demonstrate that absence of galectin 9 leads to a significant increase in neutrophils recruitment into the brain during NCC, particularly at the peak of inflammation.

Inflammatory cytokines and chemokines expression in the CNS after *M. corti* infection.

To determine whether Galectin $9^{-/-}$ mice exhibit differences in immune mediators in the CNS during M. corti infection, the protein level of various cytokines, chemokines, and several other signature mediators were analyzed by CBA analysis. In infected Galectin $9^{-/-}$ mice, both IL-10 and IL-17 were detected at a lower level in the CNS at 1wk p.i., whereas a reduced IL-5 was detected in these mice at 3wk p.i. compared to the WT mice (Figure.IV- 4). To our surprise, in general, there were no significant differences between WT and Galectin $9^{-/-}$ mice observed in the CNS levels of majority of the immune mediators analyzed (Figure.IV- 4). Moreover, the absence of galectin 9 did not appear to significantly influence the CNS level of neutrophil chemoattractants (CXCL1), neutrophil survival mediator (GM-CSF) in the parasite-infected- Galectin $9^{-/-}$ mice as compared to the WT infected mice.

Galectin 9-deficiency has no effect on efferocytic clearance of neutrophils.

To determine the mechanism of neutrophils accumulation in the CNS of *M. corti* infected Galectin $9^{-/-}$ mice, we examined the possible role of galectin 9 in neutrophil

clearance by efferocytosis. Macrophages from WT and Galectin $9^{-/-}$ mice with or without infection with *M .croti* were exposed to CFSE–labeled dead neutrophils from WT mice *in vitro*. Flow-cytometric analysis was performed to measure the frequency of the Ly6G-F4/80+CFSE+ cells, which represent the efferocytic macrophages that have internalized CFSE labeled neutrophils. The uptake of neutrophils by macrophages from Galectin $9^{-/-}$ mice did not display any significant difference in comparison with the macrophage from WT mice. The results indicated that galectin 9 on macrophages does not participate in neutrophils clearance. Thus, a higher neutrophil accumulation in Galectin $9^{-/-}$ brains is unlikely due to any defect in their efferocytic clearance by macrophages.

Discussion

Parasites express both nonhost-like and host-like glycan antigens (29). The expression of host-like glycans by parasitic helminths is widespread, and different host-like glycans are found in a broad range of parasites (13, 91, 102, 118). It is thus no surprise that there is ever increasing evidence supporting a role for lectins, the glycan binding receptors, in immune functions. Because lectin receptors have been implicated in a wide variety of immunological functions including first-line defense against pathogens, cell trafficking, cell differentiation and immune regulation (26, 67, 71-73, 103, 165), a better understanding of their involvement in CNS immunopathogenesis might lead to the identification of novel therapeutic strategies against neuropathological conditions. Here we found that galactose-binding lectin galectin 9 is upregulated robustly within 7 days of infection in the CNS of mice infected i.c. with *M. corti*. We hypothesized that this early upregulation of galectin 9 is involved in providing necessary signals for regulating the immune responses in the CNS parasite-infected mice. Indeed, the results from present

studies strongly support the influence of galectin 9 on the host immune response affecting the pathogenesis of the CNS parasitic disease.

Infection of the CNS by *M. corti* is characterized by the initial accumulation of parasites and inflammatory cells in extra-parenchymal areas of the brain, which include ventricles, meninges, and subarachnoid spaces. Macrophages represent the predominant leukocyte populations in the parasite-infected brain both initially and during prolonged infection (7, 81, 166, 167). In contrast, among the infiltrating immune cells, neutrophils are detected at a very low level both early and at later times of infection in the CNS during murine neurocysticercosis (33). To our surprise, we saw a larger accumulation of neutrophils in the CNS at the peak of inflammation (3wk p.i.) in M. corti-infected galectin 9-deficient mice. Neutrophil-mediated responses are essential for combating microbial infection, however, their direct role in protection to helminth parasite infections is limited. Persistent accumulation of neutrophils can lead to bystander tissue destruction, owing to their noxious cargo (168). Thus, it is likely that the increased accumulation of neutrophils in the CNS of parasite-infected Galectin $9^{-/-}$ mice is contributing to the exacerbated disease severity. Furthermore, the fact that most of the hyper-inflammatory mediators detected at similar level correlating with an unchanged parasite burden, further supports a direct effect of neutrophil-associated tissue damaging mediators contributing to the mortality of Galectin $9^{-/-}$ mice. However, if galectin 9 absence eventuates in increased nervous tissue pathology in parasite-infected Galectin $9^{-/-}$ mice remains to be determined.

Accumulation of large numbers of neutrophils in the CNS microenvironment of parasite-infected Galectin $9^{-/-}$ mice could either be due to modulation of expression of associated inflammatory mediators; due to a defect in their clearance by efferocytosis; or a

combination of both. Indeed, accumulating evidence suggests galectins can function as pattern recognition receptors (PRRs) in innate immunity as they bind both endogenous glycans as well as bind exogenous glycans on the surface of potentially pathogenic microbes, parasites, and fungi (50, 84). However, our findings strongly suggest galectin 9 is unlikely functioning as a PRR in regulating neutrophil turnover in the CNS microenvironment. If galectin 9 is functioning as a PRR, we would expect to observe a significantly upregulated expression of chemokines and cytokines involved in neutrophil functions in the CNS of Galectin $9^{-/-}$ mice. Instead, we found no significant difference in the level of neutrophil chemoattractants (CXCL1) and neutrophil survival mediators (GM-CSF) in brain homogenates of parasite-infected WT and Galectin $9^{-/-}$ mice. Moreover, IL-17 was detected at a reduced level in the CNS of Galectin $9^{-/-}$ mice at 3wk p.i. As IL-17 activates pathway involved in neutrophils recruitment, our results further suggest an unlike role of Galectin 9 as a PRR, whose activation by parasite molecules controls the neutrophil recruitment. On the other hand, we also found no difference in efferocytic clearance of neutrophils by galectin 9 deficient macrophages from Galectin $9^{-/-}$ mice inoculated with *M. corti* or PBS. In similar settings, deficiency of another related galectin, the galectin 3 on macrophages significantly decreases their capacity to efferocytosis neutrophils. Indeed, accumulation of large numbers of neutrophils in the brains of parasite-infected Galectin $3^{-/-}$ mice is due to a defect in efferocytic clearance of neutrophils by the galectin 3 deficient macrophages. In light of no observed role of galectin 9 as PRR or in efferocytosis, we are currently investigating a possible novel role of this lectin as a negative regulator of neutrophil influx into the CNS by performing adoptive transfer experiment. Relevant to our hypothesis, mouse MGL that binds galactose and galactosamine containing glycans,

has been reported to impede migration of immature dendritic cells (DCs) (112) as well as neutrophils (112). Indeed, MGL-1 has recently shown by us to negatively regulate neutrophils infiltration into the lungs of *Klebsiella pneumonia* infected mice (112). In toto, notwithstanding the mechanisms involved, this study shows that galectin 9 plays an important role in negatively regulating the neutrophil influx into the CNS, which can have major implications in the therapeutic measurements of inflammation associated CNS disorders.

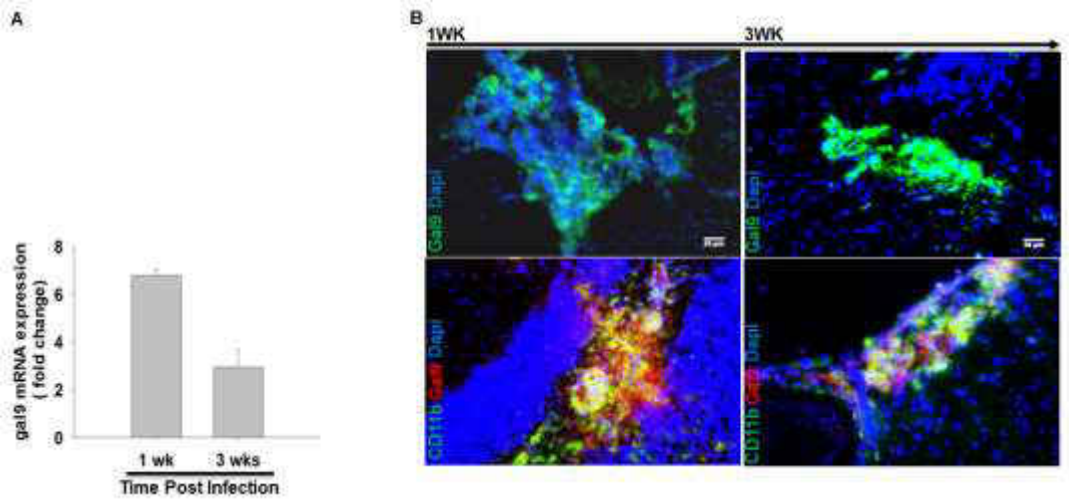

Figure.IV- 1: Expression and distribution of galectin 9 in the parasite-infected brains

(A) The total RNA was extracted from the brains at 1wk and 3wk post inoculation and

mRNA expression of galectin 9 was measured by qRT-PCR as described in Methods. The fold changes in parasite-infected brains were calculated over the levels in mock control mice using the formula 2−(ΔΔCt). Data shown are the mean ± SEM of 3–4 mice per time point in two independent experiments. (B) Double immunofluorescence staining was performed on brain tissue cryosections from mock or parasite- infected C57/BL6 mice. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Galectin 9 was stained using an affinity purified anti-mouse galectin 9 goat IgG followed by Alexa Fluor® 546 labeled (Red) Goat anti-Rabbit IgG. galectin 9 expression was co-visualized with macrophages using an affinity purified anti-mouse CD11B Alexa Fluor® 488 labeled (Green). Images shown are representative of 3 independent experiments with 3–4 mice each. Magnification of 20X

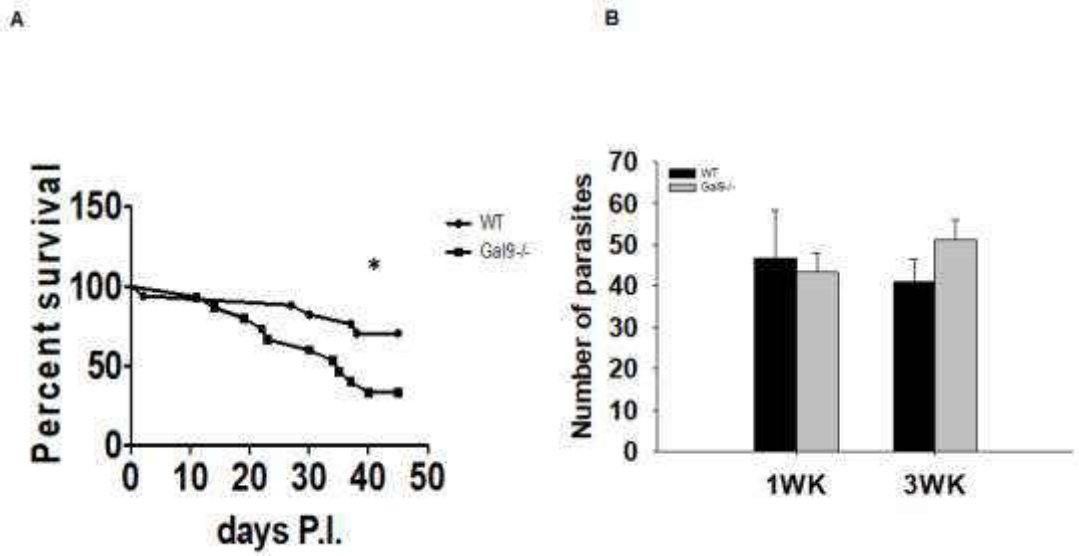

Figure.IV- 2: Galectin $9^{-/-}$ mice exhibit reduced survival and increased brain pathology despite exhibiting similar parasite burden as the WT mice.

(A) WT and Galectin $9^{-/-}$ mice were i.c. infected with 60 M. corti and were assessed daily for disease severity. The survival was monitored for 3 weeks post inoculation. Statistical comparison of susceptibility was done by Log-rank (Mantel-Cox), $P<0.05$,n=9-10. (B) Number of parasites at 1wk and 3wk p.i. in parenchymal (P) and

extra parenchymal (EP) regions in individual Galectin $9^{-/-}$ and WT brains were calculated by microscopic examination of serial H&E-stained brain sections. The bars show average +/- SEM parasites from 5-6 mice per group

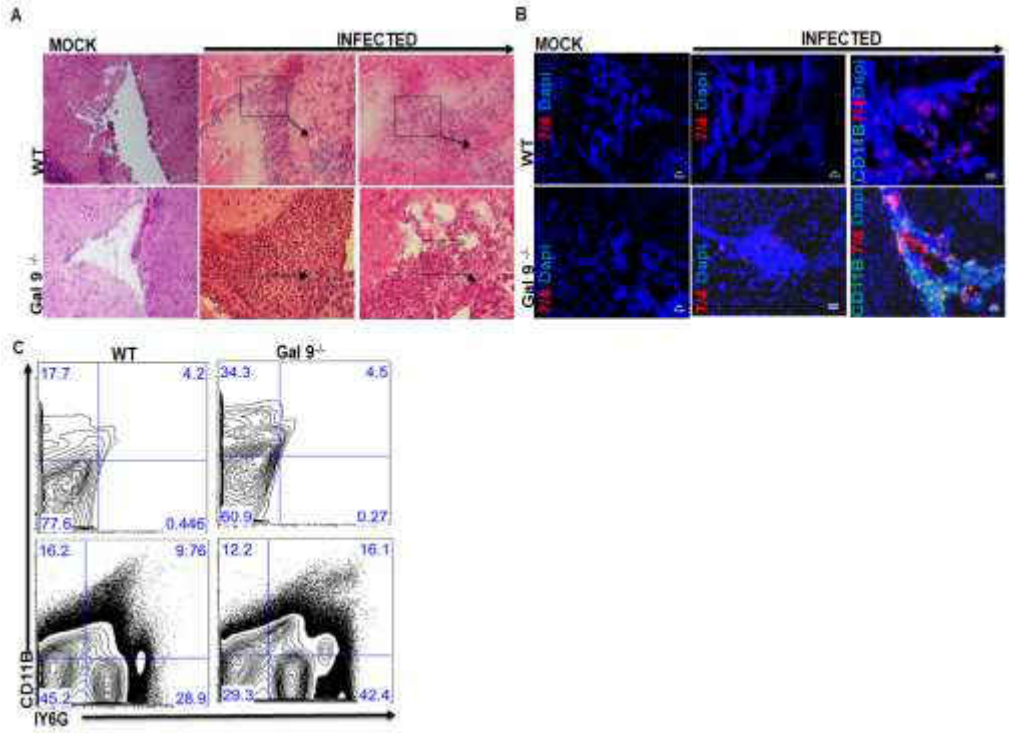

Figure.IV- 3: Parasite-infected Galectin $9^{-/-}$ mice exhibit increased neutrophil accumulation in brain.

IF microscopy on brain cryosections from WT and Galectin $9^{-/-}$ mice i.c. injected with HBSS (mock) or with 60 parasites at 1wk and 3wk p.i. Neutrophils (7/4+) were detected by using an affinity purified anti-mouse 7/4 goat IgG followed by Alexa Fluor® 546

labeled (red). Nuclei (blue) were stained with 4'6' diamidino-2-phenylindole-dilactate (DAPI). Images shown are representative of 3 independent experiments with 3–4 mice each group. (B) Flow cytometry analysis of CD11b and Ly6G on brain mononuclear cells harvested from mock control and M. corti infected WT and Galectin $9^{-/-}$ mice at 1wk and 3wk p.i. The cells were double-stained with anti-Ly6G-APC and anti-CD11b-Pacific Blue antibodies as markers for neutrophils. Representative contour plots show CD11b+Ly6G+ neutrophils in brains of parasite-infected WT and Galectin $9^{-/-}$ mice. The bar graph on the right shows mean ± SEM of percent of CD11b+Ly6G+ neutrophils in brains of M. corti infected WT and Galectin $9^{-/-}$ mice each from 3 independent experiments.

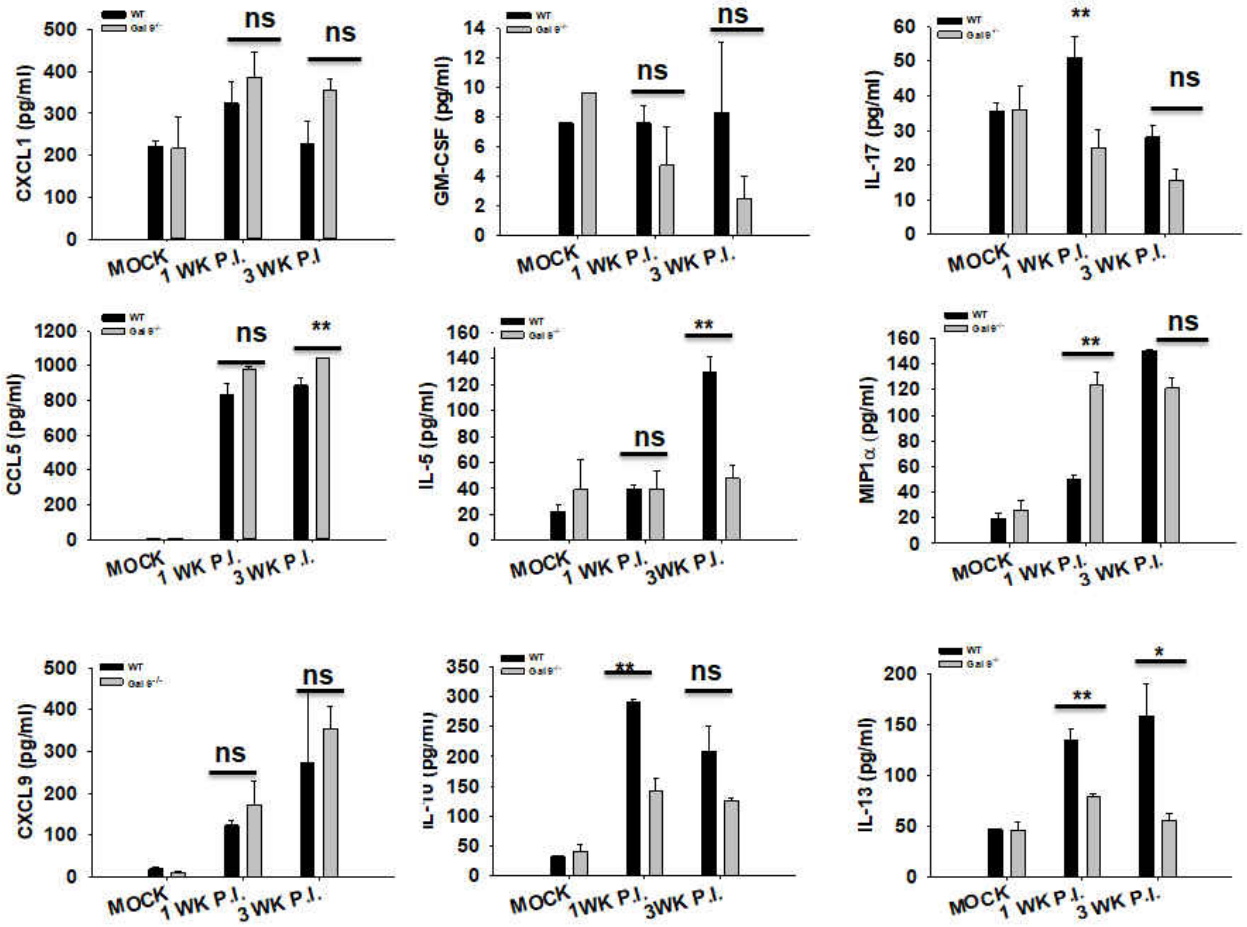

Figure.IV- 4: **Inflammatory mediators in the CNS of parasite-infected Galectin $9^{-/-}$ and WT mice.**

(A) The brains from mock control and M. corti infected WT and Galectin $9^{-/-}$ mice were harvested at 1wk and 3wk p.i., homogenized, and the protein level of host immune mediators measured by CBA using flow cytometric analysis (BD Biosciences). Results shown are mean ± SEM of 3–4 each infected and mock control

mice from 2–3 independent experiments. Statistical significances are denoted by asterisks (*, $p<0.05$; **, $p<0.005$).

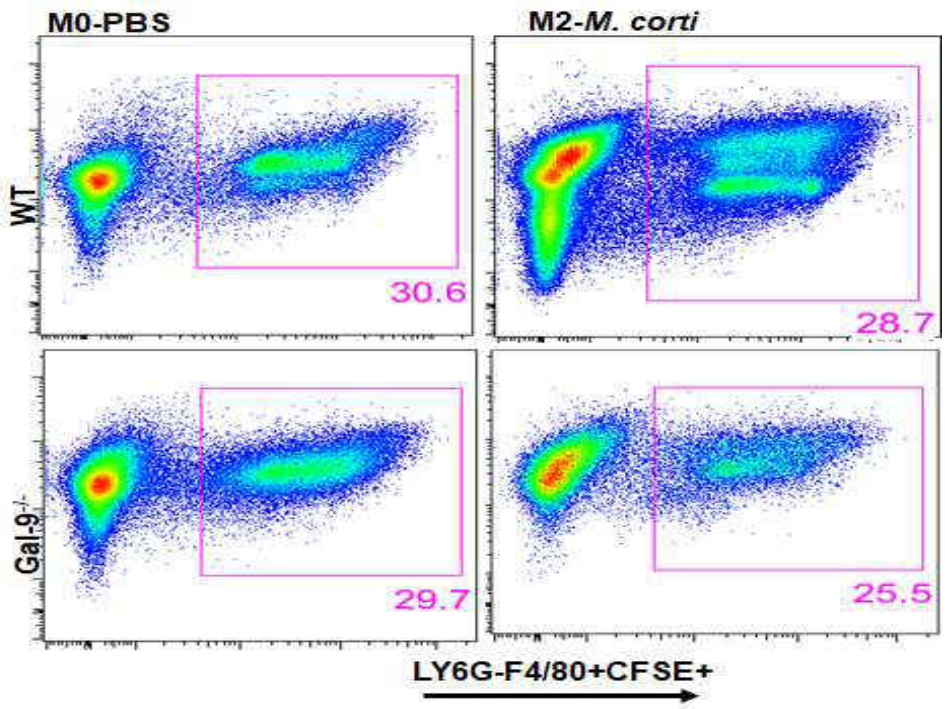

Figure.IV- 5: Galectin $9^{-/-}$ M2-macrophage do not exhibit defect in efferocytosis of neutrophils *ex-vivo*.

WT and Galectin $9^{-/-}$ mice were either infected with *M. corti* , or PBS.(A) Efferocytosis of CFSE+ neutrophils by macrophages from WT and Galectin $9^{-/-}$ mock control mice that received vehicle (M0-PBS) or *M. corti* parasite (M2- *M. corti*) intraperitoneally. The percent of Ly6G-F4/80+CFSE+ macrophages that have internalized CFSE labeled neutrophils are shown by the scatter plot.

CHAPTER V

GENERAL DISCUSSION

The major goal of these studies was to elucidate the protective correlates in NCC, focusing on mechanisms shaping trafficking of M2 macrophages into the CNS, and effector molecules expressed in M2 cells regulating CNS immunopathology. As in most other neuroinflammatory diseases, clinical presentations of NCC are thought to be influenced by chronic inflammatory responses causing neuropathology. In the absence of a classically defined lymphatic system in the CNS, macrophages and microglia functions as innate immune responders to infection. The initial recognition of microbial molecules by these cells induce the initial local inflammatory response and effector functions as well as shaping the subsequent pathogen-specific adaptive immune response. In this regard, it has been established that the M1 inflammatory functional phenotype in macrophages/ microglia generally promote the destruction of microbial pathogens, but also can cause widespread nervous tissue damage as well as impair CNS repair process (93). In contrast, emerging evidence suggests that M2 activation phenotype of these cells promote nervous tissue repair and restoration (95, 96, 99). However, direct mechanistic evidence showing the protective function of M2- macrophages/ microglia in the CNS microenvironment is limited. In addition, mechanisms specifically involved in M2 macrophage infiltration into the CNS is yet to be understood. In this regard, our work has shown before that in mouse model of NCC, M2 macrophages are first to infiltrate into the parasite-infected CNS. They seemed to play essential/ necessary roles in containing neuropathology and disease severity as the reduced number of cells with M2 activation phenotype in the CNS of STAT6$^{-/-}$ mice

was associated with heightened disease severity (none of the mice survived after 2wk p.i.). The objective of the proposed studies was to elucidate the M2 associated protective correlates in mouse model of NCC, focusing on; a) characterizing the mechanism/s regulating trafficking of M2 macrophages into the parasite-infected CNS, and b) effector molecules expressed in M2 cells regulating CNS immunopathology. In this regard, a growing body of evidence indicates that glycans from a number of pathogens including helminths act as PAMPs. Previous studies have shown that *M. corti* parasite infection is associated with the release of glycans in the CNS microenvironment. Particularly, the parasites release glycans containing terminal galactose/ galactosamine, both robustly and early during infection (40). In this study, we extended these findings and analyzed the expression and distribution of galectins, which are the most common PRRs that recognize galactose containing molecules, in normal and parasite-infected brains. We found a upregulate expression of galectin 3 and galectin 9 (infiltrating macrophages), and galectin 7 (brain endothelial cells) in the parasite-infected brains. Our initial first hypothesis was that galectin 3 and galectin 9 expressed in M2 macrophages would contribute to the M2 macrophages –mediated anti-inflammatory, immune-regulatory, and/ or tissue-protective responses to prevent the inflammation-induced pathology in parasite-infected brains. Our second hypothesis was that the parasite-infection induced expression of galectin 7 on brain endothelial cells would influence the trafficking of M2 macrophages into the CNS.

Although, galectin 3 have been detected in both immune cells, including dendritic cells, macrophages, and neutrophils (5, 50, 51, 53, 169) and nervous tissue cells such as microglia and astrocytes during different infection (57), in the helminth infected brains

galectin 3 was specifically detected in the cytosol of infiltrating M2 macrophages. Galectin 3 has been demonstrated with diverse functions in host immunity. It acts both as PRRs to recognize pathogen structures but also can function by itself as a danger-associated molecular pattern (DAMP). It modulates the migration of immune cells to the sites of inflammation as well as augment the effector functions of these infiltrating immune cells. The diverse functions of galectin 3 thought to due to the difference in its cellular localization (extracellular, membrane-bound, or cytoplasmic), oligomerization, as well as the molecules it interacts with (72, 78, 103, 120). It has been shown to involve in bacterial clearance by macrophages (5) as well as modulating dendritic cells function to parasite infection (169). The importance of the galectin 3 in host immunity and pathogenesis is evident in several clinical settings. For example, infections of Galectin $3^{-/-}$ mice in microbial infection involving *Streptococcus pneumonia*, *H. pylori,* and *E. coli* resulted in more severe disease outcome. Interestingly, the increased disease severity of Galectin $3^{-/-}$ infected with *Streptococcus pneumonia* and *H. pylori* was due to the reduced neutrophil trafficking in the absence of galectin 3 contributing to an impairment of pathogen clearance. In contrast, we recently showed in *F. novicida* infection galectin 3 function as DAMP/ alarmin, and a reduced neutrophilic inflammation in Galectin $3^{-/-}$ mice was correlated with reduced pathology and improved survival compared to the excessive inflammation associated sepsis signs in WT infected mice. Similarly, infection with *Leishmania major*, an intracellular parasite, an impaired neutrophil response and reduced inflammatory cytokine production contributes to an increase in parasite burden and susceptibility in Galectin $3^{-/-}$ mice (170). In a model of a different parasitic infection, infection of mice with *Toxoplasma gondii*, galectin 3-deficient mice showed a reduced

inflammatory response with increased parasite burden in both systemic organs as well as in brain, correlated with an increased disease severity in them as compared to the infected WT mice (171). Thus, galectin 3 seems to play a role in promoting inflammation, including neutrophil-mediated response that improves survival to microscopic bacterial and parasitic infections. However, the results from our experiments demonstrated that deficiency of galectin 3 leads to increased accumulation of neutrophils and M2 macrophages in the helminth infected brains correlated with increased neuropathology and overall disease outcome. Importantly, the adoptive transfer of M2 macrophages from WT mice into Galectin $3^{-/-}$ NCC mice reduced CNS inflammation in terms of decreased neutrophils accumulation, which coincided with lower infiltrating immune cell death. Thus, it is likely during infection with macroscopic helminth parasites, the abundant expression of galectin 3 in M2 macrophages controls the neutrophilic accumulation. In the absence of galectin 3 mediated function of M2 macrophages in reducing neutrophil accumulation leads to tissue pathology. Due to the sensitive nature of the nervous tissue, neutrophil-associated pathology in the absence of galectin 3 in M2 macrophages leads to severe clinical symptoms with fatal consequences in murine NCC.

One of the possibilities for the observed increased accumulation of neutrophils in the CNS of parasite-infected Galectin $3^{-/-}$ mice could be due to a defect in efferocytosis of neutrophils by Galectin $3^{-/-}$ M2 macrophages. This is somewhat supported by a recent study showing galectin 3 secreted by neutrophils facilitates efferocytosis of these cells by interacting with its ligand on macrophage surface (61). However, the data from our studies clearly suggested that galectin 3 in the cytosol of M2 macrophages regulates neutrophil turnover possibly by efferocytosis in the CNS microenvironment. In this regard,

efferocytosis is an important process by which phagocytic cells engulf and clear apoptotic cells (172-175). The multi-step efferocytosis process involves recognition of “find me” signals/ molecules presented by apoptotic cells. Some these “find me” molecules are ATP, UTP, CX3CL1, and lipid lysophosphatidylcholine (LPC), which allow the phagocytes cells to migrate towards the apoptotic cells. Some of the well-characterized receptors on macrophages such as P2Y2 receptor and G2A facilitates phagocytic cells interaction with these “Find me” signals (172-176). However, expression of “eat me” signals on the dying/ dead cells such as the phosphatidylserine (PS) by the phagocytic cells lead to uptake of the dying/ dead cells by phagocytes (172-176). In this regard, receptors such as Tim-3, Tim-4, as well as Stabilin 2 have been known to interact with the “eat me” ligands, and leading to the cytoskeleton rearrangement and internalization of the dying cells showed that Galectin 3 plays a critical role in the clearance of dead neutrophils during NCC. Efferocytosis in critical to control inflammation. Although, the data from our studies clearly suggested a key role for galectin 3 in M2 macrophages associated efferocytosis, however, the mechanisms involved needs to be identified.

During murine NCC, similar to the galectin 3 expression profile, galectin 9 was primarily expressed in the infiltrating myeloid cells. We speculated that galectin 9 will function similarly as galectin 3 in regulating neutrophil turnover and associated inflammatory pathology. This was also supported by some of our recent works involving *F. novicida* intranasal infection, where both galectin 3 and galectin 9 found to function as alarmins and mice deficient of either galectin 3 or galectin 9 intra-nasally infected with *F. novicida*, displayed reduced neutrophil accumulation and tissue pathology in the lungs (107, 177). This was correlated with a decreased systemic inflammation and improved

survival in Galectin $3^{-/-}$ and Galectin $9^{-/-}$ mice. Similar to our findings in Galectin $3^{-/-}$ NCC mice discussed above, an increased accumulation of neutrophils in the CNS of parasite-infected Galectin $9^{-/-}$ mice, albeit the effect was relatively less pronounced as compared to that observed with galectin 3 deficiency. Moreover, infected Galectin $9^{-/-}$ mice displayed higher neuropathology and increased susceptibility to *M. corti* infection of the CNS. Mechanistically, however, in contrast to the role of galectin 3 in M2-macrophage mediated efferocytosis, M2 macrophages from Galectin $9^{-/-}$ mice displayed no defect in efferocytosis of neutrophils. Notwithstanding the mechanisms involved, galectin 9 promotes anti-inflammatory/ immune-regulatory role by controlling neutrophils turnover in the parasite-infected CNS to prevent the inflammation-induced pathology. Perhaps, Galectin 9 by binding to its ligand Tim-3 or CD44 facilitates apoptotic clearance of neutrophils as observed for galectin 9 mediated immune cell clearance reported in other pathological settings (8, 63). Indeed, neutrophils express both CD44 and Tim-3 on their surface. Another possibility is that galectin 9 presence on neutrophil surface regulates its turnover and thereby contributing to the observed disease severity in galectin 9-deficient mice.

A considerable body of evidence supports the view that microglia/macrophages are potent modulators of the outcome of neuropathological conditions such as in brain infections, stroke, traumatic brain injury, and spinal cord injury (5, 6). For instance, M2 macrophage/ microglia-induced response is thought to be important in nervous tissue repair as well as containment of neuropathology. However, most of our knowledge of these important findings are derived from *in vitro* studies or *in vivo* studies where M2 cells are depleted using chemicals. Here using a murine model of NCC, we further provided direct mechanistic evidence showing the protective function of M2- macrophages to decrease

neuropathology and improved mortality during CNS helminth infection. Galectin 7 plays an important role in initial influx of M2 macrophages into the CNS as indicated by reduced numbers of these cells observed in the CNS microenvironment of the parasite-infected Galectin $7^{-/-}$ mice. Importantly, adoptive transfer of galectin $7^{+/+}$ (WT) M2 macrophages failed to migrate into the CNS of parasite-infected galectin 7-deficient mice further indicating galectin 7 as a central regulator of M2 cells transmigration into the CNS. The findings suggests that galectin 7 on brain endothelial cells a critical role in regulating M2-macrophages influx into the CNS, and in its absence the reduced accumulation of M2 cells contribute to the lethality of Galectin $7^{-/-}$ mice, may offer important mechanisms to target in CNS inflammatory disease conditions. On the other hand, galectin 3 and galectin 9 upregulation were found predominantly in infiltrating macrophages and infiltrating myeloid cells respectively. As an increased neutrophilia was observed in the CNS of Galectin $3^{-/-}$ and Galectin 9 mice, suggesting they play immune regulatory functions in the CNS microenvironment. In toto, our studies make an important contribution in establishing the novel protective mechanisms of M2 macrophages functions in the CNS pathological conditions. Our studies provide a better understanding of the immunopathogenesis of NCC which should lead to new therapeutic strategies in other CNS inflammatory disorders as well.

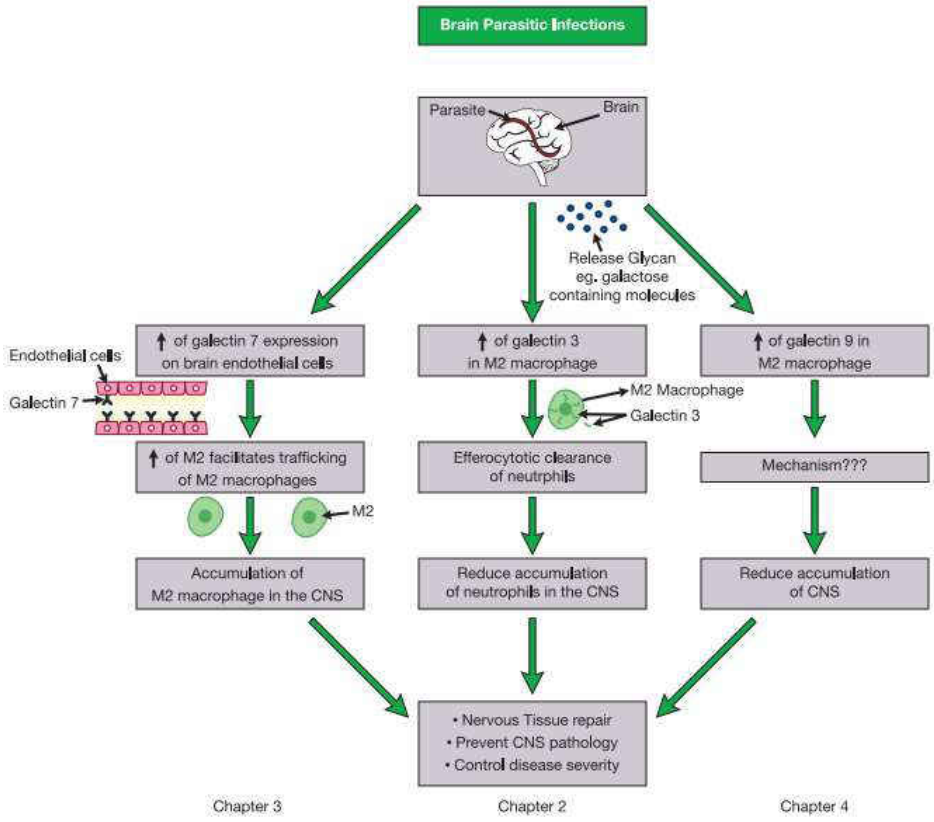

Figure.V: Working model

Murine NCC are believed to release parasitic glycans in the CNS microenvironment. The glycans induce the differential expression of galectins that recognize galactose moieties, in endothelial cells play a critical role in immunopathology by modulating trafficking of M2 macrophages into the CNS, whereas galectins expressed in M2 cells controls the protective functions of these cell types including Nervous tissue repair, preventing NCS pathology and controlling disease severity.

References

1. Franco R, Fernandez-Suarez D. 2015. Alternatively activated microglia and macrophages in the central nervous system. Progress in neurobiology 131:65-86.
2. Giles KM, Ross K, Rossi AG, Hotchin NA, Haslett C, Dransfield I. 2001. Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. The Journal of Immunology 167:976-986.
3. Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M, Allison JP, Allen JE. 2007. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. J Immunol 179:3926-36.
4. Mishra BB, Gundra UM, Teale JM. STAT6(-)/(-) mice exhibit decreased cells with alternatively activated macrophage phenotypes and enhanced disease severity in murine neurocysticercosis. J Neuroimmunol 232:26-34.
5. Sano H, Hsu DK, Apgar JR, Yu L, Sharma BB, Kuwabara I, Izui S, Liu FT. 2003. Critical role of galectin-3 in phagocytosis by macrophages. J Clin Invest 112:389-97.
6. Shin T. 2013. The pleiotropic effects of galectin-3 in neuroinflammation: a review. Acta Histochem 115:407-11.
7. Alagesan S, Griffin MD. 2014. Alternatively activated macrophages as therapeutic agents for kidney disease: in vivo stability is a key factor. Kidney Int 85:730-3.
8. Sada-Ovalle I, Chavez-Galan L, Torre-Bouscoulet L, Nava-Gamino L, Barrera L, Jayaraman P, Torres-Rojas M, Salazar-Lezama MA, Behar SM. 2012. The Tim3-galectin 9 pathway induces antibacterial activity in human macrophages infected with Mycobacterium tuberculosis. J Immunol 189:5896-902.
9. Alvarez JI, Rivera J, Teale JM. 2008. Differential release and phagocytosis of tegument glycoconjugates in neurocysticercosis: implications for immune evasion strategies. PLoS Negl Trop Dis 2:e218.
10. Gundra UM, Mishra BB, Wong K, Teale JM. Increased disease severity of parasite-infected TLR2-/- mice is correlated with decreased central nervous system inflammation and reduced numbers of cells with alternatively activated macrophage phenotypes in a murine model of neurocysticercosis. Infect Immun 79:2586-96.
11. Mishra PK, Morris EG, Garcia JA, Cardona AE, Teale JM. Increased accumulation of regulatory granulocytic myeloid cells in mannose receptor C type 1-deficient mice correlates with protection in a mouse model of neurocysticercosis. Infect Immun 81:1052-63.
12. White AC, Jr. 1997. Neurocysticercosis: a major cause of neurological disease worldwide. Clin Infect Dis 24:101-13; quiz 114-5.
13. Gundra UM, Mishra BB, Wong K, Teale JM. 2011. Increased disease severity of parasite-infected TLR2−/− mice is correlated with decreased central nervous system inflammation and reduced numbers of cells with alternatively activated macrophage phenotypes in a murine model of neurocysticercosis. Infection and immunity 79:2586-2596.

14. White AC, Jr. 2000. Neurocysticercosis: updates on epidemiology, pathogenesis, diagnosis, and management. Annu Rev Med 51:187-206.
15. White AC, Jr., Robinson P, Kuhn R. 1997. Taenia solium cysticercosis: host-parasite interactions and the immune response. Chem Immunol 66:209-30.
16. Woodhall D, Jones JL, Cantey PT, Wilkins PP, Montgomery SP. Neglected parasitic infections: what every family physician needs to know. Am Fam Physician 89:803-11.
17. Alvarez JI, Mishra BB, Gundra UM, Mishra PK, Teale JM. 2010. Mesocestoides corti intracranial infection as a murine model for neurocysticercosis. Parasitology 137:359-72.
18. Garcia HH, Del Brutto OH, Cysticercosis Working Group in P. 2005. Neurocysticercosis: updated concepts about an old disease. Lancet Neurol 4:653-61.
19. Del Brutto OH, Salgado P, Lama J, Del Brutto VJ, Campos X, Zambrano M, Garcia HH. 2015. Calcified neurocysticercosis associates with hippocampal atrophy: a population-based study. Am J Trop Med Hyg 92:64-8.
20. Singhi P, Suthar R. 2015. Neurocysticercosis. Indian J Pediatr 82:166-71.
21. Del Brutto OH, Del Brutto VJ. 2012. Calcified neurocysticercosis among patients with primary headache. Cephalalgia 32:250-4.
22. Del Brutto OH. 2014. Neurocysticercosis in a European traveler returning from India, primarily suspected as neoplasia. J Travel Med 21:75.
23. Del Brutto OH. 2012. Neurocysticercosis: a review. ScientificWorldJournal 2012:159821.
24. Del Brutto OH. 2014. Clinical management of neurocysticercosis. Expert Rev Neurother 14:389-96.
25. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 140:805-20.
26. Vasta GR. 2012. Galectins as pattern recognition receptors: structure, function, and evolution. Adv Exp Med Biol 946:21-36.
27. Maizels RM, Yazdanbakhsh M. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nature reviews Immunology 3:733.
28. Gundra UM, Mishra BB, Wong K, Teale JM. 2011. Increased disease severity of parasite-infected TLR2-/- mice is correlated with decreased central nervous system inflammation and reduced numbers of cells with alternatively activated macrophage phenotypes in a murine model of neurocysticercosis. Infect Immun 79:2586-96.
29. Hewitson JP, Grainger JR, Maizels RM. 2009. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. Molecular and biochemical parasitology 167:1-11.
30. Cardona AE, Restrepo BI, Jaramillo JM, Teale JM. 1999. Development of an animal model for neurocysticercosis: immune response in the central nervous system is characterized by a predominance of γδ T cells. The Journal of Immunology 162:995-1002.
31. Steinman L. 2014. Immunology of relapse and remission in multiple sclerosis. Annu Rev Immunol 32:257-81.

32. Cardona AE, Gonzalez PA, Teale JM. 2003. CC chemokines mediate leukocyte trafficking into the central nervous system during murine neurocysticercosis: role of γδ T cells in amplification of the host immune response. Infection and immunity 71:2634-2642.
33. Mishra PK, Li Q, Munoz LE, Mares CA, Morris EG, Teale JM, Cardona AE. 2016. Reduced Leukocyte Infiltration in Absence of Eosinophils Correlates with Decreased Tissue Damage and Disease Susceptibility in DeltadblGATA Mice during Murine Neurocysticercosis. PLoS Negl Trop Dis 10:e0004787.
34. Chauhan A, Sun Y, Pani B, Quenumzangbe F, Sharma J, Singh BB, Mishra BB. 2014. Helminth induced suppression of macrophage activation is correlated with inhibition of calcium channel activity. PLoS One 9:e101023.
35. Fleury A, Trejo A, Cisneros H, Garcia-Navarrete R, Villalobos N, Hernandez M, Villeda Hernandez J, Hernandez B, Rosas G, Bobes RJ, de Aluja AS, Sciutto E, Fragoso G. 2015. Taenia solium: Development of an Experimental Model of Porcine Neurocysticercosis. PLoS Negl Trop Dis 9:e0003980.
36. Gonzalez AE, Bustos JA, Jimenez JA, Rodriguez ML, Ramirez MG, Gilman RH, Garcia HH, Cysticercosis Working Group in P. 2012. Efficacy of diverse antiparasitic treatments for cysticercosis in the pig model. Am J Trop Med Hyg 87:292-6.
37. Mitchell GF, Handman E. 1977. Studies on immune responses to larval cestodes in mice: a simple mechanism of non-specific immunosuppression in Mesocestoides corti-infected mice. Aust J Exp Biol Med Sci 55:615-22.
38. Cardenas G, Guevara-Silva E, Romero F, Ugalde Y, Bonnet C, Fleury A, Sciutto E, Nunes CM, Soto-Hernandez JL, Shankar SK, Mahadevan A. 2016. Spinal Taenia solium cysticercosis in Mexican and Indian patients: a comparison of 30-year experience in two neurological referral centers and review of literature. Eur Spine J 25:1073-81.
39. Cardona AE, Teale JM. 2002. γ/δ T cell-deficient mice exhibit reduced disease severity and decreased inflammatory response in the brain in murine neurocysticercosis. The Journal of Immunology 169:3163-3171.
40. Mishra BB, Gundra UM, Teale JM. 2008. Expression and distribution of Toll-like receptors 11-13 in the brain during murine neurocysticercosis. J Neuroinflammation 5:53.
41. Abplanalp AL, Morris IR, Parida BK, Teale JM, Berton MT. 2009. TLR-dependent control of Francisella tularensis infection and host inflammatory responses. PLoS One 4:e7920.
42. Mishra BB, Gundra UM, Teale JM. 2011. STAT6(-)/(-) mice exhibit decreased cells with alternatively activated macrophage phenotypes and enhanced disease severity in murine neurocysticercosis. J Neuroimmunol 232:26-34.
43. Mishra BB, Gundra UM, Wong K, Teale JM. 2009. MyD88-deficient mice exhibit decreased parasite-induced immune responses but reduced disease severity in a murine model of neurocysticercosis. Infect Immun 77:5369-79.
44. Takeda K, Akira S. 2005. Toll-like receptors in innate immunity. Int Immunol 17:1-14.
45. Iwasaki A, Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol 5:987-95.

46. Mishra BB, Gundra UM, Teale JM. 2008. Expression and distribution of Toll-like receptors 11–13 in the brain during murine neurocysticercosis. Journal of Neuroinflammation 5:53.
47. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep 6:13.
48. Rodero MP, Khosrotehrani K. 2010. Skin wound healing modulation by macrophages. Int J Clin Exp Pathol 3:643-53.
49. Wynn TA, Vannella KM. 2016. Macrophages in Tissue Repair, Regeneration, and Fibrosis. Immunity 44:450-462.
50. Murray PJ, Wynn TA. 2011. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 11:723-37.
51. Su X, Yu Y, Zhong Y, Giannopoulou EG, Hu X, Liu H, Cross JR, Ratsch G, Rice CM, Ivashkiv LB. 2015. Interferon-gamma regulates cellular metabolism and mRNA translation to potentiate macrophage activation. Nat Immunol 16:838-849.
52. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41:14-20.
53. Wynn TA. 2015. Type 2 cytokines: mechanisms and therapeutic strategies. Nat Rev Immunol 15:271-82.
54. Ruckerl D, Allen JE. 2014. Macrophage proliferation, provenance, and plasticity in macroparasite infection. Immunol Rev 262:113-33.
55. Roszer T. 2015. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. Mediators Inflamm 2015:816460.
56. Loke P, MacDonald AS, Robb A, Maizels RM, Allen JE. 2000. Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. Eur J Immunol 30:2669-78.
57. Jang JC, Nair MG. 2013. Alternatively Activated Macrophages Revisited: New Insights into the Regulation of Immunity, Inflammation and Metabolic Function following Parasite Infection. Curr Immunol Rev 9:147-156.
58. Mishra BB, Gundra UM, Teale JM. 2009. Toll-like receptors in CNS parasitic infections. Curr Top Microbiol Immunol 336:83-104.
59. Cummings RD, Nyame AK. 1996. Glycobiology of schistosomiasis. The FASEB Journal 10:838-848.
60. Khoo K-H, Dell A. 2001. Glycoconjugates from parasitic helminths: structure diversity and immunobiological implications. The Molecular Immunology of Complex Carbohydrates—2:185-205.
61. Van Die I, Cummings RD. 2009. Glycan gimmickry by parasitic helminths: a strategy for modulating the host immune response? Glycobiology 20:2-12.
62. van Die I, Cummings RD. 2006. Glycans modulate immune responses in helminth infections and allergy, p 91-112, Parasites and allergy, vol 90. Karger Publishers.
63. Cummings RD. 2009. The repertoire of glycan determinants in the human glycome. Molecular BioSystems 5:1087-1104.

64. Nyame AK, Lewis FA, Doughty BL, Correa-Oliveira R, Cummings RD. 2003. Immunity to schistosomiasis: glycans are potential antigenic targets for immune intervention. Experimental parasitology 104:1-13.
65. Mukaro VR, Bylund J, Hodge G, Holmes M, Jersmann H, Reynolds PN, Hodge S. Lectins offer new perspectives in the development of macrophage-targeted therapies for COPD/emphysema. PLoS One 8:e56147.
66. Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. Cell 140:805-820.
67. Vázquez-Mendoza A, Carrero JC, Rodriguez-Sosa M. 2013. Parasitic infections: a role for C-type lectins receptors. BioMed research international 2013.
68. Norling LV, Perretti M, Cooper D. 2009. Endogenous galectins and the control of the host inflammatory response. J Endocrinol 201:169-84.
69. Römer CE, Elling L. 2011. Galectins: Structures, binding properties and function in cell adhesion, Biomaterials-Physics and Chemistry. InTech.
70. Vasta GR. 2009. Roles of galectins in infection. Nat Rev Microbiol 7:424-38.
71. Vasta GR, Ahmed H, Nita-Lazar M, Banerjee A, Pasek M, Shridhar S, Guha P, Fernandez-Robledo JA. 2012. Galectins as self/non-self recognition receptors in innate and adaptive immunity: an unresolved paradox. Front Immunol 3:199.
72. D'Haene N, Maris C, Rorive S, Decaestecker C, Le Mercier M, Salmon I. 2014. Galectins and neovascularization in central nervous system tumors. Glycobiology 24:892-8.
73. Leffler H, Carlsson S, Hedlund M, Qian Y, Poirier F. 2002. Introduction to galectins. Glycoconj J 19:433-40.
74. Rabinovich GA, Baum LG, Tinari N, Paganelli R, Natoli C, Liu FT, Iacobelli S. 2002. Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? Trends Immunol 23:313-20.
75. Sato S, St-Pierre C, Bhaumik P, Nieminen J. 2009. Galectins in innate immunity: dual functions of host soluble beta-galactoside-binding lectins as damage-associated molecular patterns (DAMPs) and as receptors for pathogen-associated molecular patterns (PAMPs). Immunol Rev 230:172-87.
76. Cummings RD. 2014. T cells are Smad'ly in love with galectin-9. Immunity 41:171-3.
77. Steelman AJ, Li J. 2014. Astrocyte galectin-9 potentiates microglial TNF secretion. J Neuroinflammation 11:144.
78. Breuilh L, Vanhoutte F, Fontaine J, van Stijn CM, Tillie-Leblond I, Capron M, Faveeuw C, Jouault T, van Die I, Gosset P, Trottein F. 2007. Galectin-3 modulates immune and inflammatory responses during helminthic infection: impact of galectin-3 deficiency on the functions of dendritic cells. Infect Immun 75:5148-57.
79. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS. 2007. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature 450:430-4.
80. Lalancette-Hebert M, Swarup V, Beaulieu JM, Bohacek I, Abdelhamid E, Weng YC, Sato S, Kriz J. 2012. Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. J Neurosci 32:10383-95.

81. Jouault T, El Abed-El Behi M, Martinez-Esparza M, Breuilh L, Trinel PA, Chamaillard M, Trottein F, Poulain D. 2006. Specific Recognition of Candida albicans by Macrophages Requires Galectin-3 to Discriminate Saccharomyces cerevisiae and Needs Association with TLR2 for Signaling. The Journal of Immunology 177:4679-4687.
82. Wright RD, Souza PR, Flak MB, Thedchanamoorthy P, Norling LV, Cooper D. 2017. Galectin-3-null mice display defective neutrophil clearance during acute inflammation. J Leukoc Biol 101:717-726.
83. Biron-Pain K, Grosset AA, Poirier F, Gaboury L, St-Pierre Y. Expression and functions of galectin-7 in human and murine melanomas. PLoS One 8:e63307.
84. Demers M, Magnaldo T, St-Pierre Y. 2005. A novel function for galectin-7: promoting tumorigenesis by up-regulating MMP-9 gene expression. Cancer Res 65:5205-10.
85. Saussez S, Kiss R. 2006. Galectin-7. Cellular and molecular life sciences 63:686-697.
86. Wu C, Thalhamer T, Franca RF, Xiao S, Wang C, Hotta C, Zhu C, Hirashima M, Anderson AC, Kuchroo VK. 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. Immunity 41:270-82.
87. Ransohoff RM. 2016. How neuroinflammation contributes to neurodegeneration. Science 353:777-83.
88. An C, Shi Y, Li P, Hu X, Gan Y, Stetler RA, Leak RK, Gao Y, Sun BL, Zheng P, Chen J. 2014. Molecular dialogs between the ischemic brain and the peripheral immune system: dualistic roles in injury and repair. Prog Neurobiol 115:6-24.
89. Gadani SP, Walsh JT, Lukens JR, Kipnis J. 2015. Dealing with Danger in the CNS: The Response of the Immune System to Injury. Neuron 87:47-62.
90. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, Chen J. 2015. Microglial and macrophage polarization-new prospects for brain repair. Nat Rev Neurol 11:56-64.
91. Girgis NM, Gundra UM, Loke P. 2013. Immune regulation during helminth infections. PLoS Pathog 9:e1003250.
92. Gundra UM, Girgis NM, Ruckerl D, Jenkins S, Ward LN, Kurtz ZD, Wiens KE, Tang MS, Basu-Roy U, Mansukhani A, Allen JE, Loke P. 2014. Alternatively activated macrophages derived from monocytes and tissue macrophages are phenotypically and functionally distinct. Blood 123:e110-22.
93. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. 2010. Mechanisms underlying inflammation in neurodegeneration. Cell 140:918-934.
94. Nakagawa Y, Chiba K. 2015. Diversity and plasticity of microglial cells in psychiatric and neurological disorders. Pharmacol Ther 154:21-35.
95. Franco R, Fernandez-Suarez D. 2015. Alternatively activated microglia and macrophages in the central nervous system. Prog Neurobiol 131:65-86.
96. Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J. 2012. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke 43:3063-70.
97. David S, Kroner A. 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nat Rev Neurosci 12:388-99.

98. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958-69.
99. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, van Wijngaarden P, Wagers AJ, Williams A, Franklin RJM, Ffrench-Constant C. 2013. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci 16:1211-1218.
100. Mahanty S, Garcia HH, Cysticercosis Working Group in P. 2010. Cysticercosis and neurocysticercosis as pathogens affecting the nervous system. Prog Neurobiol 91:172-84.
101. Nash TE, Garcia HH. 2011. Diagnosis and treatment of neurocysticercosis. Nat Rev Neurol 7:584-94.
102. Dell A, Haslam SM, Morris HR, Khoo K-H. 1999. Immunogenic glycoconjugates implicated in parasitic nematode diseases. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1455:353-362.
103. Cooper D, Iqbal AJ, Gittens BR, Cervone C, Perretti M. 2012. The effect of galectins on leukocyte trafficking in inflammation: sweet or sour? Ann N Y Acad Sci 1253:181-92.
104. Cooper DN. 2002. Galectinomics: finding themes in complexity. Biochim Biophys Acta 1572:209-31.
105. MacKinnon AC, Farnworth SL, Hodkinson PS, Henderson NC, Atkinson KM, Leffler H, Nilsson UJ, Haslett C, Forbes SJ, Sethi T. 2008. Regulation of alternative macrophage activation by galectin-3. J Immunol 180:2650-8.
106. Ivashkiv LB. 2013. Epigenetic regulation of macrophage polarization and function. Trends Immunol 34:216-23.
107. Mishra BB, Li Q, Steichen AL, Binstock BJ, Metzger DW, Teale JM, Sharma J. 2013. Galectin-3 functions as an alarmin: pathogenic role for sepsis development in murine respiratory tularemia. PloS one 8:e59616.
108. Mishra BB, Mishra PK, Teale JM. 2006. Expression and distribution of Toll-like receptors in the brain during murine neurocysticercosis. J Neuroimmunol 181:46-56.
109. Sun Y, Chauhan A, Sukumaran P, Sharma J, Singh BB, Mishra BB. 2014. Inhibition of store-operated calcium entry in microglia by helminth factors: implications for immune suppression in neurocysticercosis. J Neuroinflammation 11:210.
110. Gundra UM, Girgis NM, Gonzalez MA, San Tang M, Van Der Zande HJP, Lin JD, Ouimet M, Ma LJ, Poles J, Vozhilla N, Fisher EA, Moore KJ, Loke P. 2017. Vitamin A mediates conversion of monocyte-derived macrophages into tissue-resident macrophages during alternative activation. Nat Immunol 18:642-653.
111. Girgis NM, Gundra UM, Ward LN, Cabrera M, Frevert U, Loke P. 2014. Ly6C(high) monocytes become alternatively activated macrophages in schistosome granulomas with help from CD4+ cells. PLoS Pathog 10:e1004080.
112. Jondle CN, Sharma A, Simonson TJ, Larson B, Mishra BB, Sharma J. 2016. Macrophage Galactose-Type Lectin-1 Deficiency Is Associated with Increased Neutrophilia and Hyperinflammation in Gram-Negative Pneumonia. J Immunol 196:3088-96.

113. Bystrom J, Evans I, Newson J, Stables M, Toor I, van Rooijen N, Crawford M, Colville-Nash P, Farrow S, Gilroy DW. 2008. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. Blood 112:4117-27.
114. Fernandez-Boyanapalli R, Frasch SC, Riches DW, Vandivier RW, Henson PM, Bratton DL. 2010. PPARgamma activation normalizes resolution of acute sterile inflammation in murine chronic granulomatous disease. Blood 116:4512-22.
115. Wang G, Zhang J, Hu X, Zhang L, Mao L, Jiang X, Liou AK, Leak RK, Gao Y, Chen J. 2013. Microglia/macrophage polarization dynamics in white matter after traumatic brain injury. J Cereb Blood Flow Metab 33:1864-74.
116. Ransohoff RM, Kivisakk P, Kidd G. 2003. Three or more routes for leukocyte migration into the central nervous system. Nat Rev Immunol 3:569-81.
117. Shi C, Pamer EG. 2011. Monocyte recruitment during infection and inflammation. Nature reviews Immunology 11:762.
118. Sperandio M, Gleissner CA, Ley K. 2009. Glycosylation in immune cell trafficking. Immunol Rev 230:97-113.
119. Ge XN, Ha SG, Liu FT, Rao SP, Sriramarao P. 2013. Eosinophil-expressed galectin-3 regulates cell trafficking and migration. Front Pharmacol 4:37.
120. Jia W, Kidoya H, Yamakawa D, Naito H, Takakura N. 2013. Galectin-3 accelerates M2 macrophage infiltration and angiogenesis in tumors. Am J Pathol 182:1821-31.
121. Nash TE, Singh G, White AC, Rajshekhar V, Loeb JA, Proano JV, Takayanagui OM, Gonzalez AE, Butman JA, DeGiorgio C, Del Brutto OH, Delgado-Escueta A, Evans CA, Gilman RH, Martinez SM, Medina MT, Pretell EJ, Teale J, Garcia HH. 2006. Treatment of neurocysticercosis: current status and future research needs. Neurology 67:1120-7.
122. Rivest S. 2009. Regulation of innate immune responses in the brain. Nature Reviews Immunology 9:429-439.
123. Cherry JD, Olschowka JA, O'Banion MK. 2014. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. Journal of neuroinflammation 11:98.
124. Ramesh G, MacLean AG, Philipp MT. 2013. Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. Mediators of inflammation 2013.
125. Kielian M. 2014. Mechanisms of virus membrane fusion proteins. Annual review of virology 1:171-189.
126. Glass CK, Natoli G. 2016. Molecular control of activation and priming in macrophages. Nature immunology 17:26-33.
127. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. The Journal of pathology 229:176-185.
128. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41:14-20.
129. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nature reviews immunology 8:958-969.

130. David S, Kroner A. 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nature Reviews Neuroscience 12:388-399.
131. Lammertse D, Jones L, Charlifue S, Kirshblum S, Apple D, Ragnarsson K, Falci S, Heary R, Choudhri T, Jenkins A. 2012. Autologous incubated macrophage therapy in acute, complete spinal cord injury: results of the phase 2 randomized controlled multicenter trial. Spinal cord 50:661-671.
132. Kreider T, Anthony RM, Urban JF, Gause WC. 2007. Alternatively activated macrophages in helminth infections. Current opinion in immunology 19:448-453.
133. Springer TA. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76:301-314.
134. Sturge J, Todd SK, Kogianni G, McCarthy A, Isacke CM. 2007. Mannose receptor regulation of macrophage cell migration. Journal of leukocyte biology 82:585-593.
135. Nieminen J, St-Pierre C, Bhaumik P, Poirier F, Sato S. 2008. Role of galectin-3 in leukocyte recruitment in a murine model of lung infection by Streptococcus pneumoniae. The Journal of Immunology 180:2466-2473.
136. Fukumori T, Takenaka Y, Yoshii T, Kim H-RC, Hogan V, Inohara H, Kagawa S, Raz A. 2003. CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. Cancer research 63:8302-8311.
137. Cooper D, Norling LV, Perretti M. 2008. Novel insights into the inhibitory effects of Galectin-1 on neutrophil recruitment under flow. Journal of leukocyte biology 83:1459-1466.
138. Van Kooyk Y, Geijtenbeek TB. 2002. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. Immunological reviews 186:47-56.
139. van Vliet SJ, Paessens LC, Broks-van den Berg VC, Geijtenbeek TB, van Kooyk Y. 2008. The C-type lectin macrophage galactose-type lectin impedes migration of immature APCs. The Journal of Immunology 181:3148-3155.
140. Wu Z, Hofman FM, Zlokovic BV. 2003. A simple method for isolation and characterization of mouse brain microvascular endothelial cells. Journal of neuroscience methods 130:53-63.
141. Cardona AE, Restrepo BI, Jaramillo JM, Teale JM. 1999. Development of an animal model for neurocysticercosis: immune response in the central nervous system is characterized by a predominance of gamma delta T cells. J Immunol 162:995-1002.
142. Cardona AE, Teale JM. 2002. Gamma/delta T cell-deficient mice exhibit reduced disease severity and decreased inflammatory response in the brain in murine neurocysticercosis. J Immunol 169:3163-71.
143. Shechter R, London A, Varol C, Raposo C, Cusimano M, Yovel G, Rolls A, Mack M, Pluchino S, Martino G. 2009. Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. PLoS medicine 6:e1000113.
144. Nikolakopoulou AM, Dutta R, Chen Z, Miller RH, Trapp BD. 2013. Activated microglia enhance neurogenesis via trypsinogen secretion. Proceedings of the National Academy of Sciences 110:8714-8719.

145. Ekdahl CT, Claasen J-H, Bonde S, Kokaia Z, Lindvall O. 2003. Inflammation is detrimental for neurogenesis in adult brain. Proceedings of the National Academy of Sciences 100:13632-13637.
146. Koh TJ, DiPietro LA. 2011. Inflammation and wound healing: the role of the macrophage. Expert reviews in molecular medicine 13.
147. Lu K-T, Sun C-L, Wo PY, Yen H-H, Tang T-H, Ng M-C, Huang M-L, Yang Y-L. 2011. Hippocampal neurogenesis after traumatic brain injury is mediated by vascular endothelial growth factor receptor-2 and the Raf/MEK/ERK cascade. Journal of neurotrauma 28:441-450.
148. Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J. 2012. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke 43:3063-3070.
149. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. 2009. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. Journal of Neuroscience 29:13435-13444.
150. Perego C, Fumagalli S, De Simoni M-G. 2011. Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice. Journal of neuroinflammation 8:174.
151. Finkelman FD, Shea-Donohue T, Morris SC, Gildea L, Strait R, Madden KB, Schopf L, Urban JF. 2004. Interleukin-4-and interleukin-13-mediated host protection against intestinal nematode parasites. Immunological reviews 201:139-155.
152. Gause WC, Urban JF, Stadecker MJ. 2003. The immune response to parasitic helminths: insights from murine models. Trends in immunology 24:269-277.
153. Reyes J, Terrazas L. 2007. The divergent roles of alternatively activated macrophages in helminthic infections. Parasite immunology 29:609-619.
154. Alvarez JI, Teale JM. 2006. Breakdown of the blood brain barrier and blood-cerebrospinal fluid barrier is associated with differential leukocyte migration in distinct compartments of the CNS during the course of murine NCC. J Neuroimmunol 173:45-55.
155. Cummings RD, Nyame AK. 1996. Glycobiology of schistosomiasis. FASEB J 10:838-48.
156. Khoo KH, Dell A. 2001. Glycoconjugates from parasitic helminths: structure diversity and immunobiological implications. Adv Exp Med Biol 491:185-205.
157. Dell A, Haslam SM, Morris HR, Khoo KH. 1999. Immunogenic glycoconjugates implicated in parasitic nematode diseases. Biochimica Et Biophysica Acta 1455:353-62.
158. Sotelo J, Del Brutto OH. 2002. Review of neurocysticercosis. Neurosurg Focus 12:e1.
159. Garcia-Vallejo JJ, van Kooyk Y. 2009. Endogenous ligands for C-type lectin receptors: the true regulators of immune homeostasis. Immunol Rev 230:22-37.
160. Sakaguchi M, Imaizumi Y, Okano H. 2007. Expression and function of galectin-1 in adult neural stem cells. Cell Mol Life Sci 64:1254-8.
161. Yan YP, Lang BT, Vemuganti R, Dempsey RJ. 2009. Galectin-3 mediates post-ischemic tissue remodeling. Brain Res 1288:116-24.

162. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. 2008. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol 9:1179-88.
163. Nieminen J, St-Pierre C, Bhaumik P, Poirier F, Sato S. 2008. Role of galectin-3 in leukocyte recruitment in a murine model of lung infection by Streptococcus pneumoniae. J Immunol 180:2466-73.
164. Fukumori T, Takenaka Y, Yoshii T, Kim HR, Hogan V, Inohara H, Kagawa S, Raz A. 2003. CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. Cancer Res 63:8302-11.
165. Vasta GR, Ahmed H, Bianchet MA, Fernandez-Robledo JA, Amzel LM. 2012. Diversity in recognition of glycans by F-type lectins and galectins: molecular, structural, and biophysical aspects. Ann N Y Acad Sci 1253:E14-26.
166. Faz-López B, Morales-Montor J, Terrazas LI. 2016. Role of macrophages in the repair process during the tissue migrating and resident helminth infections. BioMed research international 2016.
167. Kreider T, Anthony RM, Urban JF, Jr., Gause WC. 2007. Alternatively activated macrophages in helminth infections. Curr Opin Immunol 19:448-53.
168. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. 2012. Neutrophil function: from mechanisms to disease. Annual review of immunology 30:459-489.
169. Rőszer T. 2015. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. Mediators of inflammation 2015.
170. Bhaumik P, St-Pierre G, Milot V, St-Pierre C, Sato S. 2013. Galectin-3 facilitates neutrophil recruitment as an innate immune response to a parasitic protozoa cutaneous infection. The Journal of Immunology 190:630-640.
171. Bernardes ES, Silva NM, Ruas LP, Mineo JR, Loyola AM, Hsu DK, Liu F-T, Chammas R, Roque-Barreira MC. 2006. Toxoplasma gondii infection reveals a novel regulatory role for galectin-3 in the interface of innate and adaptive immunity. The American journal of pathology 168:1910-1920.
172. Mares CA, Sharma J, Li Q, Rangel EL, Morris EG, Enriquez MI, Teale JM. Defect in efferocytosis leads to alternative activation of macrophages in Francisella infections. Immunol Cell Biol 89:167-72.
173. Martin CJ, Peters KN, Behar SM. Macrophages clean up: efferocytosis and microbial control. Curr Opin Microbiol 17:17-23.
174. Ravichandran KS. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. Immunity 35:445-55.
175. Ravichandran KS, Lorenz U. 2007. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol 7:964-74.
176. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 14:166-80.
177. Steichen AL, Simonson TJ, Salmon SL, Metzger DW, Mishra BB, Sharma J. 2015. Alarmin function of galectin-9 in murine respiratory tularemia. PloS one 10:e0123573.