The Gal-lectin and innate host defenses against *Entamoeba histolytica*

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ABSTRACT

Entamoeba histolytica, etiological agent of amebiasis, continues to be a significant threat to human health worldwide. The disease affects 10% of the world's population and leads to an estimated 100, 000 deaths a year. The parasite's surface Gal-lectin is an immunodominant protein that also mediates colonization and pathogenicity. The Gal-lectin is the most promising vaccine candidate against amebiasis. However, the immune mechanisms involved in protection against disease remain unclear. The objective of this study was to characterize the immunological basis of the host defense mechanisms using a Gal-lectin based vaccine. Exposure of the Gal-lectin with immature dendritic cells increased cell maturation and activation and upregulated co-stimulatory molecules and pro-inflammatory cytokines production. Dendritic cell activation was dependent on NF-κB and MAPK activation. In vaccination studies, the adjuvant effect of CpG-ODN, a synthetic oligodeoxynucleotide capable of stimulating Th1 immune responses enhanced the immune response to the Gal-lectin when administered systemically or mucosally. Protected animals had elevated anti-Gal-lectin serum and stool IgA antibodies capable of blocking parasite adherence *in vitro*. Analysis of cytokine responses in vaccinated and protected animals revealed increased IFN-γ production compared to controls. Finally, *E. histolytica* DNA was shown to activate macrophages in a TLR9 and MYD88-dependent manner. Immunized gerbils with Gal-lectin and *E. histolytica* DNA induced protective immunity against a challenge infection. Taken together, these findings underscore the importance of multivalent subunit vaccines in Th1 mediated immune responses in host defense against amebiasis.

ABRÉGÉ

Le parasite protozoaire *Entamoeba histolytica*, l'agent étiologique de l'amibiase, demeure toujours une menace. On admet actuellement que la prévalence de la maladie est de 500 millions de cas, causant 100,000 décès par an. La molécule de surface Gal-lectin est nécessaire pour la colonisation et l'invasion de l'intestin par *E. histolytica*. La Gal-lectin est une molécule idéale pour un vaccin contre l'amibiase. Les caratéristiques immunologiques de la Gal-lectin sont inconnues. L'objectif de cette étude était de caractériser les mécanismes immunologiques de l'hôte induient par un vaccin Gal-lectin. Les cellules dendritiques exposées à la Gal-lectin maturent et produisent des lymphokines inflammatoires. Cette maturation implique l'activation de NF-κB et des MAP kinases. Les oligodeoxynucléotides non-méthylés CpG sont des immunostimulants qui engendre une réaction immunitaire dans les mammifères. La vaccination avec CpG-ODN a augmenté la réponse immunitaire envers la Gal-lectin soit par injection parentérale ou mucosale. Les animaux protégés avaient des taux élevés d'anticorps IgG et IgA contre la Gal-lectin, capablent de bloquer l'adhérence du parasite *in vitro*. L'analyse des cytokines a démontré que les animaux protégés avaient une augmentation des taux d'INF-γ comparé aux contrôles. Finalement, l'ADN d'*E. histolytica* peut activer les macrophages via TLR9 et MDY88. L'immunisation des gerboises avec la Gal-lectin et l'ADN du parasite induit une protection contre l'aimbiase hépatique. Pris ensemble, ces résultats suggèrent une stratégie de vaccination qui profite du déclenchement de la réponse immunitaire par les produits dérivés du pathogène.

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LIST OF ABBREVIATIONS

Ab-	Antibody
ALA-	Amebic liver abscess
APC-	Antigen presenting cell
BMDC-	Bone marrow derived DC
CD-	Complementary differentiation
CHO-	Chinese hamster ovary
ConA-	Concanavalin A
CpG-	Cytosine-phosphate-Guanosine
Ct-	Cholera toxin
DC-	Dendritic cell
DNA-	Deoxy ribonulceic acid
Eh-	*Entamoeba histolytica*
ELISA-	Enzyme-linked immunosorbent assay
ER-	Endoplasmic reticulum
ERK-	Extracellular signal transduction kinase
FACS-	Fluorescence activated cell sorting
Gal-lectin-	Galactose/N-acetyl-D –galactosamine inhibitable lectin
GM-CSF-	Granulocyte-macrophage colony stimulating factor
HEK-	Human embryonic kidney
Hsp-	Heat shock protein
IFN-	Interferon

Ig-	Immunoglobulin
IL-	Interleukin
i.n.-	Intranasal
iNOS-	Inducible nitric oxide synthase
i.p.-	Intraperitoneal
IRAK-	Interleukin-1 receptor-associated kinase
JNK-	Jun-NH_2 terminal kinase
kDa-	Kilo Dalton
LPS-	Lipopolysaccharide
MAb-	Monoclonal antibody
MAP kinase-	Mitogen activated protein kinase
MHC-	Major histocompatibility complex
MLR-	Mixed leukocyte reaction
MyD-	Myeloid differentiation
NADPH-	Nicotinamide adenine dinucleotide phosphate
NF-κB-	Nuclear factor -κB
NK-	Natural killer
NO-	Nitric oxide
ODN-	Oligodeoxynucleotide
PAMP-	Pathogen associated molecular pattern
PRR-	Pathogen recognition receptor
PCR-	Polymerase chain reaction
RNA-	Ribonucleic acid

ROS-	Reactive oxygen species
SAP-	Soluble amebic proteins
SCID-	Severe combined immunodeficient
Th-	T helper
TIR-	Toll/IL-1 receptor
TLR-	Toll-like receptor
TNF-	Tumor necrosis factor
TRAF-	Tumor necrosis factor receptor-associated factor
TRIF-	Toll/IL-1 receptor domain-containing adaptor inducing IFN-β

THESIS OFFICE STATEMENT

As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the students is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

Candidates have the option of including, as parts of the thesis, the text of one or more paper submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with the respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression form one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

The thesis must conform to all the other requirements for the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (1) a table of contents (2) a brief abstract in both English and French (3) an introduction which clearly states the rational and objectives of the research (4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper)

(5) a final conclusion and summary (6) a thorough bibliography and (7) an appendix containing an ethics certificate in the case of research involving humans or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

In general, when co-authored papers are included in the thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all authors of the co-authored papers.

STATEMENT OF ORIGINALITY

Manuscript I

This manuscript describes the activation of dendritic cells by *E. histolytica* Gal-lectin. This is the first report of this vaccine candidate directly modulating dendritic cell maturation and the ensuing protective immune response. These observation help elucidate the immunological basis for the protective effects of the Gal-lectin.

Manuscript II

This study evaluates the adjuvant potential of CpG-ODN in a parenteral Gal-lectin vaccine. The results demonstrate that CpG-OND adjuvants contributed to the complete protection of gerbils from amebic challenge infection. This is the first report of a Gal-lectin vaccine with adjuvants that demonstrate promising use in humans. Furthermore, this study confirmed the crutial role of IFN-γ in protection from invasive amebiasis.

Manuscript III

This paper reports the use of CpG-ODN in a mucosal Gal-lectin vaccine. This is the first report of a Gal-lectin mucosal vaccine without conventional adjuvants, that could induce antigen specific fecal IgA and confer protective systemic immunity.

Manuscript IV

This manuscript details the identification of *E. histolytica* DNA as a parasite derived molecule that can activate macrophages via TLR9. The immunological properties of *E. histolytica* DNA on immune cells and in conjunction with the Gal-lectin are reported here for the first time.

STATEMENT OF AUTHORSHIP

This thesis consists of four manuscripts co-authored with my supervisor Dr. Kris Chadee. Dr. Chadee provided financial support for the laboratory work, advice with regards to experimental design and corrections of the thesis and manuscripts. Kathy Keller and Elaine deHeuvel (research technicians) helped with animal handling in the vaccination studies and Michael Prystajecky (summer student) helped with preliminary real time PCR results in the fourth manuscript.

SECTION I: LITERATURE REVIEW

INTRODUCTION

Entamoeba histolytica, the etiological agent of human amebic dysentery and amebic liver abscess, is named for its ability to destroy tissues. Despite the availability of effective anti-amebic chemotherapy, this protozoan parasite continues to be a significant threat to human health around the world and leads to 100,000 deaths annually [1]. Amebiasis is the third leading cause of parasitic death after malaria and schistosomiasis.

In contrast to other protozoan parasites with complex life cycles, *E. histolytica* cycles only from the infective cyst stage to the mobile trophozoite stage. The parasite has a direct life cycle with humans as the only relevant hosts. These characteristics of the parasite simplify vaccine development and make vaccination a feasible intervention to reduce morbidity rates of amebiasis [2]. Acquired immunity to *E. histolytica* has been repeatedly demonstrated in laboratory animals, and recent epidemiological studies have correlated anti-amebic IgA responses with resistance to infection [3]. In addition, patients drug-cured of invasive amebiasis with metronidazole are also resistant to subsequent *E. histolytica* infections. This evidence supports the possibility of an effective amebiasis vaccine, which could prevent parasite colonization and invasive disease.

The leading candidate antigen for an amebiasis vaccine is the Galactose/N-acetyl-D-galactosamine inhibitable lectin (Gal-lectin). The Gal-lectin is a major surface adhesin of the parasite, essential for binding to colonic mucins for colonization and adherence to host cells [4]. The Gal-lectin is a heterodimer composed of heavy and light subunits linked by disulfide bonds, of which the heavy subunit is known to be required in adherence and cytotoxicity as it contains the carbohydrate recognition domain (CRD) [5]. The Gal-lectin is an ideal vaccine candidate because it is antigenically stable, immunogenic and essential for parasite biological functions. *In vitro* studies have shown that the Gal-lectin can stimulate macrophage production of TNF-α, nitric oxide (NO) and IL-12. Vaccination trials in animal models of experimental amebiasis have demonstrated the protective potential of the Gal-lectin and identified the requirement of cell-mediated responses to protect from disease progression [6, 7]. The immunological basis for the protection observed with Gal-lectin immunization is not known, and the challenge lies in developing a vaccine that could prevent colonization and disease in the case of parasite invasion. An ideal amebiasis vaccine then would contain the Gal-lectin in order to induce mucosal IgA antibodies that could block parasite binding to colonic mucins, but also Gal-lectin specific cellular immune response that could clear invading trophozoites.

Recently, as our knowledge about innate immunity increases, the choice of adjuvant in vaccine development has become as important as the choice in vaccine antigen. It is imperative that the vaccine formulations induce the correct response to ensure protection. Dendritic cells have a crutial role in this vaccine response as they direct

the capture of antigens and the ensuing responses. In fact, antigen capture in the absence of concurrent dendritic cell maturation leads to immune tolerance and not immunity [8]. An emerging field in vaccination research is the use of TLR ligands in immunizations to ensure the proper adjuvant response. CpG-ODNs are recognized by TLR9 expressed on sub-sets of dendritic cells and induce maturation as well as potent Th1 cytokine production [9]. CpG-ODNs have been used in animal models of various diseases and shown consitent adjuvant properties both with parenteral and mucosal vaccines [10]. On going clinical trials with CpG-ODN have investigated the use of these TLR ligands as adjuvants and demonstrated that they are well tolerated by humans and produce the desired improved immune responses.

The Gal-lectin is a prime candidate for an amebiasis vaccine, however its role in dendritic cell activation has been hitherto unexplored. Moreover, given the immunogenic Th1 response and protection observed to date with the Gal-lectin, efforts should be made to improve vaccine efficacy. This has lead to the current study with the following specific objectives:

1) *To determine the immunological activation potential of the Gal-lectin in dendritic cells*
2) *To augment Gal-lectin Th1 immunity using CpG-ODNs in parenteral and mucosal vaccines*
3) *To identify the protective potential of the Gal-lectin and other Th1 inducing amebic components*

Our studies showed that the Gal-lectin induces dendritic cell maturation, activation, and the production of Th1 cytokines. This is important in terms of vaccination, demonstrating that the Gal-lectin can induce dendritic cell maturation and direct the ensuing protective Th1 response *in vivo*. For the first time we have tested the Gal-lectin with CpG-ODN adjuvant and observed protective immunity in gerbils characterized by increased IFN-γ production. When administered mucosally, Gal-lectin and CpG-ODN induced antigen specific fecal IgA and could also protect gerbils against systemic challenge infection in the liver. We identified *E. histolytica* DNA as another immunogenic parasite molecule and demonstrated its protective effect in combination with the Gal-lectin. These results have illustrated the safe and effective use of CpG-ONDs in an amebiasis vaccine with the Gal-lectin.

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CHAPTER 1: AMEBIASIS AND *ENTAMOEBA HISTOLYTICA*

1.1 *Entamoeba histolytica* morphology and life cycle

Entamoeba histolytica is an enteric protozoan parasite belonging to Subclass: Gymnamoebia; Class: Lobosea; Order: Amoebida; Family: Endamoebidae. The amoeba was first described in 1875 by Fedor A. Lösch in St. Petersburg [1]. The physician studied the amebic trophozoites from the stool of a farmer with a fatal case of amebiasis. However, the association of amebae with pathogenesis was only established in 1903 by Schaudinn when he named the species *E. histolytica* for its ability to lyse tissues [2].

E. histolytica has a simple life cycle, with human beings as the only natural hosts. The parasite exists either as a mobile trophozoite or the infective cyst form. Cysts are typically round, between 10-15 μm in diameter, and contain four nuclei, glycogen and chromatoidal bodies (crystallized ribonucleoproteins). The infective cyst stage is ingested through fecally contaminated food or water. The cyst withstands gastric passage, travels through the small intestine where it excysts and colonizes the colon. After excystment, the cytoplasm and nuclei divide to give rise to eight daughter amebae, which will further divide by binary fission. The amebic trophozoites range in width from 20-30 μm, although some can be as small as 10 μm or as large as 60 μm. The parasites multiply within the mucosal crypts of the large intestine, actively moving by the extension of their pseudopodia.

Trophozoites have a thin clear ectoplasm easily distinguished from their granular endoplasm, which contains the nucleus and food vacuoles. The nucleus is clear, but after staining with iron-hematoxylin, a dark central endosome with radiating achromatic fibrils can be seen. Trophozoites will feed on intestinal bacteria and food particles, multiply and encyst in the colon. The cysts are excreted into the environment with feces, thereby completing the life cycle.

1.2 Epidemiology

The World Health Organization estimates that *E. histolytica* infects 500 million people worldwide and leads up to 100, 000 deaths a year [3]. *E. histolytica* is a cosmopolitan parasite, but is most prevalent in subtropical areas like Mexico, Africa, India, and South America. Higher prevalence in those areas is due to lower standards of sanitation leading to greater contamination of food and water. All races are equally susceptible to infection, but highrisk groups include travelers to endemic areas [4], homosexuals [5] and immunocompromised individuals [6]. Following this last point, severe cases of amebiasis are more common in young children, pregnant women and elderly individuals [7].

The majority of infected individuals remain asymptomatic, with the parasite residing in the large intestine as a commensal. For yet undetermined reasons, an estimated 10 % of infected humans will develop symptoms of invasive disease. The factors that determine whether an infected individual will develop invasive disease are not known. A recent study conducted in an endemic area of Bangladesh suggests that

there may be an association between the parasite genotype and risk of complication [8]. There may also be host factors that could predict the outcome of disease, however these remain unidentified to date. One study associated susceptibility to amebic liver abscess formation with HLA-DR3 and complotype SC01 in some Mexican populations [9], however there are still unexplained differences in rates of diseases between populations. **To summarize, amebiasis continues to be a global threat to human health and can be attributed to poor sanitation and inadequate water quality.**

1.3 Clinical symptoms and diagnosis

Approximately 10 % of individuals infected with *E. histolytica* will develop invasive amebiasis [10]. The two major clinical manifestations of disease are amebic colitis and extraintestinal amebic liver abscess.

Amebic colitis is characterized by gradual dysentery with symptoms including abdominal pain, painful bowel movements and bloody diarrhea [11]. Individuals at risk of developing fulminant amebic colitis include pregnant woman, immunocompromised persons, and patients treated with corticosteroids. Toxic megacolon is the most feared complication of amebic colitis appearing in 0.5% of cases. This condition is an acute dilation of the colon, associated with 40% mortality due to sepsis [12]. The most common extraintestinal representation of disease is the amebic liver abscess. The abscess arises from the dissemination of trophozoites via the portal circulation once the parasite breaches the colonic mucosa. Some patients

have concurrent amebic colitis, but in most cases the individual has no intestinal symptoms. Individuals with amebic liver abscesses will complain of fever, upper right quadrant pain, and hepatic tenderness [13]. These symptoms are acute but can be chronic, along with anorexia and weight loss. In rare cases the trophozoites may spread to other soft organs, such as the lungs and the brain. Patients with pleuropulmonary amebiasis may develop a cough, pleuritic chest pain and in the case of the formation of a bronchial fistula, large amounts of sputum may be coughed up. In less than 0.1% of amebic liver abscess cases, a concurrent brain abscess may form. Symptoms include headaches, vomiting, seizures and changes in mental condition.

Diagnosis of amebic colitis requires the identification of *E. histolytica* trophozoites in the stool or intestinal mucosa. Historically, a gut infection was diagnosed by light microscopy, by identification of hematophagous throphozoites in the stool. Although these trophozoites would indicate an infection, the occurrence of these hematophagous trophozoites is rare [14]. Furthermore, the identification of the pathogenic *E. histolytica* is difficult because of the presence the non-pathogenic *E. dispar*, which is morphologically identical. However, alternative techniques have facilitated the identification of an *E. histolytica* infection.

Isoenzyme analysis is considered the "gold standard" for disease diagnosis, as it accurately makes the distinction between the pathogenic *E. histolytica* and non-pathogenic *E. dispar* [15]. A type II pathogenic zymodene observation is positive for amebiais. This method involves the culture of trophozoites from stool samples and

requires laboratory facilities and several weeks to complete. This method, therefore, is not practical in the field or developing endemic countries. Other molecular tests include enzyme-linked immunosorbent assays (ELISA), like the TechLab *E. histolytica* test that can distinguish *E. dispar* from the pathogenic ameba by using monoclonal antibodies against the Galactose/N-acetyl-D-galactosamine inhibitable lectin (Gal-lectin) [16]. Although the ELISA tests are sensitive, results are usually confirmed by another method, such as PCR [17-19]. These PCR reactions amplify and detect *E. histolytica* DNA in stool samples or from trophozoites after culture from the samples. Serological methods can be used to determine prior infection with either *E. histolytica* or *E. dispar*. Symptomatic patients develop serum anti-amebic antibodies whereas patients with *E. dispar* do not. Levels of serum IgG anti-amebic antibodies remain high for years, which can indicate prior exposure to the parasite but is not useful for the diagnosis of acute amebiasis in endemic areas. In a clinical setting, the simplest method for diagnosis of amebic colitis, when an *E. histolytica* infection is suspected and to rule out non-infectious causes, is a colonoscopy or a colonic biopsy to confirm the presence of trophozoites [20]. In the case of an amebic liver abscess, stool sample microscopy is not recommended as most patients do not have associated dysentery. Therefore, diagnosis relies on positive serology for *E. histolytica* and the identification of the abscess through imaging. Ultrasound, magnetic resonance, CT scans and tomography can all be used to detect a liver abscess [21,22].

1.4 Treatment

The drugs of choice for treatment of amebiasis are the nitroimidazole derivatives, like metronidazole. The compound is activated in anaerobic organisms, such as *E. histolytica*, by reduction of the 5-nitro group. The reduced compound is then capable of damaging amebic DNA [23]. Treatment of amebic colitis starts with metronidazole therapy, followed by luminal agents to eliminate parasite colonization. Luminal agents like paromomycin, which inhibit protein synthesis, are poorly absorbed by the gut and are therefore used for clearing susceptible protozoa from the intestinal lumen [24]. Fulminant colitis is treated similarly with the addition of antibiotics for intestinal microflora. Due to the risk of invasive disease, asymptomatic patients are treated with luminal agents to clear infections and to reduce the spread of infective cysts. In most cases, treatment of amebic liver abscess can be accomplished with a single dose of metronidazole [25]. Drainage is not required and not recommended as it risks contamination of the peritoneal cavity [26, 27]. Aspiration of abscess fluids should be reserved for patients that do not respond to conventional treatment or those with a large abscess at risk of rupturing. Patients with amebic liver abscess are also treated with luminal agents to eradicate intestinal colonization.

1.5 Pathogenesis of amebiasis

The pathogenesis of amebiasis is a complicated process involving three distinct stages and multiple parasite virulence factors. The parasite is acquired by the ingestion of the cyst stage in contaminated food or water, however infection and disease progression requires these three stages: colonization, mucus depletion and damage of

host cells. The parasite must overcome the innate mucosal defenses of the host, including both physical barriers and immune mechanisms.

The first step in pathogenesis of amebiasis is the colonization of the host's colon by the mobile amebic trophozoites. The human gastrointestinal tract is protected by a gelatinous mucus layer, which is composed of carbohydrate rich mucin molecules. Colonization of the parasite to this mucin layer is mediated by a single molecule, the Gal-lectin. The Gal-lectin binds to Galactose and N-acetyl-D galactosamine residues of colonic mucins [28]. The Gal-lectin is a major surface protein, which contains the carbohydrate recognition domain involved in adherence to the terminal sugar moieties of colonic mucins and host cells [29]. Adherence to colonic mucins allows the parasite to multiply and prevents it from being carried away, thus establishing infection. The *E. histolytica* Gal-lectin has been shown to have high binding affinity (K_d= 8.2 x 10^{-11} M^{-1}) to purified rat and human colonic mucins [30]. At the same time, however, mucins provide a protective role, preventing parasite contact with underlying epithelial cells.

In the second stage, the parasite overcomes this physical barrier, by both depletion and destruction of mucins. Colonic mucins are produced by goblet cells in the intestine and it is believed that *E. histolytica* can induce hypersecretion in these cells. The parasite can induce significant mucous secretion from globlet cells. In fact, studies in gerbils suggested that the trophozoites release a mucous secretagogue, which would lead to the eventual depletion of mucin secretion from globet cells

[31,32]. The exact nature of the secretagogue and the mechanisms involved in this depletion process are not known. There is evidence that prostagladin E_2 (PGE_2) might be involved as it demonstrates mucous secretagogue activity on rat and human colonic epithelial cells [33]. Furthermore, *E. histolytica* is known to have the cyclooxygenase (COX)-like enzyme, which catalyzes the conversion of arachidonic acid to PGE_2 and could therefore produce its own prostaglandin [34]. In conjunction with mucous depletion is the process of mucous destruction by the parasite, which occurs through the activity of cysteine proteases. It has specifically been shown that *E. histolytica* cysteine protease 5 (EhCP5) can directly cleave the C-terminus of the MUC2 polymer, thus destroying the gel forming properties of mucins [35]. In fact, EhCP5 deficient trophozoites are less virulent both *in vivo* and *in vitro* [36]. It is interesting to note that the non-pathogenic *E. dispar* does not express this protease. While cysteine proteases have a significant role in mucin destruction, parasite glycosidases might also contribute to this stage of pathogenesis. *E. histolytica* is known to produce an array of glycosidases, such as β-N-acetylhexosaminidase, α-D-glucosidase, β-D-galactosidase and β-D-glucosidase [37]. These glycosidases would aid in the degradation of mucin sugars and may facilitate cleavage by cysteine proteases, thereby removing the protective mucous layer.

Upon removal of this protective mucous barrier, the parasite comes in contact with host intestinal epithelial cells. As mentioned above, parasite adherence is mediated by the Gal-lectin, and so this molecule is also required for binding to host cells. Adherence to host cells is followed by cytolysis thus leading to tissue destruction

from which the parasite gets its name. The importance of Gal-lectin mediate cell contact has been illustrated *in vitro* with a Chinese Hamster Ovary (CHO) cells deficient in terminal galactose/N-acetyl galactosamine residues, which were resistant to amebic cytolytic activity compared to normal cells [38]. It has also been demonstrated *in vivo* that Gal-lectin deficient trophozoites are less virulent, causing very little pathology [39]. Cytotoxicty is observed within 5-15 minutes after parasite contact, and involves both apoptosis and necrotic cell death [40-42]. This cell death is thought to be associated with the action of amoebapore proteins [43,44]. Amoebapores are pore-forming proteins that are inserted by the trophozoites into the host cell membrane upon contact and cause cell lysis. A recent study, however suggested a role for amoebapores in amebic liver abscess formation and not in amebic colitis [45].

The mechanisms of target cell death induced by *E. histolytica* are still under investigation. Studies *in vitro* using either CHO cells or Jurkat T-cells demonstrate a 20-fold increase in intracellular calcium levels immediately after target cell contact with the parasite [40]. This event is then followed by membrane blebbing and cell death, however it is still unclear whether calcium is acting as a secondary messenger initiating downstream events that would lead to this cell death. Apoptosis is also believed to be involved in cell death due to amebic contact with host cells. As mentioned above both necrosis, involving cell swelling and rupture, and apoptosis, characterized by specific cellular changes, are observed in target cell death related to amebiasis. There is increasing evidence of apoptotic events after cell contact with

amebic trophozoites. It was first reported that DNA fragmentation occurred in murine myeloid cells exposed to the parasite and this event is not inhibited in cells overexpressing Bcl-2 [46]. Amebic liver abscess models in knockout mice have also reported that hepatocyte death is independent of Fas ligand or TNF receptor [47]. In fact, gene array analysis in a mouse model of amebiasis reported the up-regulation of genes associated with programmed cell death, apoptosis, upon amebic infection [48]. Some *in vitro* studies with Jurkat T-cells suggest a role for caspases in apoptotic target cell death due to exposure to amebae. This cell death was dependent on caspase-3 but not caspases 8 and 9 [49,50]. Another study supports the role of caspases in pathogenesis, as using a general caspase inhibitor could reduce amebic liver abscess formation by 70% in a mouse model of the disease [51]. Other evidence for apoptosis after parasite contact includes host cell exposure of the apoptotic marker phosphatidyl serine [52], and the fact that ameba preferentially ingest/phagocytose apoptotic cells over necrotic cells [41].

Although contact-dependent cytolysis by the parasite is an important aspect of pathogenesis, the parasite may also cause damage by altering normal intestinal physiology. The cysteine proteases mentioned above are known to also degrade extracellular matrix proteins [53], laminin [54] and intestinal epithelial cell tight junctions [55]. Tight junctions link adjacent epithelial cells and maintain cell polarity, separating apical and basolateral regions. These tight junctions regulate the passage of solutes from the lumen and thus are involved in homeostasis. It has been shown *in vitro* that *E. histolytica* can disturb epithelial tight junction integrity and

increase cellular permeability [55]. Therefore alterations in normal gut physiology by the presence of the parasite could inadvertently facilitate trophozoite invasion and progression of disease.

The pathogenesis of amebiasis is a multifactorial process and recent evidence also suggests a role for host immune responses in disease progression. Infiltration of host immune cells at the site of infection can lead to extensive damage, and in fact this is observed in amebic colitis [31]. Neutrophils are the first cell recruited at the site of infection and this is believed to be due to the release of chemoattractants by host epithelial cells in response to parasite exposure. Contact-dependent killing of epithelial cells leads to the release of IL-1α, which can further induce the secretion of IL-8 by neighboring cells [56]. Furthermore, our lab has demonstrated that IL-8 secretion from intestinal epithelial cells can occur in the absence of direct contact with the parasite, as soluble amebic proteins on their own can induce this chemokine release [57]. Apart from the potent effect of these soluble amebic proteins, PGE_2 is known to induce IL-8 secretion in these cells following amebic infection [58,59]. As previously mentioned, *E. histolytica* express COX-like enzyme and is capable of producing PGE_2 which could act on host epithelial cells. IL-8 is a potent neutrophil chemoattractant produced during amebic infection, but the amebae themselves are also known to produce a neutrophil chemoattractant [60]. Other immune cells such as plasma cells, lymphocytes and macrophages are also recruited to the lesion [61]. Tissue destruction is exacerbated by the lysis of this infiltrate by the parasite, specifically macrophage and neutrophil lysis. Upon cell lysis, these cells release toxic

contents, which may include proteases, cathepsins, lysozymes and reactive oxygen species or nitrogen intermediates, all of which can lead to intestinal tissue damage [62,63]. The role of neutrophils in pathogenesis is further supported by and *in vivo* model study where there was decreased tissue damage as well as decreased colonic ulceration after neutrophil depletion [64]. Amebae are resistant to neutrophil attack [65], however the mechanisms of this resistance are not known. Resistance could be due to the presence of superoxide dismutase NADPH: flavin oxidoreductase in *E. histolytica* [66], which could neutralize reactive oxygen species.

In the cases of amebic colitis, parasite factors and immune cell infiltration lead to extensive tissue damage and may facilitate parasite invasion into the sub-mucosa. This invasion is typically identified by the formation of flask-shaped ulcers in the mucosal tissue. At this stage tissue dwelling parasites seem to inhibit further immune cell recruitment by secreting monocyte locomotion-inhibitory factor [67[, which interferes with leukocyte migration and displays anti-inflammatory properties. [68]. If the colitis is left untreated the amebae may eventually perforate through the muscle and reach the blood vessels and be disseminated by the circulatory system to soft tissue organs. In these organs pathogenesis may be different than at the intestinal level, but still involves the Gal-lectin for contact-dependent cell death, amoebapores and cysteine proteases. **To summarize, the pathogenesis of amebiasis can be divided into three phases: colonization, mucin degradation and host cell damage. Diseases progression is multifactorial and involves the actions of both host and parasite factors.**

1.6 Host response and immunity to *E. histolytica*

The distinct clinical manifestations of disease with *E. histolytica*, mainly amebic colitis and amebic liver abscess, also have distinct immune responses. Amebic colitis is characterized by a prominent pro-inflammatory response, whereas amebae in the liver do not cause hepatitis but an abscess surrounded by connective tissue to segregate healthy liver parenchyma from diseased areas. Despite this pathophysiological difference, there are many common immune components active during amebic colitis and amebic liver abscess.

The parasite first encounters intestinal epithelial cells and as a result gut inflammation in amebic colitis is the result of epithelial cell release of pro-inflammatory cytokines IL-8 and IL-1β [56]. As mentioned above, these mediators can attract neutrophils and macrophages to the site of infection. Neutrophils are also the first host defense cell to contact amebae in the liver, indicating recruitment of these cells to the site of infection [69]. Neutrophils were found to play a role in early defense against liver abscess in SCID mice [70]. Depletion of neutrophils in this model lead to the formation of larger abscesses and the absence of the ring of inflammatory cells, which normally surround the necrotic region [71]. In the case of amebic colitis, however, it would appear that neutrophil recruitment exacerbates inflammation through the release of their toxic contents [72].

The inflammatory response in epithelial cells is dependent on NF-κB, the key transcription factor regulating pro-inflammatory cytokines such as IL-6, IL-1β, IL-8 and TNF-α. Human intestinal xenografts treated with antisense oligonucleotide to p65 sub-unit of NF-κB and then infected with *E. histolytica* trophozoites demonstrated lower levels of IL-8 and IL-1β than controls [72]. It has also been reported that patients with amebic colitis who are mistakenly administered corticosteroids that inhibit NF-κB display increased disease severity and risk of complication [73]. Although inflammation in amebic colitis definitely contributes to tissue damage it may also be required to limit infection.

Macrophages have been associated with cell-mediated immunity against *E. histolytica.* The activation of these effector cells depends on environmental cues and the inflammatory stimuli. The major priming signal for macrophage activation is IFN-γ. Naive bone marrow derived macrophages exposed to *E. histolytica* proteins demonstrate increased expression of *c-fos*, which is involved in activation, and increased TNF-α mRNA [74]. Macrophages can effectively kill amebae *in vitro* in a nitric oxide (NO)-dependent manner [75]. TNF-α production by activated macrophages can enhance iNOS mRNA for increased NO production [76]. The ability of *E. histolytica* proteins to stimulate TNF-α could be central in activating primed macrophages for amebicidal activity. In fact, in a SCID mouse model of amebic liver abscess where the genes for IFN-γ receptor and iNOS were disrupted it was found that animals developed greater disease severity [77]. In the case of amebic liver abscess formation, IFN-γ is important to prime macrophages for activation and

iNOS is essential to mount a proper amebicidal response. On the other hand, in amebic colitis most of the damage is due to the inflammatory response. When a human intestinal xenograft model was used to determine the role of TNF-α and IL-1, it was found that depletion of these cytokines resulted in less inflammation and reduced pathology when infected with *E. histolytica* [78]. **To summarize, the response of the human intestine and the human liver to *E. histolytica* is fundamentally different.**

1.7 Moduation of host response by *Entamoeba histolytica*

Perhaps the difference in pathology associated with amebic colitis and amebic abscess is due to the ability of the parasite to vary its virulence in the two organs. *E. histolytica* has an array of virulence factors involved in pathogenesis and has also demonstrated the ability to modulate host immune responses in order to promote parasite survival. For example, parasite cysteine proteases have been shown to cleave complement components C3a and C5a to counteract immune cell recruitment thus suppressing the inflammatory response [79]. Furthermore cysteine proteases can cleave immunoglobulins and abolish antibody-mediated protective effects [80]. More importantly, the parasite has the ability to suppress monocyte and lymphocyte functions, thus limiting the extent of the immune response against it. Macrophages from gerbils displaying acute amebic liver abscess are refractory to the effects of IFN-γ, are no longer able to generate a respiratory burst and therefore have lost the ability to kill trophozoites [81]. The mechanisms of this macrophage suppression are unclear, however, there are potential mediators involved such as macrophage

migration inhibitory factor (MIF) and PGE_2. MIF is secreted by the parasite and is found to inhibit monocyte locomotion, inhibit respiratory burst in immune cells and inhibit T-cell hypersensitivity reaction [82,83]. In addition PGE_2 is believed to have a role in immune suppression as inhibition of this prostaglandin with indomethacin results in the reduction of abscess size [84]. It is known that PGE_2 is produced during amebic liver abscess development in the liver [58,59] and that this molecule suppresses macrophage MHC class IIa expression and TNF-α production in response to IFN-γ [85]. Immune suppression by *E. histolytica* is not restricted to macrophages; in fact T-lymphocytes functions are attenuated by the parasite in a similar fashion. Similarly, pre-treatment of murine splenocytes with amebic antigens results in a decreased subsequent proliferative response to the T-cell mitogen concanavalin A (ConA), as well as phytohemaglutinin and LPS [86]. Is has also been extensively shown that serum from amebic liver abscess (ALA) patients or gerbils with acute ALA can suppress T-cell proliferative responses to conA [87]. This specific suppression is mediated by a decrease in IL-2 production [88]. **To summarize, *E. histolytica* has evolved mechanisms to circumvent the host defense and this feature could play role in the outcome of infection.**

Although the host reponse to *E. histolytica* is complicated and may even be modulated by the parasite, there is evidence that humans develop immunity after amebic infection. It is known that individuals infected with *E. histolytica* develop antibodies against the parasite. Patients who develop amebic liver abscess develop anti-amebic antibodies detectable within a week of the occurrence of symptoms [89,

90], however these serum antibodies do not appear to have any effect on the course of abscess development. This observation suggests that antibodies may play a role earlier at the onset of infection or protect against subsequent infections. It has been demonstrated in laboratory studies that passive immunization of hamsters with human immune serum can partially protect the animals from intrahepatic challenge with live *E. histolytica* trophozoites [91]. Another similar study performed passive immunization with anti-amebic antibodies from humans with amebic liver abscess and found that this could protect against SCID mouse abscess development when challenged with the parasite [92]. Since SCID mice lack functional T and B cells, this study suggests a role for pre-existing anti-amebic antibodies in protection from liver abscess formation. Humans also develop serum IgM antibodies against the *E. histolytica* Gal-lectin, 55% of amebic colitis patients, 77% of amebic abscess patients and 13.4% of asymptomatic patients having detectable antibody titers [93].

Considering that *E. histolytica* is an intestinal parasite, it is not surprising that mucosal antibodies could play a role in protective immunity. In fact, anti-amebic IgA antibodies have been detected in the saliva of patients diagnosed with amebic colitis [94]. Furthermore, these human salivary IgA against the *E. histolytica* can inhibit parasite adherence to target cells *in vitro*, suggesting the ability to prevent colonization [95]. Recent epidemiological studies have illustrated the importance of mucosal immunity in resistance to *E. histolytica* infection. These studies were conducted in preschool Bangladeshi children where there is a 55% *E. histolytica* infection rate and colitis is diagnosed in 4% of these cases of infection [96]. These

studies determined that children with anti-amebic stool IgA had 64% fewer infections and the occurrence of these antibodies was thought to prevent parasite colonization [97]. In most cases the anti-amebic stool IgA was detectable near the time of resolution. While the presence of stool IgA was associated with protection from reinfection, the absence of serum IgG against the parasite was also associated with innate resistance to *E. histolytica* infections [96]. Upon closer examination it was determined that the presence of stool IgA specifically against the Gal-lectin was protective, associated with 86% less new infections over the following year. Protective immunity after amebic infection appears to be mediated by mucosal IgA against *E. histolytica.*

Protective immunity against amebiasis is not limited to mucosal responses. There is evidence that cell-mediated immunity also plays a role, however there is a need for more humans studies in this aspect of immunity against *E. histolytica.* Cell-mediated immunity appears to have a greater role in amebic liver abscess, where the tissue dwelling parasite is in contact with immune cells. Lymphocytes from patients cured from amebic abscess that are stimulated *in vitro* with soluble amebic antigens produce large amounts of IFN-γ, which is required for macrophage amebicidal activity [98]. It was also demonstrated *in vitro* that these stimulated lymphocytes could become amebicidal, demonstrating cell-contact dependent cytotoxicity against *E. histolytica.* Most evidence of cell-mediated responses being protective is from animal studies where drug cured animals are resistant to subsequent hepatic challenge infection [99] or immunization with amebic antigens can protect against amebic abscess challenge

[100]. Prior exposure to the parasite seems to produce immunity to reinfection mediated by elimination of the *E.histolytica* trophozoites. A recent epidemiological study has correlated IFN-γ with reduced risk of reinfection [101]. In this study peripheral blood mononuclear cells from Bangladeshi children were isolated and exposed to soluble amebic antigens to eveluate cytokine responses. Those children whose lymphocytes produced higher amounts of IFN-γ had longer survival without symptoms of disease and less risk of reinfection. IFN-γ was also associated with nutritional status and correlated to a reduced susceptibility to disease. Based on the physiological role of IFN-γ, which is to activate macrophages for innate and adaptive responses, it is interesting that this cell-mediated cytokine was correlated with resistance to amebic infection. **To summarize, mucosal immunity plays an important role in resistance to the establishment of *E. histolytica* infection and cell-mediated immunity may be important in clearing infections.**

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CHAPTER 2: *ENTAMOEBA HISTOLYTICA* GAL-LECTIN

2.1 Gal-lectin structure and gene expression

The major surface protein of *E. histolytica* is the Gal-lectin. Gal-lectin is a 260 kDa heterodimeric glycoprotein composed of a heavy and light subunit. The heavy subunit is 170 kDa and is linked by disulfide bonds to the light subunit [1]. The light subunit is 31 or 35 kDa depending on post-translational modification including a GPI-anchor. These two sub-units are non-covalently linked to an intermediate sub-unit of 150 kDa (Figure 2.1). The disulfide bonds are key to Gal-lectin conformation, as they fold it into a protease-resistant structure [2]. The Gal-lectin is only sensitive to proteolytic attack once it has been reduced and the cysteine residues alkylated.

The heavy subunit can be divided into specific regions (see Figure 2.2). The heavy sub-unit has an amino-terminal hydrophobic signal sequence, a large extracellular cysteine-rich domain, a transmembrane domain and a cytoplasmic domain [3,4]. The extra-cellular domain can be further divided into a cysteine-tryptophan region at the amino-terminal, followed by a cysteine-poor region and then the larger cysteine-rich region. This last region contains a pseudorepeat portion, sites for N-linked glycosylation of the Gal-lectin as well as the carbohydrate recognition domain (CRD) and sequence homology to human CD59, conferring complement resistance [5,6]. The 35-kDa isoform of the light sub-unit is more highly glycosylated and lacks the carboxy-terminal GPI anchor present on the 31 kDa isoform [7,8]. The GPI-anchor is unusually short (7 amino acids) in these light subunits.

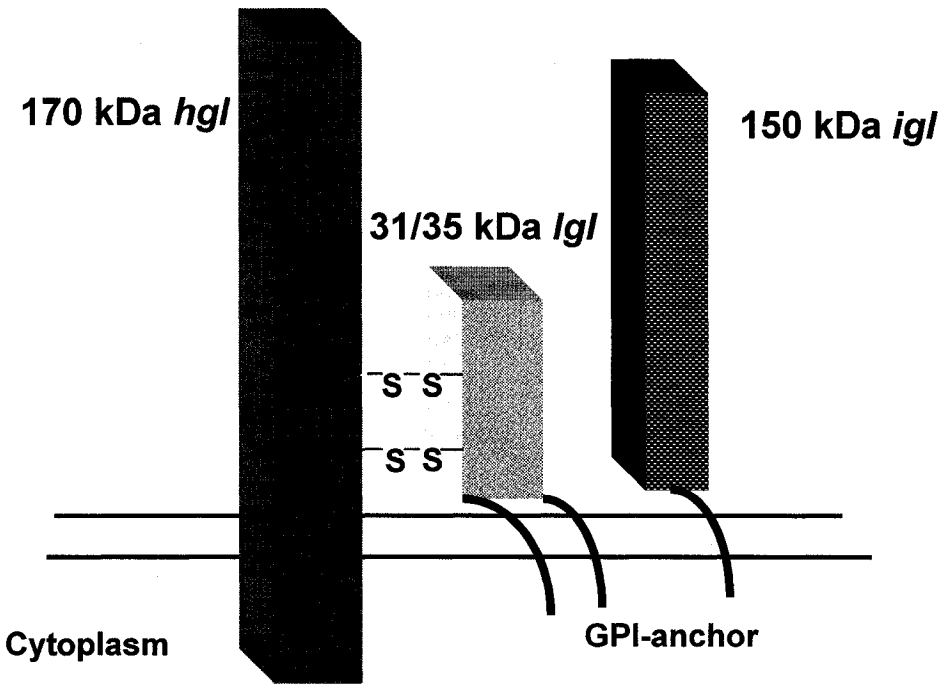

Figure 2.1: Structure of *E. histolytica* Gal-lectin. The Gal-lectin is present on the plasma membrane of the ameba and is composed of three subunits. The heavy subunit (hgl) is linked by disulfide bonds to the light subunit (lgl). The heterodimer is non-covalently associated with the intermediate subunit (igl). Both the light and intermediate subunits are GPI anchored, while the heavy subunit has a short cytoplasmic tail.

The heavy and light subunits do not seem to exist free of the heterodimer formation, as SDS-PAGE analysis of non-reduced parasite proteins demonstrate co-migration of the subunits [7]. This Gal-lectin heterodimer has a unique mechanism of membrane association, with the heavy subunit being trasmembrane and the light subunit being GPI- anchored. The importance of GPI-anchor modifications in association between subunits was illustrated with FLAG-tagged light subunits, which demonstrated that

only GPI-anchored light subunits co-precipitated with the heavy sub-unit [9]. The heterodimer interacts non-covalently with an intermediate 150-kDa subunit, which is characterized by a hydrophobic amino-terminal sequence and a GPI-anchored carboxy-terminal [10].

The heavy subunit gene family consists of 5 unlinked genes [11], of which *hgl* 1, 2 and 4 are expressed simultaneously. The products from the different *hgl* genes are 89-95% identical at the protein level, having most of the variability occurring in the cysteine-poor region. Sequencing analysis of four different *E. histolytica* isolates showed that the gene product for *hgl* 1 was highly conserved [12]. The light subunit is encoded by a gene family of 6-7 members, encoding for isoforms with different post-translational modifications [7, 8]. The *lgl* gene products have 79-85% sequence identity, showing greater variability than the *hgl* genes. **To summarize, the Gal-lectin is composed of a heavy and light subunit that have high sequence conservation.**

2.2 Gal-lectin function (parasite biology and pathogenesis)

The Gal-lectin's primary role is to mediate parasite binding to host glycoconjugates with high affinity. The Gal-lectin has 100,000-fold greater affinity for Galactose/N-acetyl-D-galactosamine (Gal/GAlNAc) containing polyvalent glycoproteins then the monosaccharides Gal or GalNAc [13]. It is believed that the Gal-lectin can tolerate wider GalNAc spacing, such as maxiclusters which are multivalent structures spaced a greater distances on a polypeptide backbone [14]. This is consistent with the Gal-

lectin having high affinity binding to colonic mucins, which display widely spaced Gal/GalNAc O-linked oligosaccharides on a mucin polypeptide backbone. Thus, it is not surprising that the *E. histolytica* Gal-lectin has been shown to have high binding affinity (K_d= 8.2 x 10^{-11} M^{-1}) to purified rat and human colonic mucins [15]. By this mechanism the Gal-lectin mediates colonization of the human colon with *E. histolytica* by binding to colonic mucin.

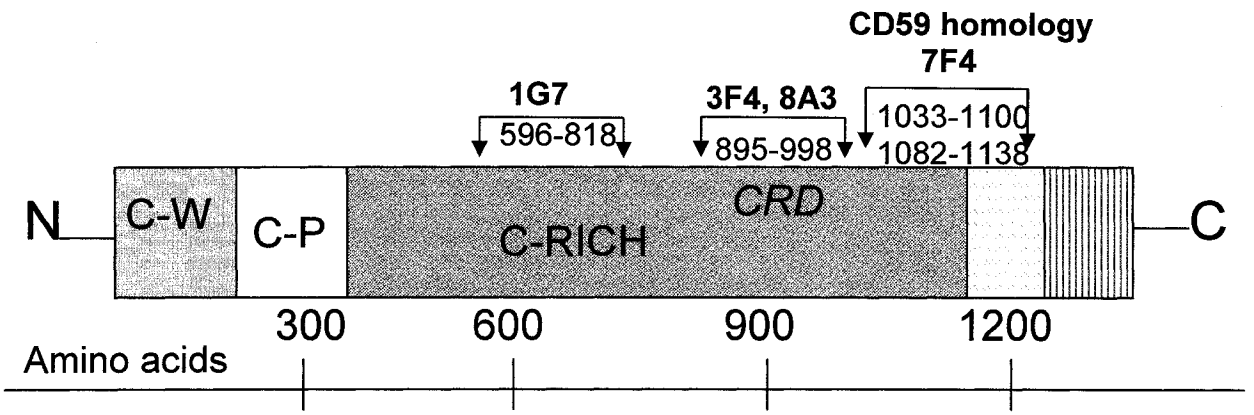

Figure 2.2: Representative diagram of the structural domains of the extracellular portion of the heavy subunit. The amino-terminal contains a cysteine-tryptophan region (C-W:1-187), followed by a cysteine-poor region (C-P: 188-377). The cysteine-rich region (C-RICH: 378-1208) contains the carbohydrate recognition domain (CRD) and a domain, which bears homology to mamaliam CD59 associated with complement resistance. Monoclonal antibodies (mAb) against the cysteine rich domain affect Gal-lectin binding activity: mAb 3F4 and 8A3 enhance adherence.

Carbohydrate recognition has been mapped to the heavy subunit of the Gal-lectin. The light subunit does not demonstrate any carbohydrate binding activity. The carbohydrate recognition domain (CRD) has been determined with the use of monoclonal antibodies (mAbs) that map to epitopes on the cysteine-rich region of the

extracellular domain on the heavy subunit (Figure 2.2). These mAbs could block parasite-binding activity and corresponded to amino acids 895-998 [16]. This sequence of 104 amino acids is highly conserved among products of the *hgl* gene family. Another group, however, expressed fragments of the *hgl* 2 protein to determine binding activity to CHO cells, and determined that amino acids 480-900 contained the CRD [17]. It is obvious from these studies that carbohydrate recognition occurs within the cysteine-rich region of the heavy sub-unit, however there may be multiple CRDs. During the mapping of the CRD, it was observed that certain mAbs (3F4 and 8A3) could enhance Gal-lectin mediated binding to CHO cells [18]. The Fab fragments alone could induce this effect therefore it was not due to antibody clustering of the lectin. In fact, the mAb binding seemed to be changing the Gal-lectin to a more active conformation. This observation is consistent with the conformational control of other eukaryote adhesins, such as LFA on T-lymphocytes [19]. The regulation of Gal-lectin conformation is thought to be mediated by inside-out signaling. The Gal-lectin cytoplasmic tail contains regions of identity with the cytoplasmic tail of β2 intergrin-like domains, this could mediate the inside-out signaling [20].

E. histolytica trophozoites *in vitro* kill a wide variety of cell lines as well as isolated human neutrophils, T-lymphocytes and macrophages. This is a contact-dependent event, which requires an intact parasite and the Gal-lectin. Contact-dependent killing of CHO cells is completely inhibited by the addition adherence blocking anti-bodies [21], and CHO glycosylation mutants lacking terminal Gal residues on N- and O-

linked sugars are resistant to amebic cytolysis [22]. The Gal-lectin is essential for cell-cell contact prior to cytolysis of the target cell. However, the Gal-lectin not only mediates adherence, it plays a part cytolytic events. When ameba and target cells are forced into contact through centrifugation, cytoxicity is still inhibited by the addition of Gal or GalNAc and does not occur if target cells lack Gal/GalNAc on their surface [23]. Furthermore, the identification of an anti-lectin monoclonal antibody (mAb) that did not block adherence but could inhibit cytotoxicity, confirmed that the Gal-lectin is implicated in this cytotolytic event [21]. It has been shown that cell killing occurs only after the Gal-lectin engages the Gal/NAc residues on O-linked oligosaccharides on the target cells [22, 24].

E. histolytica encounters the host's complement system both at the intestinal level and during the hematogenous dessimination to the liver. Trophozoites are known to activate the classical and alternative complement pathways in the absence of anti-amebic antibodies [25]. *E. histolytica* trophozoites isolated from patients with invasive disease can activate the alternative complement pathway but are resistant to C5b-9 complexes deposited on the parasite surface, while trophozoites from asymptomatic patients also activate this pathway but are killed by C5b-9 [26]. Given these opposing observations, it is not surprising that the parasite has evolved a mechanism to avoid complement attack. The Gal-lectin was found to have complement resistance activity, specifically in the heavy subunit [6]. Sequence analysis of the heavy subunit revealed a region with significant identity to human CD59. CD59 is a GPI-linked protein expressed on many cell types, which

incorporates itself into the growing membrane attack complex, inhibiting the addition of the C9 molecule. The Gal-lectin is recognized by anti-CD59 antibodies and can bind human C8 and C9. It was found that the Gal-lectin blocked the formation of the membrane attack complex in amebic membranes by inhibiting C8 and C9 insertion. **To summarize, the Gal-lectin participates in adherence to colonic mucins for colonization, adherence to target cells for contact-dependent cytoxicity and evasion of host complement system.**

2.3 Gal-lectin immunogenicity

One of the most immunogenic *E. histolytica* molecules is the Gal-lectin, which plays an important role in parasite biology. The Gal-lectin, specifically the heavy subunit, is a highly conserved antigen and is recognized by human immune sera from patients throughout the world [27]. In fact, 95% of sera from amebiasis patients have antibodies against the Gal-lectin [28, 29] and it is the major antigen recognized by pooled human immune serum [30]. Serum IgM antibody response against the Gal-lectin is observed in amebic colitis and amebic liver abscess patients [31] and patients with invasive disease also have detectable salivary IgA to the Gal-lectin [32]. Most importantly, the presence of anti-lectin mucosal IgA has been associated with a reduced risk of infection in endemic areas [33]. Acquired resistance to *E. histolytica* infection was linked to these protective intestinal IgA antibodies that are directed against the carbohydrate recognition domain of the Gal-lectin [34].

In addition to humoral responses, the Gal-lectin also demonstrates immunogenicity in cell-mediated responses. Peripheral blood monocytes isolated from individuals previously exposed to *E. histolytica* respond to the Gal-lectin, demonstrating the potency of this antigen [35]. In this study the cells proliferated and produced IL-2 as well as IFN-γ in response to treatment with purified Gal-lectin. It was also observed that T cells treated with Gal-lectin for 5 days *in vitro* became amebicidal, while cells from control individuals did not. Another study looked at the specific Gal-lectin region involved in this T cell response. Using a recombinant protein coding for amino acids 649-1202 of the cysteine-rich region to stimulate peripheral blood monocytes from amebiasis patients, it was shown that T cells proliferated [36]. These studies illustrated that there may be important T cell epitopes on the Gal-lectin responsible for the induction of cell-mediated immunity against *E. histolytica.*

Gal-lectin mediated cell immune responses have extensively been studied in macrophages. These specialized antigen-presenting cells play an important role in innate and adaptive immune responses, specifically in pathogen elimination. Activated macrophages are able to kill *E. histolytica* trophozoites *in vitro* [37]. Stimulation of murine bone marrow derived macrophages with Gal-lectin induces the production of TNF-α [38]. This TNF-α production is mediated by the cysteine-rich region as mAbs against amino acids 596-1082 could effectively block this macrophage stimulation. In another study it was demonstrated that Gal-lectin could also induce NO production in IFN-γ primed murine macrophages [39]. Macrophages were amebicidal when treated simultaneously with IFN-γ and Gal-lectin, and this

cytotoxic effect could be inhibited by mAbs against amino acids 596-818 of the cysteine-rich region. Another immunogenic property of the Gal-lectin is its ability to activate macrophage IL-12 production [40]. Human THP-1 macrophage IL-12 production by Gal-lectin stimulation could be blocked by mAbs against amino acids 596-898, demonstrating once again the importance of the cysteine-rich region in cell activation. A recent study in our laboratory has described that the Gal-lectin can also increase TLR-2 expression on macrophages [41]. **To summarize, the Gal-lectin is a potent immunogen of *E. histolytica* inducing strong humoral and cell-mediated responses.**

2.4 Animal models of amebiasis and Gal-lectin vaccine trials

E. histolytica is acquired through the ingestion of the infective cyst stage from which arise the mobile trophozoites that cause disease in humans. Unfortunately, to date there is no laboratory method to cultivate this infective cyst stage and therefore natural infection cannot be recreated in animal models. All experimentally induced amebiasis methods currently available require administration of live *E. histolytica* trophozoites into the target tissue. Although this procedure does not represent the natural course of infection, experimental amebiasis models are useful to elucidate host defense and the early stages of invasive disease.

Many animal species have been tested for the development of experimental amebiasis, including cats, dogs, monkeys, rabbits, guinea pigs, rats, mice, gerbils and hamsters [42]. One of the first and most frequently used models is the gerbil

(*Meriones unguiculatus*) model for intestinal amebiasis and amebic liver abscess. Male gerbils are anesthetized for laparotomy, and *E. histolytica* trophozoites are injected directly into the left liver lobe to induce abscess or into the cecum to induce intestinal disease. Chadee and Meerovitch [43] described the pathogenesis of this experimentally induced amebiasis. The gerbils developed amebic liver abscesses characterized by granulomas detectable as early as 5 days post infection. Hepatic lesions showed heavy granulocyte and lymphocyte infiltration, typical of acute infection. The cecal infections were studied for 10-60 days and also demonstrated granulomatous lesions as well as loss of the mucosa. In some cases, intestinal disease metastasized to the liver through portal circulation, imitating the human features of parasite dissemination to soft organs. Although gerbils are equally susceptible to intestinal and hepatic amebiasis, the intestinal model was difficult to reproduce due to the presence of naturally occurring commensals that interfered with parasite colonization. On the other hand, the gerbil model of amebic liver abscess is highly reproducible and the main model to study extraintestinal amebiasis. The main disadvantage of this model is the lack of immunological reagents to characterize the host response to *E. histolytica* infection. In our laboratory, many key gerbils cytokines were cloned including IL-18, IL-12, and GM-CSF [44-46]. Another rodent amebiasis model is the Syrian hamster (*Mesocricetus auratus*) model where animals are given experimental amebic abscesses by injection of trophozoites into the portal vein [47]. This model also lacks the immunological reagents to study immune responses and it is also less frequently used than the alternative gerbil model.

Mice disease models are advantageous because of the wide range of reagents available to dissect the immune response as well as various gene knockout strains that can allow the study of specific immune components in disease pathogenesis. In the case of amebiasis, mice have demonstrated strain dependent resistance to *E. histolytica* and have made the development of reliable mice models difficult [48]. Nonetheless, it has been shown that C3H/Hej and CBA/J mouse strains are susceptible to intestinal amebiasis, while C57BL/6 and BALB/c mice are naturally resistant. It is also important to note that mice deficient in IL-12, IFN-γ and NO are resistant to intestinal amebiasis [49]. The nature of resistance in these mice strains is unclear and complicated. Neutrophil depletion lead to increased susceptibility to intestinal amebiasis in a normally partially resistant CBA mouse strain, but had no effect on the resistant C57BL/6 mice [50]. A recent study has examined the factors involved mouse resistance using bone marrow chimeras from resistant and susceptible mice **(the following section has been published in *Trends in Parasitology 2007, 23:46-48*).** Shinjiro Hamano *et al.* [51] put forth a two tier resistance model form their chimera studies, suggesting that the first line of resistance is conferred by the intestinal epithelial lining and the second by the underlying immune cells. The authors also reported the contribution of IL-10 to innate resistance, suggesting that the cytokine had physiological effects on intestinal epithelial cells inducing a protective barrier to infection. Epithelial cells could be predisposed to intestinal invasion and would then rely on the inflammatory cells below to clear the infection. IL-10 is known to be involved in the maintenance of gut homeostasis and a deficiency in this cytokine can alter the epithelial response to luminal antigens. In

fact, IL-10 deficient mice develop chronic intestinal inflammation, which is thought to be due to an increase in T cell induced intestinal damage and Fas expression in epithelial cells [52]. Shinjiro Hamano *et al.* report that IL-10 deficient mice developed greater intestinal damage due to amebic infiltration and that this could be reproduced in the bone marrow chimera studies (IL-10-/- → IL-10 +/+). This is consistent with the IL-10 models of colitis, demonstrating that in amebiasis, the cytokine may inhibit the adverse effects of luminal amebic antigens. The bone marrow chimera studies [51] demonstrated that resistance was not due to hemopoietic cells, since transfer of resistant bone marrow into susceptible mice (C57BL/6 → CBA/J) did not reduce the intestinal damage caused by amebic challenge. This confirmed that resistance lies in the nature of the intestinal epithelial barrier. However, recent studies with *E. histolytica* cysteine proteases have demonstrated that human colonic mucins (MUC2) are cleaved by the parasite proteases in order for the amebae to gain access to the epithelial cells [53]. The major cleavage site of MUC2 by amebic proteases is sequence specific and does not seems to be present in murine mucins. It can be hyphothesized that susceptibility to infection may be due to inherent differences in intestinal mucins. The authors note that IL-10 deficiency is known to affect MUC2 synthesis, thus decreasing the natural protective gel between the parasite and target epithelial cells.

The susceptible C3H/Hej mice have been used as model for intestinal amebiasis [49]. These mice are anesthetized and a laparotomy is performed to expose the cecum in which the live *E. histolytica* trophozoites are injected. These mice develop chronic

non-healing lesions in the cecum characterized by mast cell infiltration and cecitis was marked by IL-4 and IL-13 cytokine production. Depletion of CD4+ T cells decreased parasite burden and greatly reduced inflammation associated with mast cell infiltration. In this model CD4+ cells exacerbated diseases, demonstrating the importance of host immune cells in disease progression. The disadvantage of this model is that it has a 60% infection rate and therefore requires a larger number of animals for reproducibility. In order to examine the role of T and B cells in immunity to amebiasis, severe combined immunodefient mice (SCID) have been used as a model for amebic liver abscess [54]. SCID mice develop abscesses characterized by neutrophil infiltration that surround necrotic areas containing trophozoites. The abscess is clearly separated from healthy hepatic tissue by a ring of inflammatory cells and fibrosis.

Although the C3H/Hej and SCID mouse models can illustrate immune parameters because of the availability of immunological reagents, these are limited in their ability to truly discern the interaction with a human parasite. The SCID-HU-INT model, using a human intestinal xenograft was developed to address this issue [55]. A piece of human intestinal tissue is grafted onto the sub-capsular region of a SCID mouse and allowed to integrate for 8 weeks. The graft area is then infected with *E. histolytica* and develops severe inflammation characterized by neutrophil infiltration and IL-8 as well as IL-1β secretion from epithelial cells. This model is useful to study the human immune responses to *E. histolytica*, however it is not a tool that every laboratory can use because is requires access to human tissue and also requires 2

months before experiments can take place. **To summarize, there is a lack of adequate animals to reproduce natural infection with *E. histolytica*, but useful models such as the gerbil model of amebic abscess and the C3H/Hej intestinal amebiasis model can help elucidate the mechanisms of pathogenesis and immunity.**

Acquire immunity to invasive amebiasis has been achieved repeatedly either using total amebic antigen immunizations or drug cured animals resistant to secondary infection [56, 57]. Based on the animal and epidemiological evidence that acquire immunity occurs against *E. histolytica*, the feasabiliy of an amebiasis vaccine has become more evident. To date only a few amebic proteins have been considered as vaccine candidates, including the serine-rich *E. histolytica* protein (SREHP), the 29-kDa cysteine-rich protein, and the Gal-lectin [58]. SREHP is a surface membrane protein whose function remains unclear, but which demonstrates immunogenicity both in humans and in animal models [59]. The 29-kDa cysteine-rich protein is believed to be involved in parasite detoxification of hydrogen peroxide and also demonstrates high immunogenicity with 80% of patients with amebic abscess having antibodies against this antigen [60]. The Gal-lectin, however is the most promising vaccine candidate because it is an essential parasite virulence factor an extremely immunogenic. The central role of the Gal-lectin in *E. histolytica* pathogenesis makes it a prime target for the host's immune system. Several immunization trials have used the native Gal-lectin or recombinant portions of the molecule and exhibited some degree of protection against amebic abscess or intestinal disease (Table 2.1).

Table 2.1: Gal-lectin vaccine trials and efficacies

Antigen	Form[a]	Delivery	Route[b]	% Eff[c]	Ref.
260kDa	Native	Freund's	i.p.	67[d,e]	61
aa 649-1201	Recomb. GST fusion	Freund's	i.p.	81	62
aa 767-1138	Recomb. His-tagged	Titermax	i.p.	71[d]	63
aa 482-1138	Recomb. His-tagged	Freund's	i.p.	45[d]	64
aa 482-1138	Recomb. GST fusion	*S.dublin*	orally	15.3[d]	64
aa 1-436	Recomb.	Freund's	i.p.	6.7[e]	65
aa 436-624	Recomb.	Freund's	i.p.	37.5[d]	65
aa 799-939	Recomb.	Freund's	i.p.	11.1	65
aa 939-1053	Recomb.	Freund's	i.p.	62.5[d]	65
aa 895-998	Recomb. His-tagged	Freund's	i.p.	0[d]	5
aa 1005-1029	Synthetic, KLH fusion	Freund's	i.p.	33.3[d]	66
aa 1005-1029	Recomb. CtxB fusion	none	orally	0-30[d]	66
aa 1005-1029	Recomb. CtxB fusion	Freund's	i.p.	0-55	66
260 kDa	Native	Ctx	i.n.	84-100	67
aa 578-1154	Recomb. His-tagged	Ctx	i.n.	34-91	67
260 kDa	YopE fusion	*Yersinia*	orally	NA	68

[a]All recombinant proteins were produced in *E. coli.* [b]Routes i.p .= intraperitoneally, i.n. = intranasally, orally = by oral gavage. [c]Vaccine efficacy= (number of immunized animals without disease ÷ total number of immunized animals)- (number of control animals without disease ÷ total number of control animals) x 100. [d]The lesions in non-protected animals were significantly smaller than control animals. [e]The lesions in non-protected animals were significantly larger than control animals.

The protective effects of the Gal-lectin were reported first by Petri and Ravdin [61]. In this study the native Gal-lectin was purified by immunoaffinity chromatography from detergent-solublilized *E. histolytica* amebae and combined with complete or incomplete Freund's adjuvant for immunizations. Gerbils were vaccinated with 10 μg native Gal-lectin three times at two-week intervals by intraperitoneal or subcutaneous injections and then challenged by direct intrahepatic injection of trophozoites.

All immunized gerbils developed high titers of anti-Gal-lectin antibody capable of blocking parasite adherence *in vitro*. Combining the intraperitoneal and subcutaneous immunization, the Gal-lectin demonstrated 67% vaccine efficacy, compared to sham immunized controls. The immunized gerbils that were not protected developed significantly larger abscesses than controls, however there was no correlation between antibody titers and protection. The formation of larger abscesses in these animals was believed to be due to some immunosuppressive effects of the Gal-lectin. This initial vaccination study revealed that the Gal-lectin was a potent vaccine antigen, but that it could also exacerbate disease in some instances. This brought about the notion that different regions of the Gal-lectin may be responsible for the protective and the deleterious effects observed after challenge infections. Many future vaccination trials examined the protective effects of subunits of the Gal-lectin [62-64]. A particularly interesting study by Lotter *et al.* [66] determined the protective and exacerbating regions of the Gal-lectin using four recombinant proteins corresponding to four main regions of the heavy subunit [65]. The four proteins included: the cysteine-poor region (r170CP 1-143aa), the pseudorepeat region

(r170PR 437-624 aa), two cysteine-rich regions (r170CR1 799-939 aa) and (r170CR2 940-1053). Gerbils were immunized and the formation of amebic liver abscess compared for each of the four recombinant proteins. Immunization with the r170CP was found to exacerbate disease and this due to antibody binding the amino-terminal of the molecule. The r170CR1 had no effect, while both the r170PR and r170CR2 demonstrated protection against amebic liver abscess. Passive transfer of rabbit immune serum for r170CR2 and r170PR into SCID mice also protected against abscess formation. It was also observed that 100% of serum samples from human patients with amebic disease reacted with the exacerbating r170CP protein, whereas 78% asymptomatic patient serum recognized the protective r170CR2 protein. This study identified the region of the Gal-lectin involved in protection from disease.

Although the cysteine-rich region of the Gal-lectin was protective against disease progression, at this point it was unclear whether the same effects would be observed at the intestinal level. Houpt *et al.* [67] showed that both purified native Gal-lectin and a recombinant portion (LecA 578-1154 aa) could protect against intestinal amebiasis in the C3H/Hej model. The mice were immunized intranasally with either native Gal-lectin or LecA adjuvated with cholera toxin and boosted by intraperitoneal injection with Freund's adjuvant. The mice were then challenged by intracecal injection of live *E. histolytica* trophozoites and assessed for intestinal damage by histological damage scores. The native Gal-lectin immunizations conferred protection with a vaccine efficacy of 84-100% while the recombinant LecA had a vaccine efficacy of 91% and 34% in the first and second trial respectively. Protection in this

study was associated with the presence of pre-challenge fecal anti-Gal-lectin IgA. The vaccination trial demonstrated that Gal-lectin and LecA could prevent *E. histolytica* colonization but did not seem to reduce disease severity in those unprotected animals.

Several attempts have been made to improve vaccine delivery and efficacy with the Gal-lectin. *Salmonella* strains have been used as a means of delivering antigens to the gut associated lymphoid tissue as these bacteria effectively invade Peyer's patches. This strategy could effectively induce both mucosal and systemic immunity, which could prevent parasite colonization and reduce disease severity. *Salmonella dublin* expressing the LecA fragment of the Gal-lectin was used as an oral vaccine vector [64]. Oral immunization of gerbils with 10^8-10^{10} bacteria three times provided some protection against amebic liver challenge infection, reducing abscess size compared to controls. This study demonstrated that oral immunization conferred systemic immunity against *E. histolytica.* The bacterial vector strategy was repeated recently with *Yersinia enterocolitica* expressing the Gal-lectin fused to the outer-protein [68]. Live attenuated *Yersinia* was administered orally to gerbils that were then challenged for amebic liver abscess. Oral immunization with this vector also protected gerbils against amebic liver abscess formation.

Another revolutionary vaccination strategy, DNA vaccines, can induce both humoral and systemic immune responses by directly transfecting host cells with plasmid encoding the vaccine antigen [69]. Recently in our laboratory a codon-optimized

DNA vaccine encoding the cysteine-rich region (894-1081 aa) of the Gal-lectin was developed and administered to BALB/c mice by intradermal immunization [70]. This DNA vaccine induced Gal-lectin specific IgG2a Th1-type antibodies that could reduce parasite adherence to CHO cells. Cell-mediated responses were also induced by Gal-lectin DNA vaccination, characterized by T cell proliferation to the Gal-lectin. **To summarize, the Gal-lectin is a prime vaccine candidate against *E. histolytica* and has shown protective effects in a range of vaccine trials.**

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CHAPTER 3: DENDRITIC CELLS AND INNATE IMMUNITY

3.1 Dendritic cell biology and function

Ralph Steinman found a population of dendritic shaped cells in the spleen, and was the first to describe dendritic cells [1]. Mature dendritic cells (DCs) characteristically have numerous membranous processes that extend out from the cell body, which resemble the dendrites on neurons. It was known that an immune accessory cell was necessary for the generation of a primary antibody response, but it was not until the discovery of DCs that this accessory cell was identified. DCs are now known to be professional antigen-presenting cells, highly sophisticated and potent cells able to induce primary immune responses.

Dendritic cell hematopoietic progenitors give rise to circulating precursors that can home to different tissues, where they will reside as immature dendritic cells that are very phagocytic [2-4]. DCs can originate from both lymphoid and myeloid lineages, however in humans myeloid derived DCs are considered "classical" DCs [5]. These cells arise from bone marrow CD34+ progenitor cells, which can differentiate into epidermal Langerhan cells or interstitial DCs [6, 7]. Myeloid DCs perform the typical functions, including maturation, migration and MHC-peptide complex presentation. Lymphoid DCs are found in the thymic medulla and lymph node T cell areas and are involved in immune tolerance [8]. In fact, DCs are found throughout the body and demonstrated immense heterogeneity. Mice have two distinct subsets, mainly the lymphoid and myeloid DCs. These two subsets express common markers CD11c,

MCH class II, CD86 and CD80 costimulatory molecules, and CD40. Myeloid DCs in mice can be distinguished from lymphoid DCs by the absence of CD8α expression [9, 10]. In humans, there are many levels of DCs heterogeneity. There are the precursor populations including CD14+ and CD11c+ monocytes [11]. The most diversity is found in anatomical localization with skin Langerhan cells, interstitial DCs, splenic marginal DCs, T-zone interdigitating cells, germinal-center DCs, thymic DCs, liver DCs, and blood DCs [12]. These DCs also differ in function, such as B cell proliferation or T cell differentiation. Typically DCs make up less than 1% of a give cell population, which could limit DC research. In recent years, however the generation of large numbers of DCs *in vitro* has facilitated advances in DC biology and immune regulation. When cultured with the right cytokines, precursor cells can be made to differentiate into DCs. Human blood CD14+ cells that resemble monocytes can form DCs in response to GM-CSF and TNF-α and mouse bone-marrow CD34+ cells can differentiate into DCs with GM-CSF and IL-4 [13, 14]. Lymphoid DCs can also be propagated in culture from a distinct progenitor expressing CD4 but not CD11c in the presence of IL-3 and CD40L [15].

Dendritic cells are the initiators and modulators of immune responses. DCs can accumulate rapidly to the site of infection or antigen deposition by recuitment of circulating DC precursors and immature DCs. As mentioned above, DCs are present in most tissues where they can capture and process antigens, displaying MHC-peptide complexes at their surface. They upregulate their co-stimulatory molecules (CD80, CD86) and migrate to lymphoid organs (spleen, lymph nodes) where they contact and

activate antigen specific T cells. Antigen capture is performed by DCs in the "immature" state, unable to stimulate T cells. Immature DCs are equipped with an array of pathways to take up antigens including: phagocytosis [16], macropinocytosis [17], and receptor-mediated endocytosis via C-type lectin DEC-205 [18]. DCs are known to phagocytose apoptotic and necrotic cell fragments, viruses, bacteria and some intracellular parasites [19-22]. Lamina propria DCs are involved in the uptake of gut antigens and this mechanism is specialized since it requires sampling through epithelial tight junction proteins. DCs express tight junction proteins (occluding, claudin-1and zonula occludens-1) that enables them to open tight junction and send dendrites to sample luminal antigens without compromising the epithelial barrier integrity [23]. In general, exogenous antigens are presented in the context of MHC class II (MHCII) while endogenous antigens, from viruses and intracellular parasite are processed with MHC class I. Peptide processing in DCs is rapid, due in part to the large amounts of MHC class II rich compartments in immature DCs [24]. The captured antigen is directed towards the MHCII containing HLA-DM that enhances peptide binding [25], and upon maturation peptide-loaded MHCII are exported to the cell surface where they remain stable for days [26, 27]. Cytotoxic killer T cells are generated by peptide presentation by DCs via MHCI. The endogenous MHCI pathway requires the degradation of cytosolic proteins and peptide loading onto newly synthesized MHCI molecules in the ER. Ubiquitinylated proteins are directed to the proteasome for cleavage into peptides that are then translocated to the ER and loaded onto MHCI [28, 29]. Cross-priming, a mechanism by which an APC activates a naive CD8+ T cell for specificity against an antigen from a third cell, is mediated by

an alternative MHCI pathway which involves the degradation of phagocytosed antigens [30].

Antigen and pathogen uptake, initiates DC activation and maturation. This is a complex process to produce mature DCs that will migrate to lymphoid organs to act as APCs to prime naive T cells. Activated DCs lose their phagocytic capacity, lose their tissue adhesion molecules, increase receptors for chemokines (CCR7, lymph node homing receptor) and alter their cytoskeleton for cellular mobility [31]. Upon maturation DCs strongly upregulate the expression of co-stimulatory molecules in order to prime naive T cells. T cell priming is an essential function of DCs and requires the formation of the immunological synapse. The first signal for T cell priming is the recognition of MHC-peptide complexes on DCs by the antigen-specific T cell receptor [32]. DC-T cell clustering is facilitated by the adhesion molecules intergrins β1, β2, and ICAM-3 [33, 34]. The second signal to sustain T cell activation is the interaction between co-stimulatory molecules on the DC and their respective ligands on the T cell. Co-stimulatory molecules CD80 and CD86 bind to CD28 and CD29 on T cell respectively, however CD86 is more critical in activating a T cell response [35]. DCs can be further activated by CD40 binding to CD40-ligand expressed on T cells, leading to an increased expression of CD80 and CD86 as well the production of IL-1, TNF-α, chemokines and IL-12 [36, 37]. Furthermore, engagement of RANK on DCs with its ligand TRANCE on activated T cells can also stimulate the secretion of pro-inflammatory cytokines (IL-1, IL-12 and IL-6) by DCs [38, 39]. This sequence of events leads to increased DC survival and enhances the T

cell responses by promoting longer DC-T cells interactions required for proper T cell stimulation.

DCs direct the distinct T helper cell responses generated and this is driven by the subset of DCs and the cytokine environment they create. In humans, monocyte-derived CD11c+ DCs polarize T cell toward a Th1 profile, whereas the CD11c- DCs predominantly induce Th2 type T cells [40]. The production of IL-12 by DCs directs the development of Th1 T cells [41]. IL-12 deficient mice fail to induce Th1 responses, illustrating the role of DC IL-12 determing T helper cell polarization [42]. The induction of Th2 cytokines is not well known, but seems to occur in the presence of IL-4 or in the absence of IL-12 [43]. The ability of DCs to influence the pattern of cytokine production by T cells is essential to the outcome of an immune response against an invading pathogen. Th1 cytokines may include IFN-γ, TNF-α and IL-2, which are typically associated with cell-mediated immunity to intracellular pathogens [44]. Th2 cytokines, such as IL-4, IL-5, IL-6 and IL-13 are important in response to multicellular parasitic nematodes [45]. The nature of the antigen/maturation stimulus may also determine the Th polarization following DC activation. For example, bacterial products are known to induce the production of IL-12 in DCs upon recognition by toll-like receptors (discussed in section 3.2). The microenvironment may also dictate the Th response, as IL-12 production by DCs can be enhanced by IFN-γ, while IL-10 or PGE_2 inhibit IL-12 production [46-48].

DCs are famous for their T-cell activating potential, but they also have major effects on B lymphocytes. DCs can directly activate naive and memory B cells, and help the differentiation of naive B cells into plasma cells. This is mediated by IL-12 in combination with IL-6/soluble IL-6Rα [49,50]. DCs are able to induce CD40-activated naive B cells to express surface IgA, but IL-10 is required to further differentiate these B cells into IgA secreting cells [51]. This indicates that DCs also have an important role in mucosal immunity. Germinal center DCs are unique in their ability to stimulate CD40-activated B cells to proliferate and become plasma cells in an IL-12 independent manner [52].

Dendritic cells are key players in the induction of immunity to foreign antigens and are also mediators of tolerance to self-antigens. Tolerance occurs in the thymus (central tolerance) by deletion of developing T cells, and in the lymphoid organs (peripheral tolerance) by the induction of anergy or deletion of mature T cells. In the thymic medulla DCs present self-antigens and any T-cell that has too high an affinity for these antigens are deleted (negative selection)[53]. In fact, in mice it was shown that thymic DCs could induce tolerance to myelin basic protein and thus limit the development of experimentally induced autoimmune encephalomyelitis [54]. Tolerance requires the presentation of self-antigens in the context of MHC molecules to T cells, and both DCs and macrophages are equipped with the machinery to do so, however, macrophages are not involved in T cell deletion [55].

Peripheral tolerance is specialized, having DCs capture self-antigens that are tissue specific [56]. Bone marrow-derived DCs present peptides that are derived from insulin-producing β-cells of the pancreas to T cells in the draining lymph nodes, inducing tolerance [57]. DCs have also been shown to present peptides from apoptotic cells, which could help develop tolerance to self-antigens derived from normal somatic cells, whose antigens would never reach the thymus [19]. Peripheral tolerance may also be important in keeping autoimmunity and chronic inflammation at bay when infection strikes. That is to say that during an infection, DCs would present self-antigen, environmental antigens and the pathogen's antigens but only T cell responses to the pathogen would ensue. **To summarize, DCs are professional antigen presenting cells acting as sentinels throughout the body maintaining the balance between tolerance to self-antigens and immunity against pathogens.**

3.2 Dendritic cells and Toll-like receptors

Dendritic cells are the most potent antigen presenting cells and are responsible for the initiation of immune responses. DCs have evolved to recognize signals of tissue damage or invading pathogens. The detection and capture of microorganisms by DCs triggers stimulus-specific maturation of the DCs and direct the ensuing T helper cell response. DCs express members of the Toll-like receptor family, which bind to common chemical moieties associated with pathogens.

Toll-like receptors (TLR) are pattern recognition receptors (PRR), which have evolved to recognize pathogen associated molecular patterns (PAMPs) in order to

detect in the presence of infectious agents [58]. TLRs comprise of a family of type I transmembrane receptors, characterized by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor domain (TIR) [59,60]. The leucine-rich repeats in the extracellular portion are involved in ligand recognition and signal transduction [61]. The intracellular TIR domain, is a common motif found in transmembrane and cytoplasmic proteins in animals and plants involved in host defense [62]. In fact, the first identified member of the Toll family was *Drosophila Toll*, which functions both in dorsoventral axis formation and fruitfly immunity [63, 64].

In mammals there are at least ten well-characterized TLRs and each have a distinct function in innate immune recognition (Table 3.2). TLR ligands are diverse in nature but all recognize PAMPs that can signal the presence of infection by directly binding the TLR. **TLR4** was the first characterized mammalian Toll [59] and was designated the signal-transducing receptor for LPS [65,66]. Recognition of LPS by TLR4 requires accessory molecules; first LPS binds a LPS binding protein, which transfers LPS monomers to CD14 that has high affinity for LPS [67]. MD-2 is expressed on the cell surface and associates with the outer domain of TLR4 for recognition of LPS [68]. TLR4 is also involved in recognition of other PAMPs, including lipotechoic acid, *Mycobacterium tuberculosis* heat-sensitive cell associated factor, and heat-shock protein HSP60 [69-71]. **TLR2** has the broadest range of ligands, due in part to the cooperation between TLR2 and TLR1 or TLR6, which form heterodimers [72, 73]. Some of the known TLR2 ligands include peptidoglycan, lipoproteins, and yeast cell

Table 3.2: Ligands recognized by Toll-like receptors

TLR	Ligands	Source	Notes
TLR1	Triacyl lipopeptide	Mycobacteria	with TLR2
TLR2	Triacyl lipopeptide	Mycobacteria	with TLR1
	Lipotechoid acid	Streptococcus	with TLR6
	Peptidoglycan	G+ve bacteria	
	Atypical LPS	Legionella	
	Lipoarabinomannan	Mycobacteria	with TLR6
	Porins	Neisseria	
	Diacyl lipopeptide	Mycoplasma	with TLR6
	Zymosan	*S.cerevisiae*	with TLR6
	GPI	Trypanosoma	
	HSP60, 70, 90	Host	
TLR3	Double stranded RNA	Viruses	Intracellular
TLR4	Lipopolysaccharide	G-ve bacteria	
	Envelop glycoproteins	RSV	
	Taxol	*T. brevifolia*	
	Mannan	*C. albicans*	Also by TLR2
	HSP60/70	Host	Also by TLR2
	Fibrinogen	Host	
TLR5	Flagellin	Bacteria	
TLR6	Same ligands as TLR2		with TLR2
TLR7	Viral single stranded RNA	RNA viruses	TLR8 is inactive in mice
TLR8	RNA		
TLR9	CpG-DNA	Bacteria/viruses	Intracellular
TLR10	Unknown		Absent in mice

wall [74-76]. **TLR3** is unique in that it can be expressed both on the cell surface and intracellularly, it signals via TRIF not MYD88 (discussed below) and that is it almost exclusively expressed in DCs [77-79]. TLR3 recognizes double stranded RNA, a PAMP for most viruses [80]. **TLR5** is specifically involved in the recognition of bacterial flagellin, a highly conserved protein that comprises the bacterial flagella [81]. TLR5 is also unique because it is expressed on the basolateral side of intestinal epithelial cells [82], which may help in differentiating commensal bacteria from invasive pathogens such as *Salmonella.* **TLR9** is essential in recognition of unmethylated CpG-motifs in invertebrate DNA [83] and can distinguish between motifs to induce specific immune effects (discussed in Chapter 3). TLR9 is also located intracellularly, recruited from the ER to the endosomal membrane upon CpG endocytosis [84].

On engagement with ligands, TLRs recruit specific adaptor molecules that intiate downstream signaling events. There are two distinct pathways to date that have been identified in TLR signaling [85]. The most common pathway, shared by TLR1, 2, 4, 5, and 9, requires the adaptor molecule Myeloid Differentiating factor 88 (MYD88) and leads to early activation of NF-κB and production of pro-inflammatory cytokines. MYD88 is one of three essential components to this signaling pathway, including IL-1R-associated kinase (IRAK), and TNF-receptor-associated factor 6 (TRAF6) [86-88]. MYD88 contains two protein interaction domains, a TIR domain that interacts with the TIR domain of the TLR and a death domain that binds to the corresponding death domain of IRAK. On recruitment to the receptor complex, IRAK is

autophosphorylated and associates with TRAF6, which in turn activates MAP kinase kinase 6 (MKK6) that can activate JNK, p38 MAP kinase and NF-κB [88]. The second TLR signaling pathway, is unique to TLR3 and TLR4, and is a MYD88-independent pathway. This pathway also signals through TIR on the TLR but associates with TIR domain-containing adapter inducing IFN-beta (TRIF) [89]. This pathway shares some distal intermediaries such as TRAF6 with the MYD88 dependent pathway and can induce NF-κB activation as well [90].

Dendritic cell maturation can be induced by TLRs, therefore these receptors are an essential link between innate immunity and adaptive immunity. DCs are heterogeneous in their TLR expression; myeloid DCs are known to express all TLRs, except TLR7 and TLR9, while plasmacytoid DCs selectively express TLR9 and TLR7 [91]. Stimulation of immature DCs with either LPS or CpG-DNA via TLR4 and TLR9 respectively, induces IL-12 production and enhances cell surface expression of co-stimulatory molecules [92, 93]. The maturation-inducing stimuli, such as LPS and CpG-DNA, are critical for directing T cell polarization, by supporting the DCs ability to promote a Th1 response. The TLR signaling pathway promotes NF-κB activation and the subsequent transcription of pro-inflammatory cytokines, required to mount a cell-mediated response against pathogens. TLR signaling also activates the DCs mitogen activated protein kinases (MAP kinases), which have been shown to be important in DC maturation processes [94]. DCs phenotypic maturation, cytokine production and survival are mediated by all three MAP kinases, p38, ERK and JNK [95, 96]. In fact, differentiation into a mature DC

requires specific regulation of these MAP kinases, as blocking one or more of these kinases can effectively block maturation. **To summarize, DCs express TLRs, which can effectively recognize foreign pathogen molecules and induce DC activation, leading to pro-inflammatory responses.**

3.3 Role of dendritic cells in vaccination

The ultimate aim in any vaccine design is to develop a vaccine that can induce protective immunity by appropriately targeting DCs to induce the desired response. Vaccines work by stimulating the production of long-lived effector cells and memory cells to the pathogen. Most current vaccines are efficient at inducing humoral immunity therefore there is still a great need for the development of vaccines that induce cell-mediated immunity against disease like HIV and tuberculosis.

The challenge in development of new and efficient vaccines is not only in the identification of protective antigens but also in developing a strategy that addresses key points: 1) the enhancement of antibody and T-cell mediated immune memory, 2) the improvement of the quality of the T cell responses, 3) achievment of mucosal immunity (in the case of sexually transmitted diseases) and, 4) designing both preventative and therapeutic vaccines [98]. Exploiting the normal biology of DCs could drastically improve vaccine efficicay. DCs are key players in vaccine design because they capture antigens and control the magnitude as well as the quality of the immune response. DCs have been used efficiently in mice as cellular adjuvants to elicit protective T-cell immunity against pathogens and tumors [99, 100]. Relatively

few DCs and low doses of antigen are required to induce high levels of lymphocyte proliferation and differentiation. This is due in part by the ability of a single DC to cluster with as many as 10-20 lymphocytes at a time [102]. DCs can therefore be exposed to an antigen either *in vivo*, by introducing the antigen directly, or *ex vivo* by pulsing the DCs with the antigen and subsequently administering them to a recipient. In both cases, DCs migrate to the recipient's lymph nodes and resides in the T cell areas, to select antigen-specific T cell. Bone marrow-derived DCs loaded *in vitro* with *Streptococcus pneumoniae* and transferred to naive mice induce primary Ig isotype responses against an array of baterial components [102].

Vaccine antigens, like in a natural infectious state, are not direcly presented to the immune system but must be captured, processed and intergrated into antigen-presenting molecules (MHCI or MHCII) in order to mount an immune response. DCs are the most efficient at this process and targeting this feature of DC function is a valuable strategy for vaccination. In fact, it has been shown that a peptide sequence winthin a protein delivered specifically to DCs is 100-1,000 times more efficient than a peptide administered with a non-specific adjuvant like CFA [103]. One way to directly involve DCs in vaccination is to target the DEC-205 receptor on DCs, which mediates antigen uptake and processing [18]. DCs targeted *in vivo* with the fusion antibody to DEC-205 stimulated proliferation of T cells specific for the peptide [103]. This study demonstrated two key points; first that DCs could be targeted by this strategy and second that DC maturation is essential, since endocytosis of the fusion antibody used did not induce DC maturation and effectively produced specific

tolerance and not immunity. In fact, it was established that antigen capture and presentation sets the stage for antigen-specific T cell recognition, but maturation of DCs controls the T cell response.

Vaccines must therefore contain the requisite antigens for induction of protective immunity but must also provide a maturation stimulus to ensure the development immnity and not tolerance. Several strategies can be used to overcome this issue of tolerance, by administering adjuvants that activated DCs. First there is the use of activating cytokines and second the use of activating TLR ligands. The induction of oral tolerance to ovalbumin, is enhanced if mice are pretreated with Flt3L, which expands DC populations, whereas tolerance can be reversed if mice are also administered IL-1, a cytokine known to activate DCs [104]. IFN-α can also promote antigen-specific humroal responses in mice when co-administered with Flt3L and antigen [105]. Alternatively, DCs can be activated by TLR agonists such as CpG-ODNs, which contain stimulatory motifs. Mice administered a combination of Flt3L and CpG-ODN demonstrated enhanced humoral and cellular responses to soluble proteins and develop anti-tumor immunity [106]. TLR agonists make great adjuvants if the desired response is cell-mediated immunity, since TLR activation of DCs induces the production of Th1 cytokines. It is also possible to manipulate the DC response by ensuring the production of specific cytokines, such as IL-12, a key mediator between innate immunity and cell-mediated specific immunity [107]. DCs engineered by retroviral gene transfer to produce IL-12 and pulsed with soluble *Leishmania donovani* antigen induce enhanced protective immunity against challenge

infection compared to non-engineered DCs [108]. The magnitude and kinetics of a vaccine can be improved by the cytokine milieu during the initial encounter between DCs and the antigen.

DNA vaccines are now becoming a promising strategy for the induction of strong T-cell mediated immunity. Plasmids encoding the vaccine antigen are introduced into host cells, which then process and express the protein. The success of DNA vaccines may be attributed to DCs in three ways: 1) a few DCs may be directly transfected with the vaccine plasmid and induce immune priming, 2) DCs can capture the vaccine antigen expressed by other cells that die following transfection, and 3) the CpG motifs in the plasmid backbone activate DCs [109]. **To summarize, vaccine efficacy requires the participation and activation of DCs, and can be improved by enhancing antigen capture, DC maturation and cytokines responses.**

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CHAPTER 4: CPG-DNA EFFECTS AND ADJUVANT POTENTIAL

4.1 Immunostimulatory nature of CpG-DNA

The first indication that bacterial DNA was immunostimulatory came from William Coley's use of bacterial extracts to treat cancer [1]. In fact, standard therapy for treatment of bladder cancer is the administration of attenuated extracts of mycobacteria bacillus Calmette Guerin (BCG) [2]. It was discovered that the active molecule in the BCG extract that activated natural killer cells (NK) to kill tumour cells was the DNA [3]. Other studies demonstrated that purified bacterial DNA could induce B cell proliferation and Ig secretion, but verterbrate DNA could not [4]. In order to identify the DNA structure that was responsible for the observed immunogenic effects, Krieg [5] synthesized and tested hundreds of oligodeoxynucleotides (ODN) and eventually discovered that CpG dinucleotides in particular base contexts were the key elements in B cell stimulation. All stimulatory DNA sequences contained a consensus motif of XCGY, where X is any base but C and Y is any base but G [6].

The immunostimulatory effects of the CpG motif can be attributed to the recognition of this DNA by the mammalian immune system. CpG-DNA is recognized by TLR9 on immune cells (discussed in section 3.2). Vertebrate and bacterial DNA are remarkedly different. CpG dinucleotides in veterbrate DNA are much less frequent than in bacterial DNA [7]. Futhermore, the bases flanking CpG dinucleotides in veterbrates are not random with a C preceding the motif and a G following it, thus not

in keeping with the stimulatory motif [8]. The most striking difference is the methylation patterns in bacterial and veterbrate DNA. In bacteria the CpG dinucleotide is unmethylated, while in veterbrate DNA the CpG is routinely methylated at the 5 position [7]. The importance of this methylation pattern is demonstrated by the fact that methylation of bacterial DNA with CpG methylase abrogates the immune stimulatory effects [6]. The immune effect of DNA are not restricted to bacteria, in fact stimulatory effects are observed with DNA from *Drosophila*, nematodes, mollusks, parasites and some viruses [9,10]. Some pathogens have evolved ways to overcome this immune recognition by reducing CpG frequencies [11, 12].

The immunostimulatory CpG dinucleotides and their flanking sequences have been extensively studied, and synthetic ODNs in specific sequence contexts are known to activate different immune cells. In general, CpG-DNA directly stimulates B cells, DCs and monocytes/macrophages regardless of the source of DNA or form of the ODN. ONDs can be synthesized on a phosphodiester backbone (PO), which are quickly degraded by nucleases or synthesized on a nuclease resistant phosphothioate (PS) backbone [13, 14]. Different classes of ODNs have been described based on their immune effects. CpG A-Class ODNs are defined PS poly-G motifs on the 5' and 3' ends surrounding a PO palindromic CpG sequence [15]. CpG A-Class ODNs activate NK cells and induce IFN-α from plasmacytoid DC precursors [15, 16]. CpG B-Classs ODNs have fully PS backbones with CpG motifs but no poly-G sequence [17]. These ODNs are potent B cell stimulators, but have reduced NK activation

capacity [18]. CpG C-Class ONDs combine the features of both A and B-Class ODNs, containing a hexameric CpG motif [19]. As expected, these C-Class ONDs are strong B cells stimulators but also induce strong IFN-α secretion from plasmacytoid DCs. On the other hand, some sequences are reported to be inhibitory, neutralizing the effects of stimulatory motifs [20].

CpG-DNA and CpG-ODNs activate a wide range of immune cells, stimulating both innate and acquired immne responses. CpG-DNA is a strong **B cell** mitogen, activating cells to enter the G1 phase of the cell cycle and secrete IL-6, which is required for the secretion of IgM from activated B cells [21,22]. CpG-activated B cells express increased levels of Fcγ receptor and co-stimulatory molecules [23]. CpG-DNA can also synergize with B cell receptor signals (BCR) and increase B cell proliferation and antigen specific Ig secretion [6]. CpG-DNA can prevent BCR ligation-induced B cell apoptosis by increasing NF-κB p50/c-Rel activity and maintaining *c-myc* levels [24, 25].

Dendritic cells differentially express TLR and so distinct DC subsets response to CpG-DNA. In humans, plasmacytoid DCs are directly activated by CpG-OND demonstrating key changes including: resistance to IL-4 induced apoptosis; increased surface expression of CD83, CD80, CD86, CD40, MHCII, ICAM-1; cytokine secretion of IL-6, TNF-α, IFN-α and; chemokine secretion [26-28]. In mice bone marrow-derived DCs and Langerhans cells are activated by CpG-DNA to express co-stimulatory molecules and secrete IL-12 and IL-6 [29, 30]. In mice subcutaneous

administration of CpG-DNA leads Langerhans cells to increase co-stimulatory molecule expression, secrete IL-12 and eventually migrate out of the skin [31]. CpG-DNA induces DC maturation and migration while promoting a Th-like immune activation. Murine **macrophages** respond to CpG-DNA by activation of NF-κB and expression of TNF-α and IL-12 [30, 32]. Purified human monocytes do not express TLR9, and therefore do not respond to CpG-DNA. **To summarize, CpG-DNA containing unmethylated CpG motifs, directly activates B cells and DCs and promotes the production of pro-inflammatory cytokines.**

4.2 CpG-DNA and TLR9

The cellular response to CpG-DNA is mediated by TLR9. TLR9 deficient mice are unresponsive to CpG-DNA, including inflammatory cytokine production and DC maturation [33]. Unlike most TLRs, TLR9 is located intracellularly and requires the uptake of CpG-DNA by the cell before signaling occurs. Cellular binding of CpG-DNA is unclear, but seems to required cell surface DNA binding proteins, which bind equally to any DNA types [34]. Uptake of CpG-DNA is essential for the induction of immune effects as shown by experiments where CpG-DNA was immobilized on a solid support or linked to beads (latex, magnetic, or gold) and could no longer activate lymphocytes [6, 35].

TLR9 signaling by CpG-DNA comprises a series of events that differ from other TLRs [36]. In a resting cell, TLR9 is found in the ER membranes and CpG-DNA is internalized via a clathrin-dependent endocytic pathway and rapidly moves to tubular

lysosomal compartments. Early on, TLR9 is actively recruited from the ER to the CpG-containing endosomes where there it directly binds CpG-DNA. Subsequently, the adaptor protein MYD88 is recruited from the cytoplasm to the CpG-DNA containing structures and signaling ensues. Endosomal maturation has been shown to be critical in CpG-TLR9 signaling. Blocking endosomal acidification with monensin, chloroquine or bafilomycin A can completely inhibit the stimulatory activity of CpG-DNA [37].

Signaling via TLR9 induces the activation of MAP kinases and transcription factors. B cells activated by CpG-DNA show activation of p38 and JNK within 10 minutes of exposure [38]. The p38 pathway seems to be required for cytokine production in B cells, since p38 inhibitors could block it. In macrophages, p38 and JNK are also activated following exposure to CpG-DNA [34]. The ERK pathway is activated in macrophages as well, inducing the production of TNF-α but decreasing IL-12 production [39]. CpG-DNA also triggers NF-κB activation by triggering the degradation of IκBα and IκBβ in macrophages and B cells [40, 41]. CpG-DNA can induce the production of reactive oxygen species within 5 minutes of exposure, which may also contribute to NF-κB activation [22]. CpG-DNA induces the p50/p65 heterodimer of NF-κB in macrophages while in B cells the dominant form induced is the p50/c-Rel heterodimer [25, 42]. NF-κB is the key transcription factor which regulates the expression of pro-inflammatory cytokines, and so the overall effects of CpG-DNA signaling through TLR9 is the promotion of strong Th1 immune

responses. **To summarize, CpG-DNA must be endocytosed and recognized by TLR9, which leads to downstream MAP kinase and NF-κB activation.**

4.3 CpG-DNA adjuvanticity

The type of response induced by a vaccine is crucial to its efficacy. In this respect the T helper response (Th1 or Th2) generated to an administered antigen can be directed by the type of adjuvant used. CpG-DNA is a stronger Th1-like adjuvant for the induction of B and T cell responses than the gold standard, complete Freund's adjuvant (CFA). In fact, CFA does induce Th1-dominant responses to vaccine antigens, while the incomplete FA, lacking *M. tuberculosis* induces Th2 responses [43]. However, due to undesirable side effects CFA is not safe for human vaccines. For this reason, there has been considerable interest in evaluating the efficacy as well as the safety of CpG-ODNs as adjuvants for human vaccines.

CpG motifs have been shown to improve vaccine efficacy in several ways. First, CpG-ODNs can improve the immunogenicity of protein antigens. Adding CpG-ODN to a conventional protein antigen ovalbumin, increased antibody production threefold, compared to protein alone [44]. Activity of the CpG-ODN can be further increased if it is directly linked to the antigen or co-encapsulated in liposome vesicles [45, 46]. These studies demonstrated that optimal stimulation occurs when antigen and adjuvant are presented to the immune system in close proximity. Other studies confirmed that CpG-adjuvant and antigen had to be injected in the same location, however this need not be at the same time. It was demonstrated that the antigen can

be given a week of more after the CpG-adjvant injection and still produce significant antigen-specific immune responses [47]. Second, CpG-ODN can induce both humoral and cell-mediated immune responses. Following immunization with CpG-ODN there is an antibody isotype switch to the Th1 specific IgG2a. Cytokine production upon CpG-ODN administration also shifts to a Th1 dominant response. Co-administration of CpG-ODN and ovalbumin increased the number of IFN-γ secreting spleen cells by two-fold compared to ovalbumin alone [48].

CpG-ODN has been used for both parenteral and mucosal vaccines. Many pathogens are encountered by the host at mucosal surfaces therefore there is a need for the identification of strong mucosal adjuvants. Cholera toxin is the gold standard mucosal adjuvant, but its toxicity precludes its use in humans. CpG-ODN administered with *Hepatitis* B surface antigen intranasally stimulated strong antigen-specific IgA responses throughout the mucosal system and in the serum [49, 50]. Mucosal vaccination with CpG-ODN also stimulated systemic immune responses as measured by spleen cell proliferation and IFN-γ secretion upon re-exposure to the vaccine antigen [49].

CpG-ODNs have been tested as adjuvants for a variety of pathogens and illnesses, including: bacteria, parasites, viruses, cancer and asthma. It has been shown that repeated administration of CpG-ODN alone could protect mice against lethal challenge infection with *Listeria monocytogenes* or *Francisella tularensis* [51]. This protection was correlated with high leves of IFN-γ and IL-6 production by

lymphocytes. CpG-ODNs have been extensively tested in vaccines against leishmaniasis. Live leishmania vaccines, heat-killed leishmania vaccines and leishmania protein vaccines with CpG-OND adjuvant have all demonstrated protection and challenge infection characterized by high levels of IFN-γ [52-54]. The anti-tumor effects of infections have been known for decades, demonstrating that immune activation can eradicate tumors. CpG A-Class ONDs have been tested in mice for prevention of tumor development. In this example, CpG-ODN were stabilized by the formation of complexes with cationic lipids, enabling effective NK activation and IFN-γ production [55]. Mice were given tumors by intravenous injection of carcinoma cells lines and then treated with intravenous injections of the cationic complexes. Treated mice had marked decreases metastases compared to control mice and mice with pre-existing tumors demonstrated slowed tumor progression. This protection was dependent on NK cells and IFN-γ, as mice depleted of these cells or deficient in IFN-γ production were not protected by CpG-ODN. The strong Th1 induction from CpG-ODN has been used to control the dominant Th2 inflammation seen in allergic diseases. An early study, demonstrated that Th1 CpG-induced effects could oppose the Th2 airway inflammation in a mouse asthma model [56]. CpG-ODN treatment could effectively prevent eosinophilia, IgE production and bronchial hyperreactivity. In another study, mice were sensitized to a strong Th2 stimulus, schistosome eggs, by intraperitoneal injection with or without CpG-OND [57]. Control mice developed broncho-constriction and typical allergic responses when challenged with schistosome egg antigen, while mice exposed to CpG-ODN

were protected against airway eosinophilia. In mice, CpG-OND can boost the protective efficacy of vaccines against many pathogens.

In humans, the efficacy and safety of CpG-OND continues to be tested in clinical trials. At this time, however, there have been dozens of trials in humans that can attest to the safe and effective use of CpG-ONDs. CpG 7909 is a 24 mer B-Class CpG-ODN developed by Coley Pharmaceutical Group and has been tested in different clinical trials. Engerix-B, the lisenced hepatitis B vaccine, has been tested with CpG-7909 adjuvant in clinical trials [58, 59]. In this double-blind study participants were given 1 mg of CpG-ODN with the vaccine multiple times. This combination resulted in 13-45 fold higher anti-hepatitis B antibodies among the treated individuals. This same CpG 7909 has also been tested with Fluarix vaccine and demonstrated that this adjuvant was well tolerated in all participants and could allow a reduction in vaccine dosage without losing vaccine immunogenicity [60]. In a recent phase I trial of CPG 7909 patients with previously treated lymphoma were given 3 weekly 2-hour intravenous (IV) infusions of CPG ODN 7909 at dose levels 0.01 to 0.64 mg/kg [61]. Adverse effects included nausea, hypotension, and IV catheter discomfort. NK activity generally increased in subjects and antibody-dependent cellular cytotoxicity activity increased in select cohorts. This study concluded that CPG 7909 could be given safely at these doses. CpG 7909 could also induce strong antigen specific CD8+ responses in melanoma patients given 4 monthly vaccinations of melanoma antigen and CpG [62]. Furthermore, HIV patients who are hyporesponsive to vaccinations can effectively respond to an Engerix-B and CpG 7909 immunization

without adverse effects or changes in HIV status [63]. None of the patients in these studies developed any signs or symptoms of autoimmune disease [64], which is a feared side effect of DNA immunization, as mice given large amounts of bacterial DNA can produce anti-DNA autoantibodies [65]. **To summarize, CpG-ODN have consistently shown adjuvant properties with various antigens both in animal models and in humans. CpG-DNA is an ideal vaccine adjuvant as it improves antigen presentation, improves antigen-specific humoral and cell-mediateed responses and creates a strong Th1 milieu.**

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SECTION II: MANUSCRIPTS

CHAPTER 5: MANUSCRIPT I

Activation of dendritic cells by the Gal-lectin of *Entamoeba histolytica* drives Th1 responses in vitro and in vivo

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Abstract

Amebiasis is a human disease caused by the protozoan intestinal parasite *Entamoeba histolytica*. Vaccine development has focused on the parasite's surface Gal-lectin as a protective antigen. The Gal-lectin is immunogenic and has been shown to induce Th1 cytokines *in vitro* and *in vivo*. The immunological basis of the protective immune response elicited by the Gal-lectin is unknown. In this study, we investigated the response of BALB/c bone marrow derived dendritic cells to *E. histolytica* Gal-lectin. Incubation of immature DCs with Gal-lectin resulted in activation and maturation after 24 hours. FACS analysis demonstrated an up-regulation of DC maturation markers CD80, CD86, CD40 and MHCII upon exposure to Gal-lectin. The Gal-lectin also induced dendritic cell production of IL-12 indicating a Th1 response. Gal-lectin activated DCs were able to stimulate T cell proliferation in an allogeneic mixed leukocyte reaction and adoptive transfer of Gal-lectin treated DCs into naïve mice resulted in IFN-γ producing Gal-lectin sensitized T cells. The activation of DCs by Gal-lectin was mediated by MAPK and NF-κB. These findings indicate that *E. histolytica* Gal-lectin is a potent vaccine antigen capable of directly initiating DC maturation and activation characterized by Th1 cytokine production.

Introduction

Entamoeba histolytica is an enteric human pathogen with clinical manifestations of infection characterized by amebic dysentery and amebic liver abscesses. Limitation and prevention of recurrent infection are associated with the development of an anti-amebic cell mediated immune response. Strong Th2 responses, specifically IL-4 production, are observed in animals infected with *E. histolytica*, while protection seems to be associated with a Th1 response [1,2]. In fact, studies have shown that IFN-γ activated macrophages can readily kill *E.histolytica* trophozoites *in vitro* through the production of nitric oxide [3,4]. IFN-γ has been associated with protection from amebic liver abscess in animal studies, and it is the principal cytokine involved in macrophage activation as well as a signature Th1 cytokine. The induction of this protective IFN-γ effect is an important aspect in the development of an amebiasis vaccine.

One of the leading candidates for an amebiasis vaccine is the *E. histolytica* galactose/N-acetyl-D-galactosamine inhibitable lectin (Gal-lectin). The Gal-lectin is a 260 kDa heterodimeric surface protein composed of heavy and light subunits. The heavy subunit of the Gal-lectin has an extracellular carbohydrate recognition domain involved in binding colonic mucins for colonization and binding to target cells for cytolytic activity [5,6]. The light subunit occurs in two isoforms, with or without a GPI anchor. The native Gal-lectin demonstrates high vaccine efficacy in gerbil and mouse models of amebiasis [7,8]. *In vitro*, the Gal-lectin has been shown to induce IL-2 and IFN-γ in T cells [9] and increase amebicidal macrophage activity as well as

TNF-α production [4]. No data are available on the ability of *E. histolytica* Gal-lectin to induce the production of Th1 cytokines in DCs. The immunological basis for the protective immune response elicited by the Gal-lectin in DCs is unknown.

Dendritic cells (DCs) are the most potent antigen presenting cells and are responsible for directing innate and adaptive immunity [10]. Immature DCs in peripheral tissues are specialized in antigen capture. DCs are among the first cells to encounter enteric antigens, as the gut lumen is continuously exposed to commensals and pathogens. DCs are abundant at mucosal surfaces, acting as sentinels and are also recruited during infections. Recent studies demonstrated that DCs express tight junction proteins allowing them to extend dendrites from the epithelium through tight junctions and sample luminal antigens [11]. The extent and nature of the innate or adaptive immune responses induced by DCs are related to the type of antigens sampled. DCs express broad specificity surface receptors, like mannose receptors and scavenger receptors, but also TLRs, which have evolved to recognize conserved molecular patterns in pathogens. Upon antigenic activation, DCs undergo a maturation process, involving migration to secondary lymphoid organs and presenting peptides to naïve T cells. Inflammatory cytokines, such as IL-12, IL-1β and IL-6 are also secreted upon activation of immature DCs. Therefore, DCs are the principle source of IL-12, a key mediator of innate immunity responsible for Th1 differentiation. IL-12 production by DCs was previously thought to be dependent on CD40 and CD40L binding on activated T cells [12], however, recent studies show direct IL-12 production upon DC uptake of *Leishmania* or *Plasmodium* [13,14].

As a vaccine candidate, the Gal-lectin has demonstrated invariable immunogenicity, immunodominance in humans and protective immunity in animals. Antigen capture by DCs is essential to mounting an immune response to a vaccine, but concurrent DC activation is also required for the induction of a proper response. Maturation of DCs is critical for subsequent stimulation of resting naïve T cells for the induction of a primary immune response. To this date there are no data on the interaction between DCs and the Gal-lectin. Our study shows that Gal-lectin is able to induce DC maturation and activation, characterized by pro-inflammatory cytokine production.

Materials and Methods

Animals and Reagents

Female BALB/c mice were purchased from Charles River (St.Constant, Canada). The University of Calgary Animal Care Committee approved all animal experiments. Dendritic cell activation was induced with 1μg/ml LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich, St. Louis, MO), 10 μg/ml Gal-lectin, prepared as previously described [15] or with RPMI-1640 media alone (Gibco). The Gal-lectin preparation did not contain detectable levels of endotoxin contamination as measured by the E-TOXATE assay (Sigma). MAPK inhibitors SB203580 and PD98059 were purchased from Calbiochem (San Diego, CA). Monoclonal antibodies (1G7, 3F4, 7F4, and H85) directed against the Gal-lectin heavy sub-unit were prepared from hybridoma cell supernatants [16,17] and Fab_2 fragments generated by pepsin digest.

Preparation of bone marrow derived DC

Dendritic cells were isolated from the bone marrow of naïve BALB/c mice, as previously described [18]. Briefly, femurs and tibias were removed and the bone marrow flushed using a 27gauge syringe. Red blood cells were lysed with Red Blood Cell Lysing Buffer (Sigma) and washed three times in RPMI-1640. Cells were cultured in 6 well plates (2.5×10^5 cells/ml) in RPMI-1640 media supplemented with 5% FBS, 10mM HEPES, 50 μM 2-ME, 2mM glutamine, 5 mg/ml penicillin/streptomycin sulfate and 10 ng/ml rmGM-CSF (R&D Systems, Minneapolis, MN). Non-adherent cells were removed after 5 days and remaining cells were cultured another 48hrs then sub-cultured into new plates to enrich the DC population. Sub-cultured cells were grown another 24 hrs before use in different assays. Unless otherwise stated, all experiments were performed with day 8 immature dendritic cells. Approximately 92% were positive for CD11c+ cell surface expression. T cells and B cells could not be detected in the culture. FACS analysis determined the DC population was 99% negative for CD3+ and CD19 (data not shown).

DC Flow cytometric analysis

After stimulation, DCs (1×10^6) were washed and resuspended in PBS. Expression of CD11c+, CD80, CD86, CD40 and MHCII ($I\text{-}A^d$) was quantified using FITC-, or PE-conjugated anti-mouse antibodies purchased from BD Pharmingen (San Diego, CA). Controls include isotype control antibody and cells without antibody. Samples were

collected with the BD FACSAria flow cytometer and data were analyzed using WinMDI.

Characterization of DC cytokine mRNA

DCs were stimulated with the indicated antigens or media alone for 3,6,12, or 24 hours. Total mRNA was extracted using Trizol ® (Invitrogen) following the manufacturer's instructions. 1μg of DNase treated total mRNA was reverse transcribed with MMLV enzyme and random hexamer primers (Invitrogen). Real-time PCR analysis using SyBrGreen (Qiagen) and mouse specific primers [19] was used to determine relative cytokine gene expression. Cytokine gene expression is represented as fold increase over RPMI stimulated control corrected for GAPDH house keeping gene, as determine by the $2^{\Delta\Delta ct}$ method [20]. Real-time DNA amplification assays were conducted in triplicates with the Rotor-Gene 3000 (Corbett Research).

Cytokine assays

Supernatants from DCs cultured with different antigens or media alone were collected at 3,6,12 and 24 hours and tested for IL-12 p70, IL-10 and IL-4. Cytokine levels were determined by sandwich ELISA with mouse specific antibodies (Duoset R&D Systems DY419, DY417, DY404) and mouse recombinant protein standards to calculate concentrations. Splenocyte culture supernatants from adoptive transfer experiments were collected after 60 hours and assayed for IFN-γ and IL-4 production (Duoset R&D Systems DY485 and DY404).

Mixed leukocyte reaction

T cells were purified from naïve BALB/c mice spleens using nylon wool columns. Purity was determined by FACS analysis for CD3+ (data not shown). Triplicates of 2 x 10^5 T cells per well were seeded onto 96-well plates (Corning) with titrated numbers of mytomycin C (10 μg/ml) pretreated DCs. Cells were co-cultured for 96 hours and 1μCi [methyl-^{3}H]thymidine (Amersham) was added per well for the last 18 hours. Labeled cells were harvested onto glass fiber filters with a Filtermate Harvester (Perkin Elmer) and filters were counted in a Trilux Counter LKB (Wallac: Pharmacia).

Western blots for NF-κB p65 subunit and MAPK

DCs (1 x 10^6) were stimulated with the indicated antigens for up to 60 minutes. The cells were washed and the cell pellet was collected. Nuclear and cytoplasmic extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) following the manufacturers' instructions. Nuclear or cytoplasmic proteins (30 μg) were resolved by 10 % SDS PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with a 4% milk solution, washed with T-TBS and incubated with primary antibody against NF-κB p65, phosphorylated p38, JNK, ERK or ERK 1/2(SantaCruz). Bound antibody was visualized using secondary HRP-conjugated antibody and enhanced chemiluminescence (ECL plus Reagent, Amersham).

NF-κB EMSA

DCs (1 x 10^6) were stimulated with RPMI, LPS or Gal-lectin for 30 minutes and the cells were collected for nuclear protein extraction with NE-PER reagents (Pierce). EMSAs were performed using double-stranded oligodeoxynucleotides corresponding to the NF-κB consensus sequence (5′- GGGACTTTCC-3′). Labeled probe (0.5 ng) was incubated with 5 μg of nuclear extract for 30 minutes at room temperature in binding buffer (12 mM Hepes, 60 mM KCL, 4 mM $MgCl_2$, 1mM EDTA, 1mM DTT, 12 % glycerol) with 1μg poly (dI-dC). Complexes were separated on a 6% polyacrylamide gel for 2 hours at 165 V, dried for 2 hours and exposed to Kodak BioMax XAR film (Rochester, NY) at

-80 °C.

Adoptive transfer of treated DCs

For adoptive transfer experiments, DCs were cultured as above and treated with RPMI or Gal-lectin for 24 hours. The cells were washed 3 times in PBS and resuspended at 1x 10^6 cells in 200 μl PBS. Naïve BALB/c mice were injected intraperitoneally with the DC suspensions using a 25-gauge needle. Mesenteric lymph node cells and spleen cells were recovered 5 days later and cultured at 2 x 10^6 cells/ml with RPMI, conA or Gal-lectin. Cell supernatants and mRNA were collected for cytokine assays.

Statistical analysis

Differences in the mean values between experimental groups were compared using Student's unpaired *t*-test. *P* values <0.05 are considered significant.

Results

Gal-lectin stimulates DC phenotypic maturation

DC activation by microbial products is known to induce phenotypic changes including up-regulation of cell surface co-stimulatory molecules [21]. Bone marrow cells were cultured for 8 days to produce immature DCs, which were used to determine the activation potential of amebic Gal-lectin. After 24 hours of Gal-lectin *in vitro* stimulation (Fig.5.1), DCs showed increase surface expression of CD80, CD86, CD40 and MHCII in comparison to cells cultured in media only ($p<0.05$). DCs stimulated with LPS demonstrated up-regulation of these markers to a greater extent. Up-regulation was highest for CD80 and MHCII expression on stimulated DCs. To determine the effects of Gal-lectin during the differentiation stage of dendritic cells, Gal-lectin or LPS was added to the bone marrow cultures in the presence of GM-CSF between days 5 and 7 of DC maturation. As seen when DCs are stimulated for 24hours, the addition of LPS or Gal-lectin during the generation phase also increased the cell surface expression of CD80, CD86, CD40 and MHCII (Fig.5.2) compared to cells generated with GM-CSF alone. In this experiment however, increase in CD40 expression was highest.

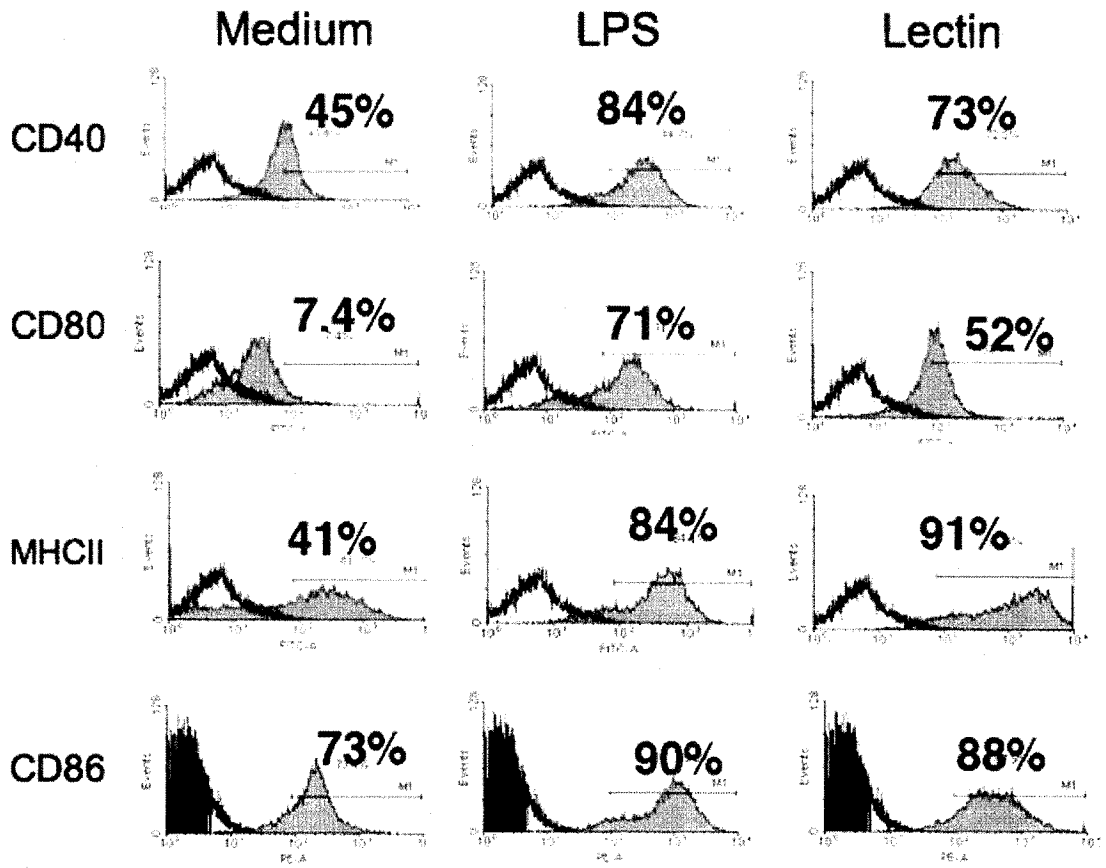


Figure 5.1. ***E. histolytica*** **Gal-lectin induces BMDC activation.** Immature DCs (day 8) were pulsed for 24 hours with medium only, LPS (1μg/ml) or Gal-lectin (10 μg/ml). Surface expression levels of CD40, CD80, MHCII and CD86 were measured by flow cytometry. Open histograms with solid lines represent isotype control, while open histograms with dotted lines represent medium only control. Filled histograms show signal for surface markers of treated cells. Numbers reflect the percent positive cells. Data from one experiment are representative of four independent experiments.

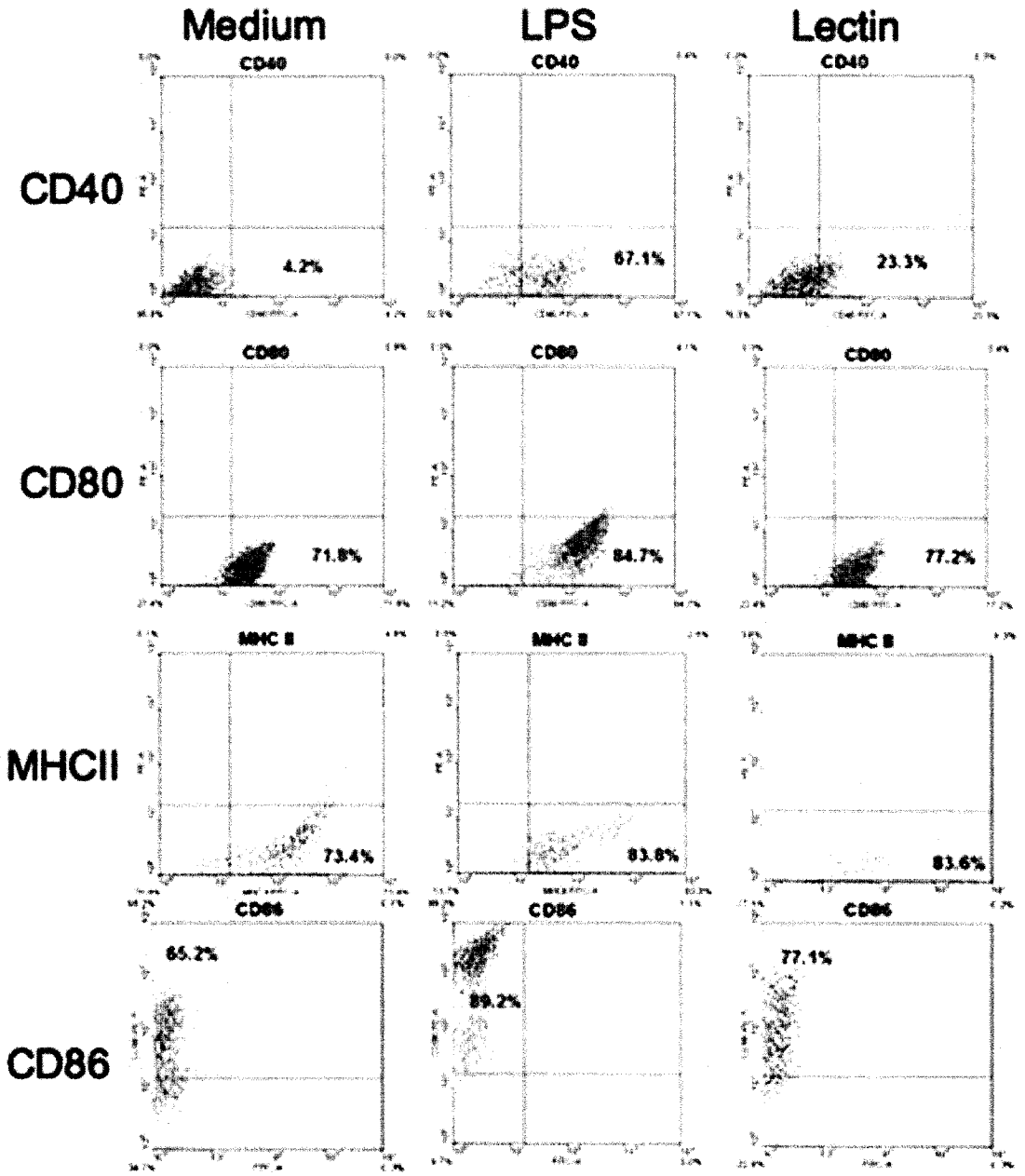


Figure 5.2. *E. histolytica* Gal-lectin activates BMDC during generation with GM-CSF. Bone marrow monocytes were cultured in the presence of GM-CSF for 7 days and LPS (1μg/ml) or Gal-lectin (10 μg/ml) were added to the culture from days 5 to 7. Surface expression levels of CD40, CD80, MHCII and CD86 were measured by flow cytometry on day 7. Numbers in quadrant represent the percent positive cells for that surface marker. Data are representative of 3 independent experiments.

Gal-lectin induces expression and secretion of cytokines by DCs

The ability of the Gal-lectin to induce cytokine expression was determined by real-time PCR with RNA collected at intervals for up to 24 hours. mRNA levels are represented as fold increases over RPMI unstimulated DCs. As shown in Figure 5.3A, IL-12p40 mRNA increased over time, peaking at 12 hours for both LPS and Gal-lectin stimulated DCs. Similar kinetics were observed with TNF-α mRNA; increasing mRNA with time, peaking at 12hours (Fig.5.3B). IL-12p70 protein secretion in the culture supernatant was detected after 3 hours incubation with LPS, compared to 6 hours incubation with the Gal-lectin (Fig.5.4A). No IL-12 was detected in unstimulated DCs.

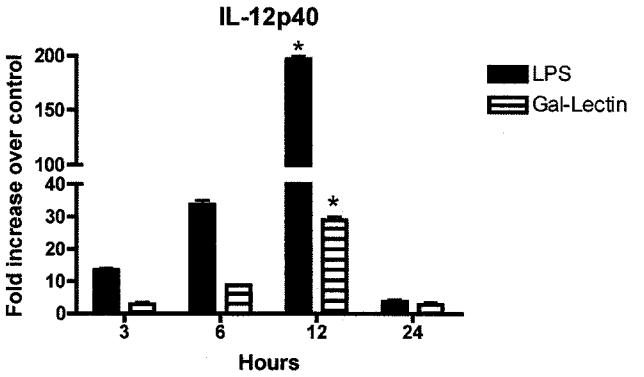
In contrast with the expression of pro-inflammatory cytokines, stimulation of DCs with the Gal-lectin or LPS did not induce detectable amounts of IL-4 (Fig.5.4B). Real-time PCR analysis revealed a decrease in IL-4 mRNA over time compared to unstimulated controls (Fig.5.3C). IL-4 protein could not be detected in culture supernatants of either Gal-lectin or LPS, although minute amounts were initially detected at the 3 hours time point. DCs incubated with media alone for 24 hours had low amounts of IL-4 (120pg/ml). No detectable levels of IL-10 were observed in the supernatants of DCs stimulated with media, LPS or Gal-lectin (data not shown). In all experiments, the kinetics of pro-inflammatory cytokine production by Gal-lectin were identical to those of LPS, however, the amount cytokine produced was greater in LPS induced DCs. To exclude the possibility that the observed DC maturation was due to LPS contamination in the native Gal-lectin preparation, we performed the

experiments using DCs derived from C3H/Hej mice, which are unresponsive to LPS. As expected, bone marrow derived DCs from C3H/Hej mice responded to the Gal-lectin in a similar fashion as the DCs from BALB/c mice, while LPS stimulation had a reduced cytokine response (data not shown).

Monoclonal antibodies against the Gal-lectin inhibit DC IL-12p40 mRNA expression and maturation

To determine Gal-lectin specificity and the region involved in IL-12 production in BMDC, we tested the inhibitory effects of a panel of non-overlapping monoclonal antibodies that correspond to epitopes on the cysteine rich region of the heavy sub-unit. As shown in Figure 5.5A both mAB 1G7 (epitope 596-818) and 3F4 (epitope 895-998) decreased IL-12p40 mRNA expression by 13% and 43% respectively ($p<0.05$). The isotype control monoclonal antibody and mAbs 7F4 and H85 had no effect on IL-12p40 expression. Gal-lectin monoclonal antibodies did not significantly reduce LPS induced IL-12p40 mRNA expression (data not shown). These inhibitory mAbs were used to determine if they could also block Gal-lectin induced DC maturation. Figure 5.5B demonstrates that mAbs 1G7 and 3F4 could reduce the activation potential of Gal-lectin in immature DCs by inhibiting the increase in surface expression of co-stimulatory molecule CD80 ($p<0.05$). Therefore amino acids 596-998 of the cysteine rich portion of the Gal-lectin contain the region responsible for maturation as well as IL-12p40 mRNA expression in dendritic cells.

A



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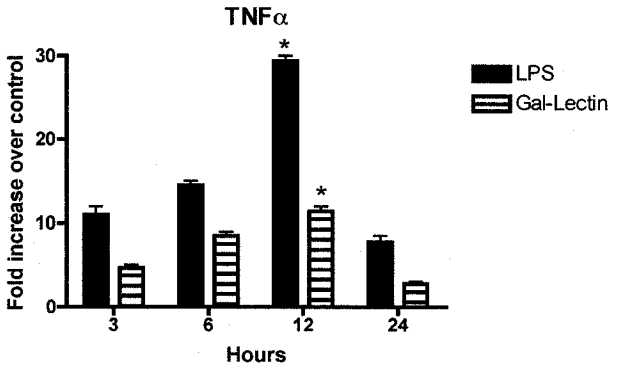

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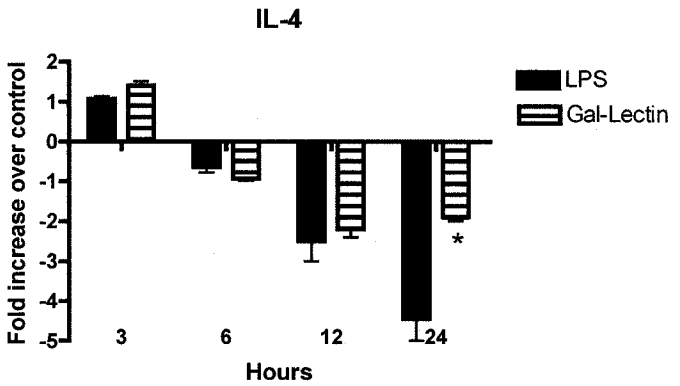

Figure 5.3. *E. histolytica* Gal-lectin increases BMDC Th1 cytokine mRNA. BMDC mRNA collected after different time points of *in vitro* incubation with medium only, LPS (1μg/ml) or Gal-lectin (10 μg/ml). Data are represented as fold increase over medium only control for mRNA expression of A) IL-12 p40, B) TNF-α and C) IL-4. Data points represent mean ± SEM from three experiments. An asterisk indicates a significant in cytokine mRNA compared to levels at the first time point ($p<0.05$).

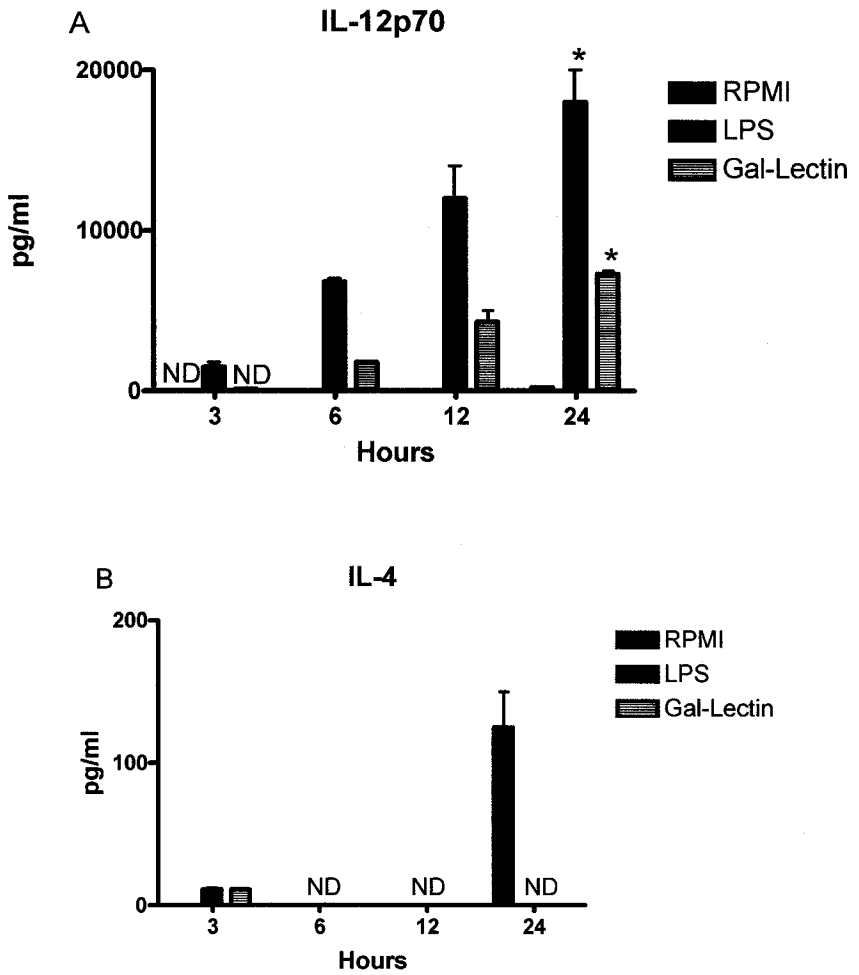

Figure 5.4. ***E. histolytica*** **Gal-lectin induces IL-12p70 production in BMDC.** Day 8 DCs were pulsed for the indicated times with medium only, LPS (1μg/ml) or Gal-lectin (10 μg/ml). Culture supernatants were collected and IL-12 p70 (A) and IL-4 (B) cytokine production determined by sandwich ELISA. Data points represent mean ± SEM from three experiments. ND indicates that the cytokine was not detectable. Asterisks represent a significant increase in protein compared to the fist time point ($p<0.05$).

DCs stimulated with Gal-lectin induce T cell proliferation in MLR

To test the functional ability of Gal-lectin treated DCs we performed a mixed leukocyte reaction. Allogeneic T cells from naïve mice were used as responder cells and proliferation was measured by [methyl-^{3}H]thymidine incorporation. Both Gal-lectin and LPS stimulated DCs were capable of inducing T cell proliferation, whereas the DCs stimulated with media alone were not capable of generating a mixed leukocyte response (Fig.5.6). Increasing the number of stimulator DCs also induced greater proliferation responses ($p<0.05$) in a dose dependent manner. LPS was confirmed to be a maturation stimulus as it induced MLR. Gal-lectin proved to be a good maturation stimulus for DCs as it generated similar MLR as LPS stimulation.

Adoptive transfer of Gal-lectin treated DCs induces Th1 response

Since Gal-lectin stimulated DCs were able to stimulate a MLR, we tested their ability to initiate a Th1 response. It is known from vaccine studies that injection of native Gal-lectin intraperitoneally in animals induces an antigen specific response. We were interested in determining whether DCs pulsed with the Gal-lectin could induce similar results. This was determined by conducting an adoptive transfer of DCs, pulsed for 24 hours with Gal-lectin or RPMI media, intraperitoneally into naïve recipient BALB/c mice. Control mice were injected intraperitoneally with native Gal-lectin only (1 μg). Mesenteric lymph nodes (MLN) cells were recovered 5 days post immunization and re-stimulated *in vitro* with ConA, RPMI or Gal-lectin to measure recall cytokine secretion.

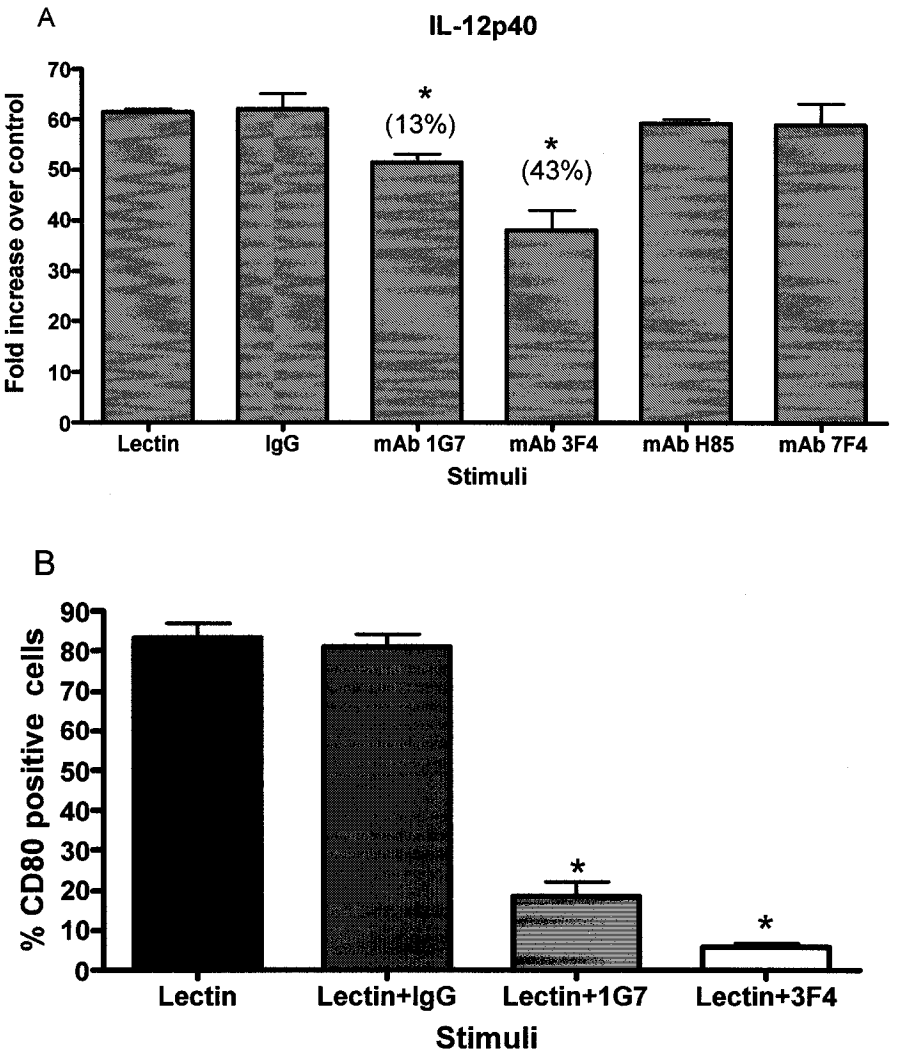

Figure 5.5. Anti-Gal-lectin monoclonal antibodies inhibit Gal-lectin induced BMDC maturation. Gal-lectin (10 μg/ml) was pretreated with 1μg/ml Fab_2 fragments overnight at 4 °C, then used to stimulate BMDCs for 3 hours at 37 °C. A) Real Time PCR analysis of mRNA transcripts is expressed as fold increase in expression over cells with media only. IgG is an irrelevant control (isotype control) which does not have specificity to the Gal-lectin. Values in parentheses correspond to percent decrease in IL-12p40 mRNA expression compared to Gal-lectin. B) FACS analysis of CD80 surface expression. Cells were treated as above but for 24 hours before analysis. Bars represent the percent positive population. Data points represent mean ± SEM from three experiments. Asterisks indicate a significant decrease in expression compared to Gal-lectin without mAb treatment ($p<0.05$).

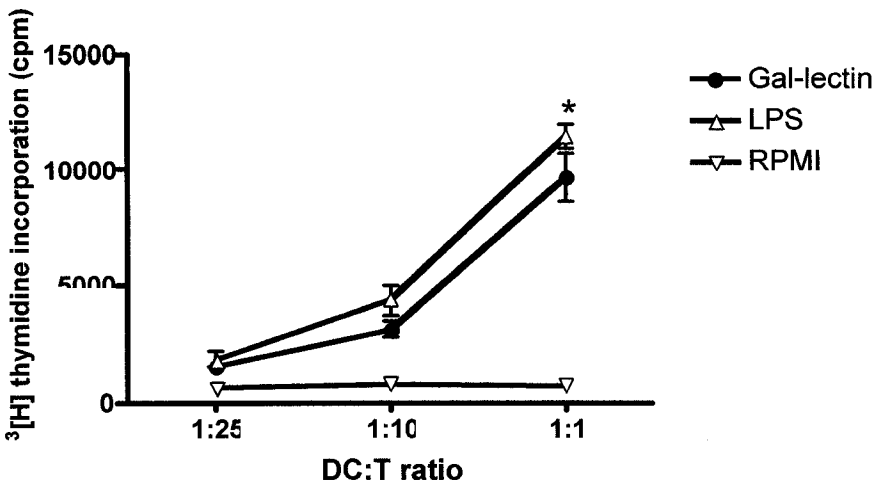

Figure 5.6. ***E. histolytica*** **Gal-lectin treatment induces proliferative allogeneic MLR.** Day 8 BMDCs were pulsed *in vitro* for 24 hrs with medium only, LPS (1μg/ml) or Gal-lectin (10 μg/ml), then co-cultured for 72 hrs with naïve allogeneic T cells (BALB/c) at graded cell densities. Proliferation responses were measured by [methyl-^{3}H]thymidine incorporation. Data points represent mean ± SEM from three experiments. Asterisks indicate a significant increase in proliferation compared to medium treated cells at that density ($p<0.05$).

MLN recovered from Gal-lectin/DC immunized mice showed a 37-fold increase in IFN-γ mRNA in response to Gal-lectin re-stimulation (Fig.5.7A). In contrast, there was no increase in IL-4 mRNA levels compared to re-stimulation with conA. MLN cells from immunized mice also produced IFN-γ (260 pg/ml), but not IL-4 in response to Gal-lectin as measured by ELISA with culture supernatants (Fig.5.7B). This indicates that Gal-lectin matured DCs have the ability to generate a Th1 Gal-lectin specific immune response *in vivo*. This response was comparable to the antigen specific response observed with native Gal-lectin injections alone.

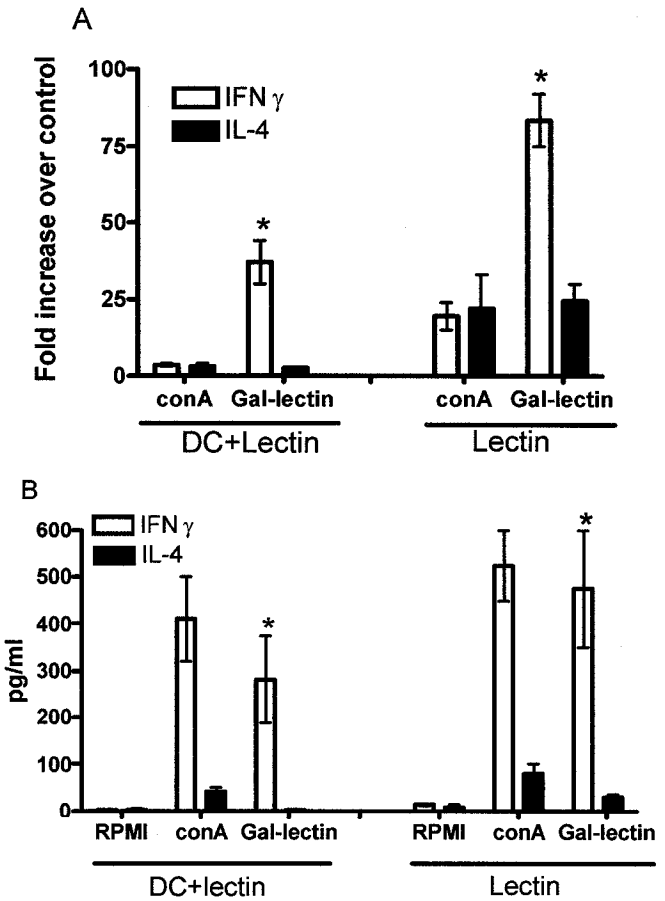

Figure 5.7. *E. histolytica* Gal-lectin stimulated BMDCs generate Th1 responses. *In vitro* T cell response to Gal-lectin was determined from mice immunized intraperitoneally with 24 hr Gal-lectin pulsed BMDCs or injected with Gal-lectin only. Mesenteric lymph node cells were isolated 5 days post injection and restimulated with medium only, conA or Gal-lectin to determine A) cytokine mRNA expression (at 24hrs) and B) IFN-γ and IL-4 cytokine production (60 hrs). Data points represent mean ± SEM from three experiments. Asterisks indicate a significant increase in cytokine levels compared to re-stimulation medium control ($p<0.05$).

DCs maturation with Gal-lectin is NF-κB mediated

The ability of DCs to regulate immunity is dependent cell maturation and activation. Maturation can occur through a variety of factors including whole bacteria, bacterial products, inflammatory cytokines and CD40 ligation. In all cases, NF-κB is central to DC maturation and in fact inhibition of NF-κB translocation blocks DC maturation

[22]. To assess the involvement of NF-κB in DC maturation with Gal-lectin, we performed an EMSA and western blot for p65 subunit with DC nuclear extracts. Stimulation of immature DCs with Gal-lectin for 30 minutes resulted in DNA binding activity of NF-κB, as did stimulation with LPS, but not with RPMI medium only (Fig.5.8A). As expected LPS and Gal-lectin showed nuclear translocation of p65 subunit into the nucleus (Fig.5.8B). In contrast, stimulation with media only did not induce p65 nuclear translocation. This indicates that Gal-lectin induces NF-κB activation and subsequent DC maturation.

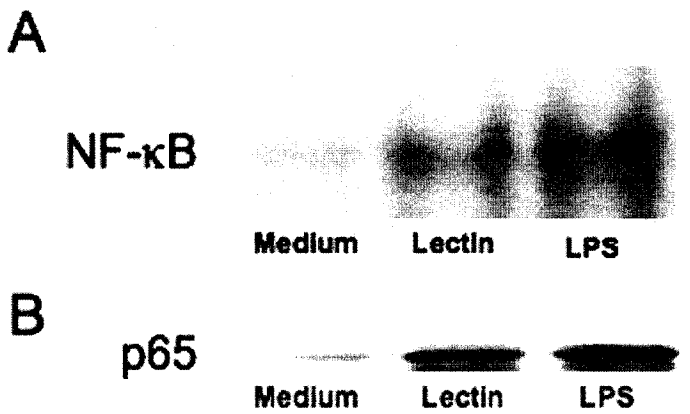

Figure 5.8. Gal-lectin induces NF-κB activation. (A) Day 8 BMDCs were pulsed for 30 minutes with medium only, Gal-lectin (10 μg/ml), or LPS (1 μg/ml), and nuclear extracts were probed in an EMSA for NF-κB DNA-binding activity or (B) electrophoretically separated and probed for NF-κB p65 subunit. Data shown are from one experiment and representative of two independent experiments.

Gal-lectin-induced MAPK signaling

To characterize the DC maturation induced by Gal-lectin, we examined the cells for changes in the MAPK signaling pathways that have been shown to be involved in DC maturation. As expected, LPS induced phosphorylation of MAPK family proteins,

p38, JNK and ERK (Fig.5.9A). Gal-lectin alone also induced the phosphorylation of these MAPK whereas incubation with media alone did not. In fact, pre-treatment of dendritic cells with p38 inhibitor SB203580 or ERK inhibitor PD98059 for 30 minutes before Gal-lectin stimulation decreased IL-12p40 cytokine mRNA expression 58 fold (Fig.5.9B), and decreased TNF-α expression 8 fold ($p<0.05$).

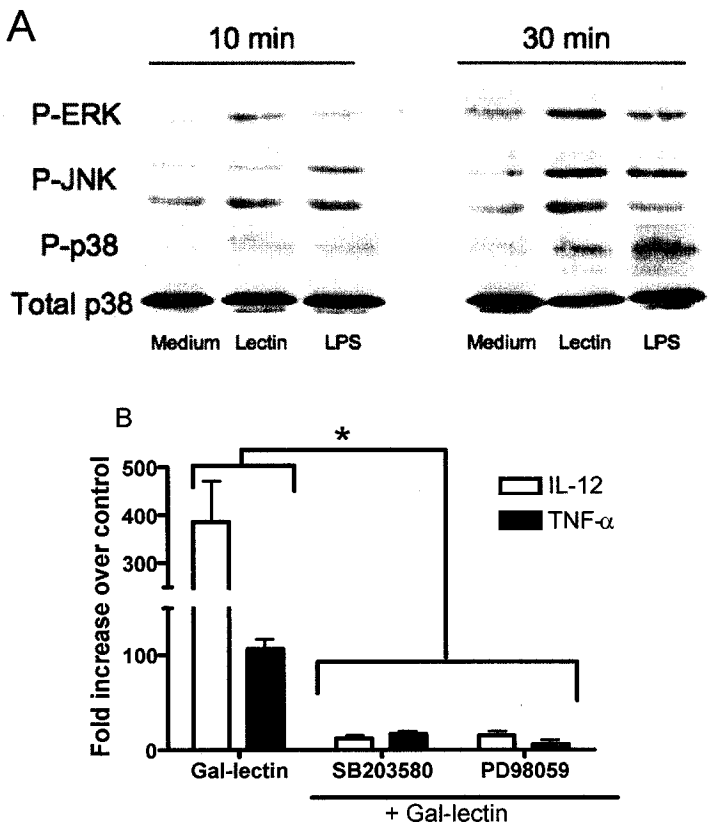

Figure 5.9. BMDC activation by Gal-lectin is mediated by MAPK. (A) Day 8 BMDCs were pulsed for 10 and 30 minutes with medium only, Gal-lectin (10 μg/ml), or LPS (1 μg/ml) and cellular extracts were separated by electrophoresis, blotted and probed with antibodies specific for phosphorylated ERK, JNK or p38. Total unphosphorylated p38 antibody was used as loading control. Data shown are from one experiment and are representative of three independent experiments. (B) Day 8 BMDCs were preincubated for 1 hour with either 25 μM SB203580 (p38 inhibitor) or PD98059 (ERK inhibitor) or media only and subsequently pulsed for 3 hours with Gal-lectin (10 μg/ml). mRNA was extracted and used in real time PCR analysis for IL-12p40 and TNFα expression. Asterisk indicates a significant decrease in mRNA levels compared to Gal-lectin treatment without inhibitors ($p<0.05$).

Discussion

We have shown that *E. histolytica* Gal-lectin can directly activate DCs and induce a Th1 immune response. The effects of Gal-lectin on DC maturation and activation are consistent with reports that Gal-lectin is immunogenic and able to engage Th1 cytokine production. The findings in this study demonstrate that *E.histolytica* Gal-lectin is able to induce DC cytokine production and up-regulate the expression of MHCII, CD80, CD86 and CD40. The effects of the Gal-lectin on immature DCs are MAPK mediated and induce NF-κB activation. It has been shown that Gal-lectin can induce the production of TNF-α in murine macrophages [23] and stimulates the production of IL-12 in human macrophage cell line THP-1 [24]. While it is well established that Gal-lectin can induce pro-inflammatory responses *in vitro*, our understanding of this Th1 induction *in vivo* has not been elucidate. This is the first report of Gal-lectin inducing dendritic cell activation characterized by a Th1 response. In this study we have demonstrated that Gal-lectin has the ability to both activate DCs and initiate the production of Th1 cytokines, both *in vitro* and *in vivo*.

DCs are the principle source of IL-12, a cytokine, which mediates early immunity by inducing cellular immunity against infectious microbes [25]. Cellular immunity is thought to be important in clearance of tissue dwelling *E. histolytica* [26]. The vaccination strategy against amebiasis is to use an amebic antigen capable of initiating a protective Th1 response, characterized by IFN-γ production for activation of macrophage amebicidal activity [4]. In this study we have shown that Gal-lectin can induce IL-12 secretion in DCs and adoptive transfer of Gal-lectin treated DCs

sensitizes T cells to produce IFN-γ upon secondary exposure to the parasite Gal-lectin. This finding may explain why IFN-γ, IL-12 and IL-2 expression is highest in Gal-lectin vaccinated gerbils and corresponds to protection from amebic challenge infection [27]. The Gal-lectin antigen initiates a pro-inflammatory response in DCs, which upon subsequent exposure to the parasite induces a Th1 response, characterized by IFN-γ, which aids in parasite clearance and inhibits IL-4 production. IL-4 has been associated with increase parasite burden; therefore IFN-γ seems to also impede parasite growth by reducing IL-4 levels [1]. Furthermore, we could not detect any IL-4 or IL-10 cytokine production in Gal-lectin stimulated DCs.

Previous studies with the Gal-lectin and murine macrophages have demonstrated that Gal-lectin stimulation induces NF-κB activation resulting in up-regulation of several genes, including TLR-2 [28]. In this study we have demonstrated that Gal-lectin induces NF-κB activation in dendritic cells. The most characterized pattern recognition receptors are the TLRs, which are expressed differently on different DC subsets and recognize pathogen associated molecular patterns. Mice have up to six defined DC subsets including myeloid and plasmacytoid DCs, which differ both phenotypically and functionally [29]. Myeloid DCs have great antigen presenting activity and are associated with T cell activation. Myeloid DCs express mostly TLRs 1 through 6, while plasmacytoid DCs express TLRs 7 and 9. These plasmacytoid DCs have less antigen presentation potential and therefore modest T cell activating properties, but are characterized by greater cytokine secretion. Bone marrow derived DC are mostly myeloid (CD11c+) and would express TLRs able to recognize

pathogen associated molecular patterns. Gal-lectin stimulation induces NF-κB activation, which is known to be a function of TLR mediated cell activation, therefore Gal-lectin could act as a pathogen associated molecular pattern, initiating beneficial pro-inflammatory responses against an *E. histolytica* infection. We tested the Gal-lectin preparation and did not find detectable amounts of endotoxin, which could have been a contaminant contributing to the maturation effects. This indicated that Gal-lectin might be capable of directly activating immune cells. We are currently studying the specific role of TLRs in Gal-lectin mediated DC activation.

MAP kinases are highly conserved cellular pathways involved in initiation of immunity as well as activation of adaptive immunity [30]. Each pathway plays a distinct role in DC maturation; however, it has been shown in human DCs that p38 is the main MAP kinase involved in DC phenotypic and functional maturation as well as IL-12 production [31]. It is believed that the three MAPK differentially regulate all aspects of DC phenotypic maturation, cytokine production and functional maturation. Our analysis of signaling in BMDCs pulsed with Gal-lectin revealed phosphorylation of all three MAP kinases: p38, ERK and JNK. Moreover, inhibition of p38 with SB203580 and ERK with PD98059 dramatically decreased Gal-lectin induced TNF-α and IL-12p40 mRNA expression. This MAPK phosphorylation by Gal-lectin stimulation is consistent with previous report of the Gal-lectin's ability to activate MAPK. In macrophages, TLR-2 upregulation in response to the Gal-lectin was shown to be dependent on p38 phosphorylation but not JNK or ERK [27], while in epithelial cells the Gal-lectin has been shown to induce p38 and ERK

phosphorylation [32]. Our findings with Gal-lectin stimulation in DCs indicates that there is phosphorylation of all MAPK, suggesting a balance of the three to promote a Th1 response and a distinct maturation state.

The Gal-lectin is a prime candidate for an amebiasis vaccine as it is antigenically stable and essential to parasite survival. The development of a Gal-lectin sub-unit vaccine requires the identification of the protective regions of the molecule. The cysteine rich region of the Gal-lectin (amino acids 379-1209) is known to contain epitopes for T and B cell stimulation [9]. It has also been repeatedly shown in macrophages that amino acids 596-998 of the cysteine rich region of the Gal-lectin contain the region that stimulates IL-12 production, TLR-2 up-regulation, TNF-α production and iNOS expression [4,24, 28]. In our studies with DCs we were able to inhibit both CD80 upregulation and IL-12p40 mRNA expression with monoclonal antibodies mapping to amino acids 596-998. This region includes to the carbohydrate recognition domain (895-998) involved in adherence to colonic mucins and target cells. Correspondingly, vaccination trials in gerbils with peptides mapped to different portions of the heavy sub-unit have demonstrated that the greatest protection from amebic liver abscess formation requires the carbohydrate recognition domain [8,15,16]. The carbohydrate recognition domain alone, however, does not induce sterile immunity therefore other portions of the heavy-subunit must be important in protection. Identifying the regions of the Gal-lectin that are protective and that are responsible for the Th1 induced responses observed in immune cells could facilitate the development of a sub-unit vaccine.

E. histolytica is exclusively a human parasite therefore the interaction of the parasite Gal-lectin with human dendritic cells may reveal different findings. However, preliminary experiments with DCs derived with GM-CSF and IL-4 from PBMC demonstrate similar activation with Gal-lectin treatment (data not shown), measured by CD83 surface expression and pro-inflammatory cytokine mRNA analysis. Not all *E. histolytica* infections result in invasive disease, but in the rare cases where the trophozoites invade the mucosal tissues, there is marked parasite induced immune suppression. During an acute infection, patients show decreased antigen-specific cell-mediated immunity, allowing the parasite to survive [26]. The ability of the Gal-lectin to induce strong Th1 responses would be useful in vaccine development against the disease, as it would provide cell-mediated immunity against potentially invasive parasites and blocking antibodies to the Gal-lectin, thus preventing parasite colonization. Indeed, using the Gal-lectin's innate ability to initiate a Th1 response could counteract the parasite's strategy to minimize cell-mediated immunity.

Acknowledgements

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CONNECTING STATEMENT I

In the first manuscript we showed that the Gal-lectin induces dendritic cell maturation and the production of Th1 cytokines. We demonstrated that Gal-lectin matured DCs can induce IL-12 production which directs a Th1 antigen-specific response, characterized by IFN-γ production. Based on the natural Th1 potential of the Gal-lectin, in the next study we evaluated the adjuvant potential of CpG-ODN to augment this IFN-γ mediated immune response. It is known that CpG-ONDs can amplify antigen-specific immune responses by stimulating innate immune cells to respond in a Th1manner.

CHAPTER 6: MANUSCRIPT II

CpG-oligodeoxynucleotide is a potent adjuvant with an *Entamoeba histolytica* Gal-lectin vaccine against amebic liver abscess in gerbils.

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Abstract

The protozoan parasite *Entamoeba histolytica* causes invasive amebiasis characterized by amebic dysentery and liver abscesses (ALA). The *E. histolytica* Gal-lectin, an immunogenic surface molecule involved in colonization and invasion, is a promising vaccine candidate against amebiasis. The Gal-lectin is known to induce Th1 cytokines in macrophages and spleen cells *in vitro* and a Th1 response is thought to be protective against ALA. In this study, we report the use of cytosine guanine oligodeoxynucleotide (CpG-ODN) as adjuvant to augment Th1 responses against Gal-lectin in the gerbil model of ALA. Gerbils were vaccinated i.m. with the native Gal-lectin + CpG-ODN or paired non-CpG control GpC-ODN and control gerbils received CpG-ODN alone. One week after the last boost gerbils were challenged intrahepatically with 10^6 amebae. Gerbils receiving CpG-ODN as adjuvant with Gal-lectin were completely protected against the development of ALA, whereas 50% of gerbils receiving GpC-ODN and Gal-lectin developed ALA and 85% of controls developed ALA. Stronger lymphoproliferation to the Gal-lectin and higher prechallenge titers of serum Gal-lectin specific antibodies (Abs), capable of blocking amebic adherence, were observed when CpG-ODN was used as adjuvant. Gerbils vaccinated with CpG-ODN and Gal-lectin also had significantly higher levels of IFN-γ, IL-12 and IL-2 mRNA than controls. These data indicate that CpG-ODN can enhance the Th1 responses, which improve the protective effects of the Gal-lectin. This is the first report of the use of CpG as potent Th1 adjuvant with the Gal-lectin to increase protection against ALA formation.

Introduction

Entamoeba histolytica, the etiological agent of amebic dysentery and ALA, leads to 100,000 deaths annually [1]. Chemotherapy is currently available, however, there remains a critical need for the development of alternate approaches to control this disease. Vaccination is a possible solution as epidemiological studies suggest the occurrence of acquired protective immunity to *E. histolytica* infections [2,3]. In contrast to other protozoan parasites with complex life cycles, *E. histolytica* cycles only between cyst and mobile trophozoite stages and humans are the only relevant hosts. These features of the parasite and the supporting epidemiological data make vaccine development an attractive therapeutic addition to chemotherapy.

One of the leading candidates for an anti-amebic vaccine is the galactose/N-acetyl-D-galactosamine inhibitable lectin (Gal-lectin). The Gal-lectin is a 260kDa heterodimer surface glycoprotein, which consists of a heavy and light subunit linked by disulfide bonds [4, 5]. The heavy subunit contains a cysteine rich carbohydrate recognition domain and monoclonal Abs directed against this region can inhibit amebic adherence to target cells. The Gal-lectin plays an important role in adherence to colonic mucins for colonization [6] and for contact-dependent killing of cells and resistance to complement attack. Apart from its biological importance, the Gal-lectin is an immunodominant and immunogenic molecule. The Gal-lectin induces the production of Th1 cytokines *in vitro* and *in vivo*, and cell-mediated immunity is important in controlling the formation of ALA in animal models. *In vitro* stimulation of macrophages with the Gal-lectin induces the production of TNF-α [7] and

splenocytes from ALA patients stimulated with Gal-lectin produce large amounts of IL-12 and IFN-γ [8]. Several vaccine trials in mice and gerbils using the Gal-lectin have shown the induction of protective immunity against ALA challenge [9-12]. In these trials, like most vaccine research with animals, the Gal-lectin was administered with Freund's adjuvant. An alternative to Freund's adjuvant is CpG-ODN, which can induce strong immune responses without the toxicity of conventional adjuvants.

Unmethylated CpG motifs are present in bacterial DNA and are recognized by the innate mammalian immune system via Toll-like receptor 9 [13]. This triggers an immune reaction characterized by Th1 cytokine expression and activation of immune cells, specifically dendritic cells and B cells [14-17]. This activation is non-specific but can be used to enhance immune responses to specific antigens. CpG–ODNs are Toll-like receptor 9 agonists, which are designed to mimic immunostimulatory sequences found in pathogens. These synthetic CpG motifs have been used as adjuvants in many systems either to enhance the immunogenicity of a vaccine or to skew the immune response from Th2 to Th1. In vaccine trials against bacterial infections, viral infections, and parasitic infections, CpG-ODNs have shown the ability to increase both innate immune responses and protective immunity [18-20]. Current clinical trials in humans testing CpG-7909 as a vaccine adjuvant are demonstrating that CpG-ODN can safely activate antigen specific immune responses [21]. The Gal-lectin has the innate ability to induce Th1 responses and in the present study we have examined the ability of CpG-ODN in combination with the Gal-lectin antigen to safely enhance protective immunity in gerbils infected with *E. histolytica.*

Materials and Methods

Native Gal-lectin

The native Gal-lectin was purified as described previously [22]. Briefly, log phase ameba (HM1:IMSS) grown in TYI-S-33 were chilled and centrifuged at 900 rpm for 5 min. The pellet was washed twice in wash buffer (75mM Tris, 65 mM NaCl, pH7.2) and then the final pellet was solubilized in solubilization buffer (150mM NaCl, 50 mM Tris pH8.3 and 0.5% Nonidet P-40) supplemented with a cocktail of protease inhibitors. The solubilized amebae were microcentrifuged at 10,000 x g at 4°C for 30 min. The supernatant was kept and run through an immuno-affinity column, generously provided by Dr. B. Mann (University of Virginia, Virginia, USA), consisting of protein-A purified anti-Gal-lectin monoclonal Abs (H85, 7F4, 5B8, 3F4 and 6D2) for 48 h at 4 °C with a peristaltic pump. The column was first washed with solubilization buffer, then with PBS and finally the Gal-lectin was eluted with elution buffer (4M $MgCl_2$, 10 mM Tris ph 7.2). The Gal-lectin was dialyzed against PBS and concentrated. Purified Gal-lectin did not contain detectable levels of endotoxin contamination as measured by E-TOXATE assay (Sigma).

ODNs

The ODNs used in this study were CpG-ODN 2006 (TCG TCG TTT TGT CGT TTT GTC GTT), and paired non-CpG control GpC-ODN 2137 (TGC TGC TTT TGT GCT TTT GTG CTT). These ODNs have nuclease-resistant phosphorothioate backbones and sequences known to be immunostimulatory in many species, including

humans. All ODNs were purchased from Coley Pharmaceutical Group (Kanata, Canada).

Vaccinations and challenge infections

Male gerbils (*Meriones unguiculatus*) 6-9 weeks old (Charles River, St.Constant, Canada) were injected i.m. in the hind legs with, either 50 μg CpG-ODN 2006 only, 50 μg CpG-ODN 2006 and 10 μg Gal-lectin or 50 μg GpC-ODN 2137 and 10 μg Gal-lectin in 100 μl PBS. Gerbils received identical booster injections at 14 and 28 days post initial injection. At day 35, gerbils were anesthetized and challenged via intrahepatic injection of 10^6 *E. histolytica* amebae (HM1:IMSS) into the left liver lobe as previously described [23]. Gerbils were sacrificed at the indicated days post challenge (2, 5, 15, 20) and their spleens and sera were collected. Livers were removed and ALA weight relative to total liver weight was measured. All protocols in this study were carried out with the approval of the McGill University Animal Care Committee.

Immunoblot

Sera from vaccinated gerbils were tested for the presence of anti-Gal-lectin Abs. Electrophoresis was performed on the native Gal-lectin in a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were probed with either a 1:1000 dilution of pooled sera from control or vaccinated gerbils or 1G7 anti-lectin monoclonal Ab. The blots were incubated with 1:10,000 HRP-conjugated anti-gerbil

IgG (Immunology Consultants Laboratory Inc. Oregon) or 1:3000 HRP-conjugated anti-mouse IgG (Amersham) and developed by ECL (Amersham).

Enzyme-linked immunosorbent Assay

A total serum IgG ELISA was performed on pooled sera from vaccinated or control gerbils. 100 ng of native Gal-lectin per well was coated on NUNC-Immuno Maxisorp 96 well plates (Falcon) in 50 µl carbonate buffer (pH 9.5). Plates were blocked overnight at 4°C in blocking buffer (PBS-1% BSA, 0.1% Tween 20), then washed three times in wash buffer (PBS-0.1% Tween 20). 100 µl of pooled sera (1:100) was added and serial diluted 1:1 in blocking buffer then incubated at 37 °C for 1 h. The plates were washed three times and incubated as above with 100 µl of HRP-conjugated anti-gerbil IgG Ab (1:10,000). After washing the plates, 100 µl of TMB (Sigma) HRP substrate was added to each well and the assay developed for 20 min. The reaction was stopped with 50 µl 2M H_2SO_4 and absorbance read at 450 nm in a Microplate Autoreader (Mandel Scientific Company Ltd/ Bio-tek Instruments).

Adherence Assay

The ability of serum Abs to inhibit amebic adherence to target cells was determined by a previously described adherence assay [24]. Briefly, log phase *E. histolytica* trophozoites were washed in M199 (Gibco) supplemented with 5.7mM cysteine, 0.5% BSA and 25mM HEPES (pH 6.8). 10^5 amebae/ml resuspended in M199 were preincubated for 1 h at 4 °C with a 1:100 serum dilution from: gerbils receiving CpG only, gerbils receiving CpG and Gal-lectin or GpC and Gal-lectin and human ALA

patient serum as positive control. During the preincubation, Chinese Hamster Ovary cells (CHO) grown in Ham's F-12 medium (Gibco) supplemented with 24mM HEPES, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, were trypsinized and resuspended at a cell density of 2.0 x 10^5/ml. Amebae (10^5) were mixed with 1 ml of CHO cell suspension and centrifuged at 900 x g for 5 min. The pellets were incubated at 4°C for 2 h undisturbed. 75% of the supernatant was decanted and the pellets resuspended by gentle vortexing. Amebic adherence was determined by light microscopy by counting the number of amebae with 3 or more CHO cells attached (positive rosette) out of 100 random amebae.

Immunofluorescence

Log phase amebae were washed and resuspended in M199 (supplemented as above) at 1.0 x 10^6 amebae/ml. Parasites were incubated for 1 h on ice with the following: M199 alone, 1G7 mAb (1:50), or prechallenge serum from vaccinated (CpG+Gal-lectin) and control (CpG or GpC+Gal-lectin) gerbils (1:100). Amebae were washed three times in fresh M199 at 150 x g and incubated with either rabbit anti-rat IgG FITC-conjugated Ab (Sigma) or goat anti-mouse IgG FITC-conjugated Ab (1:200) for 1 h on ice. After thorough washing, the amebae were pelleted at 150 x g and mounted onto glass slides with Vectashield® mounting medium. Slides were kept at 4°C until analysis through a Nikon Eclipse800 epi-fluorescence stereomicroscope. Images were collected with a 40x oil-immersion lens.

Lymphoproliferation assay

Single cell suspensions were prepared from the immunized and control gerbils' spleen by grinding the organ through a 40 μm cell strainer (Falcon) in RPMI-1640 (Gibco) supplemented with 10 mM HEPES, 50 μM 2-ME, 2mM glutamine, 5% FBS and 5mg/ml penicillin-streptomycin sulfate. Red blood cells were lysed in Red Blood Cell Lysing Buffer (Sigma) and remaining cells were suspended at 2.5 x 10^6 cells/ml in complete RPMI-1640. 100 μl of cell suspension was added to each well of a 96-well culture plate (Falcon) and equal volume of stimulus was added to the wells in triplicates. Stimuli for this experiment included: complete RPMI-1640 (no stimulus), Gal-lectin (10 μg/ml), soluble amebic proteins (SAP; 50 μg/ml) or ConA (2.5 μg/ml). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 60-72 h and 1 μCi [methyl-^{3}H] thymidine (Amersham) was added to each well in 25 μl volume RPMI-1640 for the last 18 h. Labeled cells were harvested onto glass fiber filters using an automated cell harvester (Filtermate Harvester, Perkin Elmer) and incorporated radioactivity was measured by scintillation counts (Trilux Counter LKB, Wallac: Pharmacia). Lymphoproliferation responses are expressed as a stimulation index; the ratio of cpm counts of cells receiving antigen over the cpm counts of cells without antigen.

Real-time PCR

Total RNA was isolated (RNeasy Mini Kit, Qiagen) from gerbil spleens or mesenteric lymph nodes (MLNs). During RNA purification, samples were treated with DNase (Qiagen) to remove residual genomic DNA contamination. 2.5 μg of total RNA from

each sample was reverse transcribed with Omniscript RT-Kit (Qiagen) using random hexamer primers. The resulting cDNA was subsequently diluted 1:100 and used for Real-time PCR with the Rotor-Gene 3000 system (Corbett Research). Amplification of cytokine cDNA was carried out with gerbil specific primers and Taqman probes generated by Applied Biosystems, as listed in Table 1. All amplifications were carried out with PE Biosystems Universal PCR Master Mix and the following cycling parameters: 50°C for 2 min, 95°C for 10 min, then 55 cycles of 95°C for 15 s and 60°C for 60 s. Quantitative analysis was conducted by the comparative Ct method normalized to 18s ribosomal RNA as internal control [25]. Cytokine expression levels are represented as fold increase over naïve gerbils receiving no treatment. All quantitative PCR analyses were carried out in triplicates.

Table 6.1. Gerbil specific primers and Taqman probes for Real-time PCR

Gene	Sense (5′-3′) Antisense (5′-3′)	Probe (5′-3′)[a]
18srRNA	GGCTTAATTTGACAACACGGGAAAC CACGGAATCGAGAAAGAGCTATCAA	CTCACCCCGGCCCGGACA
IL-12p40	GCTGGTCAATATACCTGCCACAAA GGAGCAGCAGACGGAAGTG	CTGAGGGTCTGGTCTCC
IL-4	ATAGCAACGAAGAACACCACAGA GCGGAGCACCCTGGTA	TCTGCAGAGGAGTCCCTT
IL-2	GCTCCTGAGAGGGATCAACAATTAC GCCTTCCTCGGCATGTAAAATTTAA	AAACTCCCCATGCTGCTCA
IFN-γ	CAGAGCAAAGCTATCAATGAACTTGT CGACTCCTTTTCCGCTTCCTTAG	CTGTCACCCAGAGTCACC

[a] Probes for Taqman Real Time PCR were labeled with 6-FAM at the 5′ terminus and a non fluorescent quencher at the 3′ terminus.

Statistical method

All animal experiments were repeated at least twice with similar results. Results are expressed as mean ± SEM of triplicate experiments. Data was analyzed using one – way ANOVA or paired-sample *t* tests. Significance was set at $P< 0.05$ for all tests.

Results

CpG-ODN increases Gal-lectin specific serum Ab levels

Since CpG-ODN 2006 is known to have a direct effect on B cells, the level of Gal-lectin specific serum Abs was determined. Pre-challenge serum from gerbils immunized with CpG only had no detectable levels of Gal-lectin Abs, while both groups receiving Gal-lectin in the vaccine had detectable Abs as seen in an immunoblot against the native protein (Fig.6.1A). CpG-ODN significantly enhanced the Ab response to the Gal-lectin compared to GpC-ODN control. In a total serum IgG ELISA, gerbils vaccinated with CpG+Gal-lectin had higher Gal-lectin specific Ab titers ($p=0.004$) than gerbils receiving control GpC+Gal-lectin (Fig.6.1B). Ab levels in the CpG+Gal-lectin group were high and titrated out at a 1:200,000 dilution. These Gal-lectin specific Ab levels remained high in the groups receiving Gal-lectin even after challenge infection, whereas the Ab levels in the CpG control group showed an increase at 2 days post infection (p.i.) and then a marked decrease at 5 days p.i followed by a slow increase in Ab titers with time (Fig.6.1C). In an *in vitro* model of the disease, serum from ALA in humans or monoclonal Abs against the Gal-lectin inhibited amebic adherence. To determine if the prechallenge anti-Gal-

lectin Abs were capable of blocking amebic adherence to target cells, we performed a CHO cell adherence assay with all sera at 1:100 dilution. As shown in figure 6.2A, immunization with CpG+Gal-lectin inhibited amebic adherence by 92%±3, which was comparable to the inhibition seen with serum derived from patients with ALA. While CpG controls did not significantly inhibit adherence, GpC+Gal-lectin group inhibited amebic adherence by 64%.

As predicted by the adherence assay, we found that CpG+Gal-lectin immunization generated immune sera, which had higher titers of adherence inhibiting and surface binding antibodies than GpC+Gal-lectin immunization (Fig.6.2B). Immunofluorescence analysis using prechallenge immune or control gerbil serum revealed that the combination of CpG-ODN with the Gal-lectin, and not either component alone, resulted in strong positive Ab binding to the Gal-lectin on the amebic surface. The amebae alone and CpG only sera had no detectable fluorescence signal. The group receiving GpC+Gal-lectin had weak fluorescence, whereas the CpG+Gal-lectin sera had strong fluorescence comparable to that of the positive control mAb 1G7.

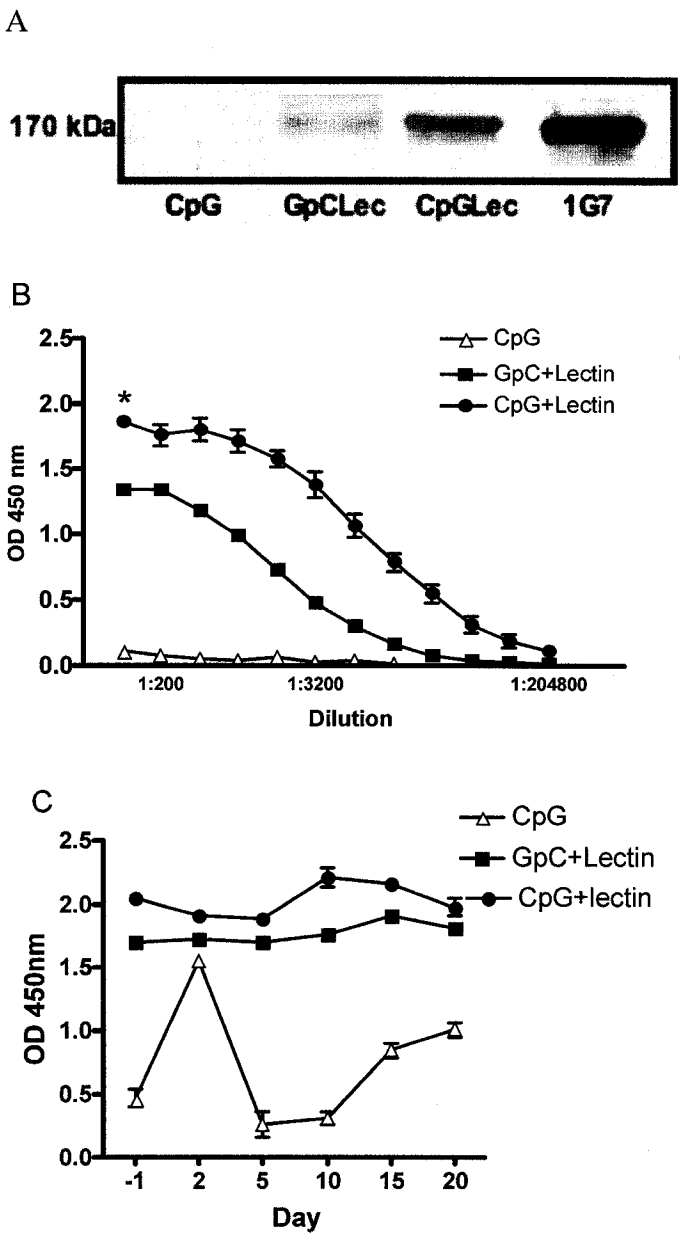

Figure 6.1. Gal-lectin speficic antibodies. (A) Immunoblot with immune or control gerbil serum (1:1000) against purified Gal-lectin. Gal-lectin specific serum IgG levels from immunized gerbils recognize the 170kDa band. Data shown are representative of three independent experiments. (B) Gal-lectin specific serum IgG levels from immunized gerbils (n=10 per group). ELISAs were performed on prechallenge serum at a starting dilution of 1:100. Points represent total IgG titers ± SEM of data from four experiments. An asterisk indicates a significant difference between groups at the starting dilution. (C) ELISA OD results from gerbil serum IgG (1:100) collected on day 2,5,10, and 15 post challenge (n=3 per group per time point) and treated as above. A significant difference in day2 Ab titers within the CpG group compared to prechallenge titers ($P<0.05$).

CpG-ODN increases Gal-lectin specific cellular responses

To determine if vaccination with CpG+Gal-lectin could increase Gal-lectin specific cellular responses, we measured spleen cell proliferation upon stimulation with the native antigen and soluble amebic proteins (SAP). As expected, gerbils vaccinated with CpG+Gal-lectin demonstrated strong proliferation to both the native Gal-lectin and SAP (Fig.6.3). This proliferation was significantly stronger ($p=0.03$) than gerbils receiving GpC+lectin, however, this last group still responded to the Gal-lectin to a greater degree than the control CpG alone. All groups exhibited strong proliferation to the T-cell mitogen ConA, demonstrating comparable T-cell viability. The higher proliferative response observed with SAP was probably a result of the higher concentration of Gal-lectin in the preparation compared to the known amount plated in the Gal-lectin wells. This experiment indicates that CpG-ODN can augment Gal-lectin specific cellular immune responses *in vitro*.

CpG-ODN adjuvant increases the protective effects of Gal-lectin

In this vaccination study our strategy was to use immunostimulatory CpG-ODN or control GpC-ODN as adjuvant with a known protective antigen, the *E. histolytica* Gal-lectin. One week after the last immunization, gerbils were anesthetized and intrahepatically challenged with 10^6 *E. histolytica* trophozoites and animals sacrificed at days 2,5,15, or 20 p.i. As shown in Table 6.2, CpG+Gal-lectin treatment protected (100%) gerbils from ALA formation, as determined by necropsy at day 20 p.i. The Gal-lectin with GpC-ODN had a vaccine efficacy of 50%, whereas 85% of CpG treated gerbils developed ALA.

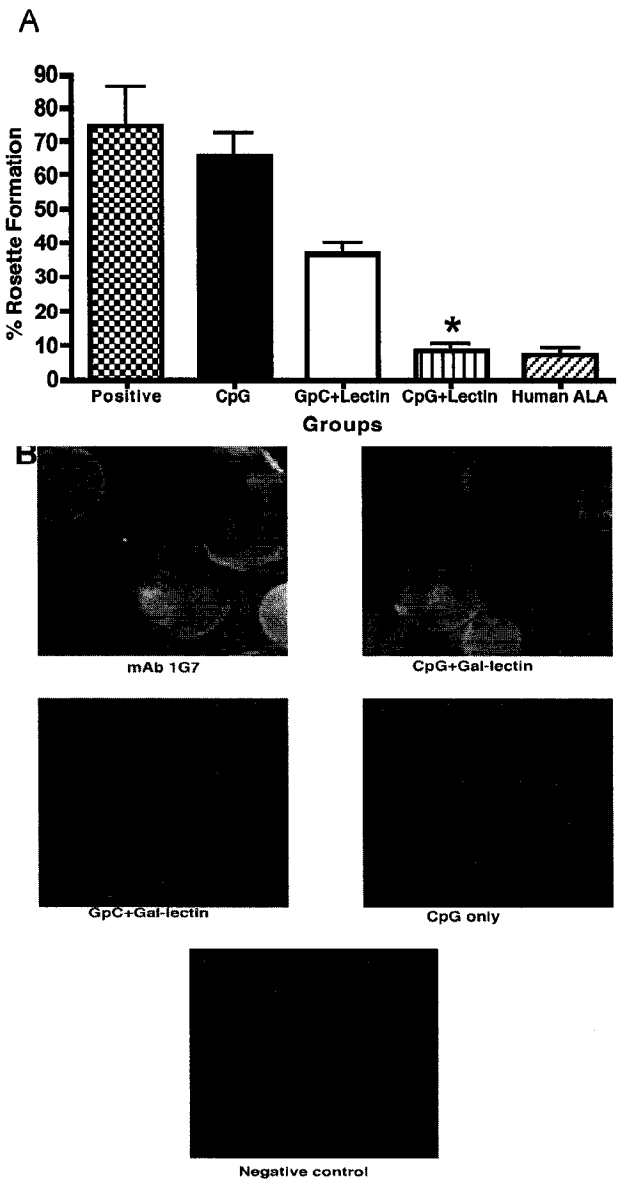

Figure 6.2. Inhibition of amebic adherence to target CHO cells by immune gerbil serum. (A) Amebae were preincubated with 1:100 immune or control serum dilutions and subsequent amebic adherence to CHO cells was determined by adherence assay. CpG+Gal-lectin immune serum significantly inhibited amebic adherence. An asterisk indicates a significant decrease in amebic adherence compared to CpG or GpC+Gal-lectin immunization ($P<0.05$). Data shown are from triplicates of three independent experiments. Bars represent the percent amebic adherence to target cells. (B) Immunofluorescence analysis of anti-Gal-lectin serum Abs on amebic surface. Amebae were incubated with immune or control gerbil sera (1:100), monoclonal Ab 1G7 (mAb) or with secondary FITC-conjugated Ab only (negative control). Images were collected at 40x oil immersion.

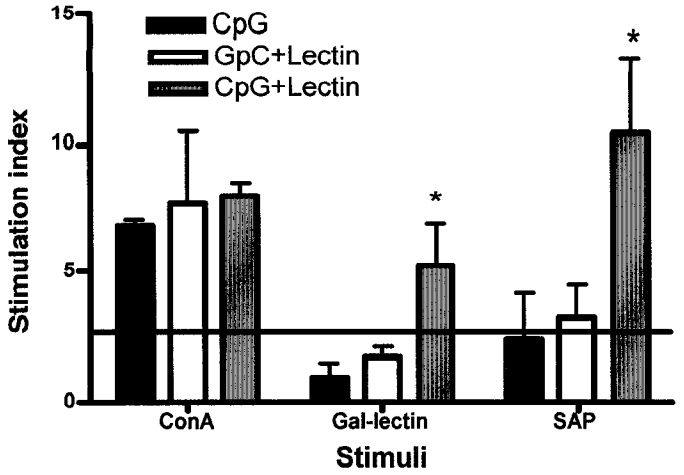

Figure 6.3. Lymphoproliferation of gerbil splenocytes in response to Gal-lectin stimulation. Immunized or control gerbil spleens (n=11 per group) were collected prechallenge infection and cells were restimulated *in vitro* for 72 h with either ConA (2.5μg), Gal-lectin (10μg) or 50 μg of soluble amebic proteins (SAP). Proliferation is represented as a stimulation index (cpms of cells with Ag/cpms of cells without Ag). A stimulation index above 2.5 is considered significant as indicated by an asterisk.

Amebic liver abscess was detectable in all groups as early as 2 days p.i. At this early time point the ALA were already smaller in the CpG+Gal-lectin group compared to controls. Figure 6.4A demonstrates the progressive development of ALA at various times after challenge infection. As shown, gerbils receiving CpG+Gal-lectin were able to clear the infection as early as 5 days p.i. This clearance continued until the abscesses were no longer detected or if detected, there were no viable trophozoites. In contrast, the CpG treated group developed larger abscesses with time while the group treated with GpC+Gal-lectin demonstrated a slight decrease in ALA size over time (Fig.6.4A). Intrahepatic challenge of trophozoites into gerbils treated with CpG resulted in the formation of very large abscesses, sometimes reaching up to 13g (Fig.6.4B). ALA size relative to total liver weight in this CpG control group was significantly larger (60% vs 20%, p=0.04) than that observed in PBS-treated controls

(data not shown). The abscesses in this group were also larger than those excised from the GpC+Gal-lectin group, indicating a CpG effect rather than an ODN effect.

Table 6.2. Prevention of ALA in gerbils by vaccination

Immunization group	ALA gerbils/number gerbils			Viable parasites[a]	% Efficacy[b]
	Exp 1	Exp2	Exp3	±	
CpG	6/8	9/9	9/11	+	N/D
GpC+Gal-lectin	4/6	4/10	5/11	+	50
CpG+Gal-lectin	0/11	0/11	0/11	-	100*

[a] ALA contents were aspirated and viable trophozoites determined by cultured *in vitro*.
[b] All gerbils were sacrificed at day 20 post infection to determine vaccine efficacy.
N/D not determined
* An asterisk indicates a significant difference between groups ($P<0.05$)

Protection against ALA is associated with increased production of Th1 cytokine mRNA. To analyze the spleen and MLN cytokine profile at different times post infection, real-time Taqman PCR analysis was performed. Cytokine mRNA levels were quantified from necropsied gerbils and compared to mRNA levels from naïve untreated, uninfected gerbils. Prechallenge (Day-1) spleen samples from the CpG+Gal-lectin group showed elevated levels of IFN-γ (4500-fold), whereas the CpG control group showed elevated levels (724-fold) of IL-12p40 (Fig.6.5A). The GpC+Gal-lectin treated group did not express elevated levels of any cytokine examined compared to the other groups at the prechallenge time point. At 2 days p.i., cytokine mRNA levels of all Th1 cytokines (IFN-γ, IL-12p40, and IL-2) were low in all groups.

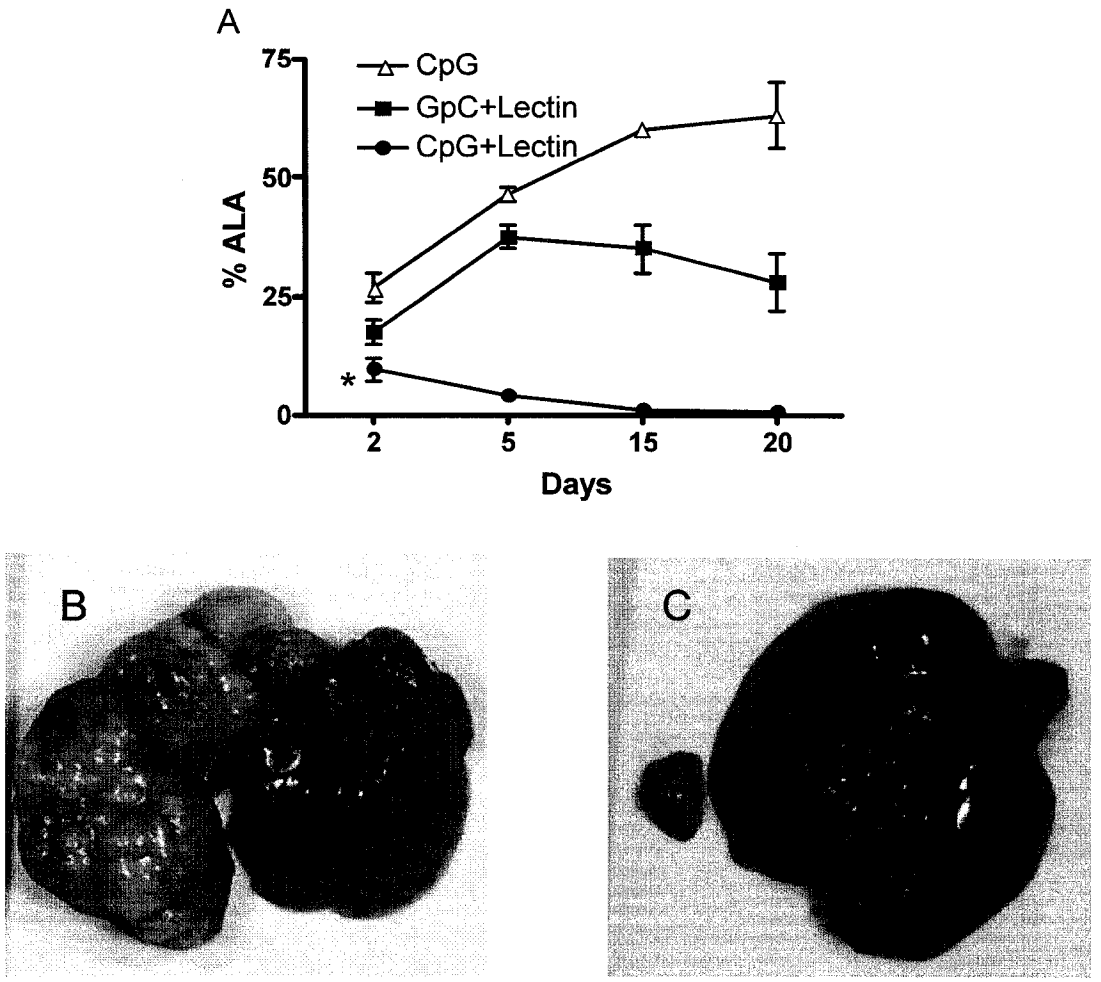

Figure 6.4. Progression of ALA formation. (A) Gerbils were sacrificed (n=3 per group per time point) at days 2, 5, 15, and 20 post challenge and their livers were examined for ALA formation. Abscess size is represented as precent of total liver weight. Asterisk indicates significantly smaller ALA in CpG+Gal-lectin group compared to the other groups at all time points ($P<0.05$). Liver pathology of CpG treated (B) and CpG+Gal-lectin treated (C) gerbils 20 days after intrahepatic challenge infection with *E. histolytica* trophozoites. Control CpG gerbils developed large multilobed abcesses whereas CpG+Gal-lectin immunized gerbils showed small abscesses or none at all. ALA pictures are representative of all challenge infection experiments.

However, at this early time point, protected gerbils still had significantly higher levels of IFN-γ, IL-12p40, and IL-2; 295-, 32- and 2.1-fold increase respectively (p=0.005). The CpG treated group showed 9000-fold increase in IL-4 mRNA at day 2 post challenge. The level of IL-4 in CpG treated gerbils remained elevated during the course of the infection, while the level of IL-2 markedly decreased (-300-fold, p=0.0014). MLN samples from the CpG+Gal-lectin treated gerbils also showed increases in Th1 cytokine mRNA compared to controls, however the expression levels and kinetics were different (Fig.6.5B).

In the CpG treated gerbils, the MLN samples again showed highest increase in IL-4 mRNA, but also in IL-2 at day 2 post challenge. However, this IL-2 expression decreased throughout the course of the infection while IL-4 levels remained elevated. The GpC+Gal-lectin control, unlike the CpG+Gal-lectin group, did not express high IFN-γ cytokine mRNA levels either in the spleen or MLN. Comparing abscessed and non-abscessed gerbils within experimental group at day 20 demonstrated that IL-4 was more elevated in abscessed animals within each group compared to protected gerbils (32-fold increase, data not shown). At this time point IFN-γ was also more elevated in abscessed gerbils (10-fold increase) as they had an active infection (data not shown).

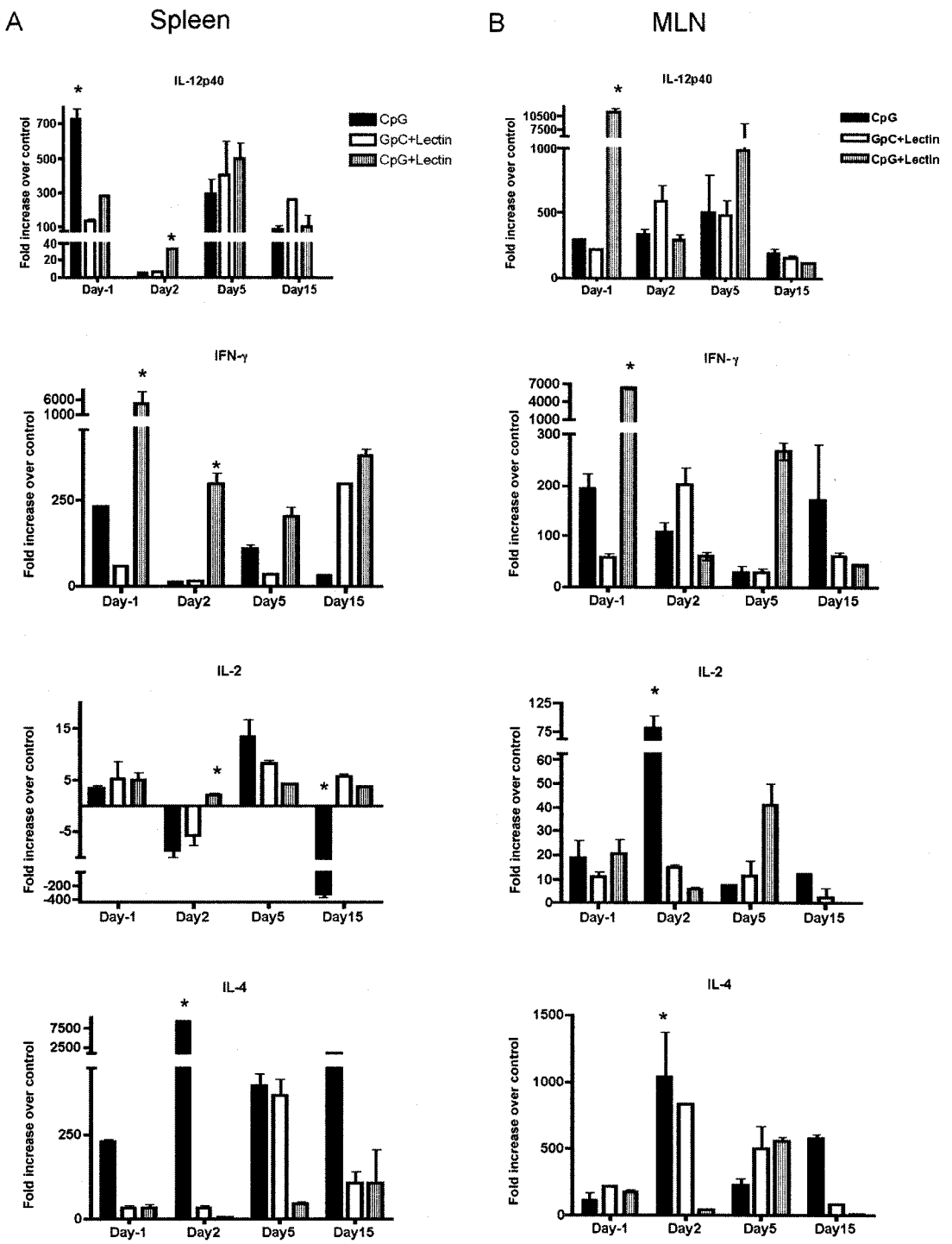

Figure 6.5. Taqman Real-time PCR analysis of spleen (A) and mesenteric lymph node (B) cytokine gene expression (n=3 per group per time point). Gene expression was normalized to 18srRNA and represented as fold increase over normal non-treated gerbil mRNA. An asterisk represents a significantly higher fold increase in the CpG+Gal-lectin group compare to other groups ($P<0.05$). Data points represent means ± SEM of results from three independent PCR reactions.

Discussion

Effective vaccine therapy requires the identification of a protective antigen and a potent vaccine adjuvant. For several reasons, the *E. histolytica* Gal-lectin is an attractive vaccine candidate as it is an important molecule involved in parasite colonization and invasion, but also an immunodominant antigen recognized by sera from amebiasis patients throughout the world [26]. CpG-ODN is a novel adjuvant, which has been shown to activate innate immune cells and enhance the vaccine efficacy of protective antigens [27,28]. In this study, we have shown that CpG-ODN adjuvant with the native Gal-lectin enhances IFN-γ production and protects gerbils from ALA. The enhanced effect can be attributed to the combination of the protective antigen and this potent Th1 adjuvant as controls with either component alone did not show comparable levels of protection.

The enhanced immune responses to the Gal-lectin with CpG-ODN adjuvant was characterized by increased IgG Ab production, T cell proliferation and more importantly production of Th1 cytokines. As a component of our vaccination studies, we examined prechallenge Ab titers and their ability to inhibit amebic functions mediated by the Gal-lectin. We used an *in vitro* adherence assay to determine the blocking effects of serum Abs. This assay revealed the ability of CpG+Gal-lectin immunization to increase the production of Abs capable of blocking the carbohydrate recognition domain on the Gal-lectin molecule, required for amebic adherence to Gal/GalNAc residues on mucins or target cells. The immunofluorescence images confirmed the observed adherence inhibition, revealing serum Abs strongly binding

the Gal-lectin on the surface of the parasite only in the group receiving adjuvant and amebic antigen. Although Abs could clearly play a role in preventing amebic adherence for colonization or cell killing, it is apparent that other immune mechanisms are involved in protection against disease. In fact, post challenge Ab titers were not significantly different between both groups that had received Gal-lectin, yet the two groups were not equally protected. The highest level of protection was demonstrated in the CpG+Gal-lectin group, which had significantly higher IL-12, IL-2 and INF-γ mRNA levels 2 days post challenge than control groups. The elevated levels of IFN-γ mRNA detected in the protected group, both prechallenge and at day 2, demonstrate the importance of this cytokine in parasite clearance. The results from gerbils sacrificed at day 20 demonstrated that IL-4 and IFN-γ were more elevated in abscessed animals within each group compared to protected gerbils (data not shown). We do not have abscessed versus non-abscessed mRNA at day 2 post infection, which would have determined the protective effects of IFN-γ within groups. Therefore we are correlating IFN-γ with protection between groups at this point and not within groups. In a previous study with the SCID mouse model of amebiasis, where the IFN-γ receptor gene was disrupted, mice had increased susceptibility to ALA formation than control SCID mice and developed significantly larger abscesses [29]. IFN-γ primed macrophages readily kill amebic trophozoites *in vitro* [30] and cell mediated immune responses have been observed in patients with ALA. In fact, humans with ALA demonstrate amebicidal IFN-γ production [8], however correlations of cell-mediated responses with immunity in humans are limited. It has been demonstrated *in vitro* that the Gal-lectin activates IFN-γ primed macrophages

for amebicidal activity via nitric oxide [7]. This report indicates that CpG-ODN in combination with the Gal-lectin safely confers protection mediated by IFN-γ, which is consistent with previous studies.

The kinetics of cytokine induction in the abscessed gerbils was marked by elevated levels of IL-4, which down-regulate the protective Th1 effects on macrophages. This reduction in macrophage activation could lead to increased parasite numbers and disease progression. The CpG control group also showed greatly reduced IL-2 mRNA levels at day 15- 20 post infection, which is considered peak infection period in the gerbil model. This corresponds to previous reports that *E. histolytica* infected gerbil serum induces a transient T cell suppression by inhibiting IL-2 production [31]. Furthermore, CpG treated gerbils developed larger abscesses than those excised from PBS treated or GpC+Gal-lectin treated gerbils (data not shown). This exacerbation of ALA could be due in part to the innate ability of CpG-ODN to activate innate immune cells, and induce cytokine secretion, which could recruit immune cells, which the amebae subsequently kill. Furthermore, in the absence of a Gal-lectin specific immune response, the amebae are able to proliferate and ALA formation goes uncontrolled. Although a previous report indicated that CpG-ODN administration can protect from bacterial challenge infections (see e.g. reference 19), this adjuvant alone could not protect gerbils from ALA and in fact lead to disease exacerbation. Unmethylated CpG DNA, which is present in bacterial and viral genomes is recognized by innate immune cells via Toll-like receptor 9. There have been reports that CpG-ODN administration alone can protect mice from subsequent

lethal challenge with bacteria sharing similar unmethylated CpG DNA patterns [32-34]. This is because the immune system recognizes these CpG motifs as foreign DNA and initiates innate responses. CpG suppression is common in vertebrate genomes, however, *E. histolytica* is a protozoan parasite with a significant underrepresentation of CpG in its genome compared to other protists [35]. In fact an *E. histolytica* 5-cytosine DNA methyltransferase has been identified and inhibition of its activity has been show to impair parasite virulence *in vivo* [36]. Therefore administration of CpG-ODN alone could not confer protection, as it does not sensitize the host to *E. histolytica* DNA. This underlines the importance of the Gal-lectin as a protective antigen in directing the proper T helper response to combat this parasitic attack.

Previous vaccination studies with the Gal-lectin have used Freund's adjuvant (complete or incomplete) and shown high protection levels characterized by cell-mediated responses against the parasite [9,10]. Freund's adjuvant cannot be used in humans due to its toxicity [37] and therefore it is important to devise alternative vaccine formulations for potential use in humans. Several groups have tested CpG-ODN as vaccine adjuvant against parasitic infections, like leishmaniasis, and shown promising results in animal models [38-40]. The degree of protection depends on the immunogenicity of the selected vaccine antigen, but all trials demonstrated the induction of Th1 responses. Currently in clinical trials is CpG 7909 "Promune" as vaccine adjuvant [21]. Theses ODNs have been shown to be safe in humans even at high doses, although adjuvant properties are observed at μg/kg doses [41]. In the

present study we tested an adjuvant, which could be administered to humans, to augment the protective effects of an immunogenic *E. histolytica* protein. We have demonstrated that CpG-ODN can enhance the protective immune responses to the Gal-lectin and prevent ALA formation in the gerbil model. The major drawback in amebiasis research is the lack of an adequate animal model for the disease. No animal has been able to reproduce the typical lesions of intestinal amebiasis. The Mongolian gerbil is the best model thus far as it can be used to mimic both intestinal and hepatic amebiasis.

E. histolytica is an intestinal parasite, and the development of a mucosal vaccine could preclude parasite colonization and invasion. Protection against parasite colonization in humans has been correlated to mucosal anti-lectin IgA antibodies [2]. Other groups have successfully developed mucosal Gal-lectin vaccines using cholera toxin B adjuvant or attenuated *Salmonella* strains for antigen delivery in the mouse model of intestinal amebiasis [42-44]. CpG-ODN has also proven to be effective as mucosal vaccine adjuvant, increasing both systemic and mucosal responses to the selected antigens [45-47]. Taken together, it would be possible to design a Gal-lectin mucosal vaccine using CpG-ODN as adjuvant, to induce both mucosal and systemic Th1 responses capable to blocking parasite colonization, thus preventing intestinal amebiasis in mice. Development of a vaccine using recombinant portions of the Gal-lectin and CpG-ODN could augment the efficacy of protective regions on the Gal-lectin. This would be more practical since it would not require the purified antigen from parasite cultures. Future work should test the mucosal vaccine efficacy of CpG-

ODN with the Gal-lectin, providing insight on the possibility of this agent as a choice for human adjuvant in an amebiasis vaccine.

Acknowledgments

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CONNECTING STATEMENT II

Our vaccination with CpG-ODN and the Gal-lectin in the second manuscript conferred protective immunity against amebic liver abscess challenge infection. This protection was correlated with elevated levels of IFN-γ and other Th1 cytokines at the time at which infection is established. The CpG-ODN adjuvant proved to be efficacious and safe when administered parenterally to gerbils. However, *E. histolytica* is an intestinal parasite and vaccination efforts are being directed toward the induction of protective mucosal responses. Thus, in the next study we addressed the ability of CpG-ODN to enhance mucosal responses to the Gal-lectin.

CHAPTER 7: MANUSCRIPT III

Intranasal immunization with Gal-lectin adjuvated with CpG oligodeoxynucleotides protects against *Entamoeba histolytica* challenge

Catherine P.A. Ivory and Kris Chadee

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Abstract

The development of an effective amebiasis vaccine could improve child health in the developing world, reducing cases of amebic colitis and liver abscess. An ideal vaccine would be comprised of a well-characterized parasite antigen and an adjuvant, which would have high potency while driving the immune response in a Th1 direction. This study describes a mucosal vaccine composed of the *Entamoeba histolytica* Gal-lectin and CpG-ODN. The Gal-lectin is a protein involved in parasite virulence and adherence and is known to activate immune cells, while CpG-ODN are known to be potent inducers of type-1 like immune responses. We demonstrate that the intranasal administration of the vaccine results in strong Gal-lectin specific Th1 responses and humoral responses. Vaccination induced the production of Gal-lectin specific T cells and the production of the pro-inflammatory cytokine IFN-γ. Vaccinated animals had detectable serum anti-Gal-lectin IgG and stool anti-Gal-lectin IgA capable of blocking parasite adherence to target cells *in vitro*. One week after immunization, gerbils were challenged intrahepatically with live trophozoites. Vaccinated gerbils had no detectable abscesses after day five, whereas control gerbil developed larger abscesses. These results show that mucosal vaccination with Gal-lectin and CpG-ODN can induce both systemic and humoral immune responses.

Introduction

Entamoeba histolytica is the enteric protozoan parasite, which causes amebic colitis and liver abscess in humans. The parasite is estimated to infect 10% of the world's population, leading to 100,000 annual deaths [1]. Amebic colitis is the most common clinical manifestation of disease as the parasite must first colonizes the colon. Intestinal infection by *Entamoeba histolytica* and progression to disease could theoretically be prevented by a vaccine intervention targeted to block parasite colonization [2,3].

E. histolytica trophozoites are able to colonize human intestine by adhering to colonic mucins and subsequently to epithelial cells via a cell surface lectin [4]. This Gal-lectin is a heterodimer containing disulfide linked light and heavy subunits, of which the latter has high binding specificity to galactose and *N*-acetyl-D-galactosamine (Gal/GalNac) [5]. The Gal-lectin is a prime vaccine candidate as it is an immunogenic molecule able to induce protection against amebic liver abscess in rodent models of the disease [6-8]. Recently it as has also been reported that there is a correlation between the presence of anti-lectin stool IgA antibodies and protection from parasite colonization in humans [9,10]. Mucosal immunity against the parasite seems to be required to prevent infection and there is substantial evidence to suggest that a colonization blocking vaccine targeting the parasite Gal-lectin could prevent trophozoite adherence and thus provide protection against subsequent invasive disease.

The development of mucosal vaccines for use in humans has been hindered by the lack of safe yet effective mucosal adjuvants. The gold standard mucosal adjuvants in animals are bacterial toxins, such as cholera toxin, however these are too toxic for use in humans. A novel class of adjuvants is CpG-ODN, synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs. These motifs are recognized by the innate immune system via TLR9, and can induce broad adjuvant effects such as the direct activation of B cells, macrophages and dendritic cells as well as IL-6 and IL-12 cytokine secretion [11-13]. CpG-ODNs contain a nuclease resistant phosphorothioate backbone, which can be co-administered with the vaccine antigen to induce specific immunity. It has been demonstrated that CpG-ODN are safe and effective adjuvants with both parenteral and mucosal vaccine administration [14-16]. Recent studies report the ability of CpG-ODNs to induce both systemic and humoral immunity upon mucosal application [16]. CpG motifs have Th1 biased immune effects due to TLR9 signaling, which can be used to augment cell-mediated immunity.

Here we evaluated CpG-ODN with intranasal delivery of purified *Entamoeba histolytica* Gal-letcin. We utilized the gerbil model of amebic liver abscess and the C3H mouse model for amebic colitis. We show that vaccination induced of both systemic and mucosal immunity against the Gal-lectin and prevented disease in the ALA model.

Materials and Methods

Animals

Male Mongolian gerbils (*Meriones unguiculatus*) 6-9 weeks old were purchased from Charles River (St.Constant, Canada). Female C3H/Hej mice 3-5 weeks old were purchased from the Jackson Laboratory (Bar Harbour, ME, USA). Animals were maintained in pathogen-free facilities at the University of Calgary. The University of Calgary Animal Care Committee approved all animal protocols for this study.

Parasites and vaccine antigens

Entamoeba histolytica strain HM1: IMSS trophozoites were grown in axenic culture in TYI-S-33 medium. Parasites were grown for 72 hours (log-phase) for use in all experiments. The native Gal-lectin was purified from log-phase amoebae on an immunoaffinity column as previously described [17]. CpG-ODN 10103 (TCG TCG TTT CGT CGT TTT GTC GTT) with a full phosphorothioate backbone was purchased from Coley Pharmaceutical Group (Kanata, Canada).

Vaccinations and challenge infections

Gerbils and C3H mice were immunized with an intranasal and intraperitoneal regimen over a 5-week period. Intranasal immunizations were given on weeks 1,3, and 5 to animals under light isoflurane anesthesia. The vaccine consisted of 10 μg of Gal-lectin and 20 μg of CpG-ODN administered to the nares in 20 μl of PBS. Intraperitoneal injections of 10 μg of Gal-lectin and 50 μg of CpG-ODN in 200 μl of PBS were delivered along with the intranasal immunization on week 5. Control

animals received intranasal delivery of 20 μg CpG-ODN in PBS only and intraperitoneal delivery of 50 μg CpG-ODN in PBS. One week following the last immunization, gerbils were anesthetized with isoflurane and challenged via intrahepatic injection of 10^6 *E.histolytica* trophozoites into the left liver lobe as previously described [18]. Gerbils were sacrificed post-challenge (day 2,5,10 and 15) and their spleens and sera were collected. Livers were excised and ALA weight was measured. Pre-challenge samples of serum, spleen, MLN were taken from gerbils and C3H mice.

Immunoblotting

Sera from vaccinated or control animals were tested for anti-Gal-lectin specific IgG. The native Gal-lectin was run on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were probed with either 1:250 pooled sera from vaccinated or control animals, or with 1G7 anti-Gal-lectin monoclonal antibody. The blots were washed with T-TBS and incubated with 1:10,000 anti-gerbil HRP-conjugated antibody (Immunology Consultants Laboratory Inc.) or with 1:3000 anti-mouse IgG (Amersham). Bound serum IgG antibodies were visualized using enhanced chemiluminescence (ECL Plus Reagent, Amersham). Stool antigens and antibodies were isolated from fresh pellets dissolved in PBS with protease inhibitors (Roche) at 1ml per 0.1 g pellet. Lectin specific IgA antibodies in the stool were detected by immunoblot against the native Gal-lectin with goat anti-mouse IgA antibodies (Sigma).

CHO cell adherence assay

As previously described [19], serum and stool antibodies against the Gal-lectin were used in an adherence-blocking assay. Briefly, log-phased amebae were pre-incubated with 1:100 dilution of pre-challenge sera or stool lysate for 1 hour at 4 °C. Chinese hamster ovary cells (CHO) were added to the amoebae, the tubes were centrifuged and incubated on ice for 2 hours undisturbed. Amoebic adherence was determined by light microscopy by identifying positive rosette formation (amoeba with 3 or more CHO cells attached). Percent rosette formation is indicated and is relative to the positive control for adherence consisting of amoebae and CHO without inhibitory serum.

Intracellular cytokine staining

Mesenteric lymph nodes cells were harvested from control and vaccinated C3H mice 5 days after the last immunization. Cells were cultured at 2 x 10^6 cells/ml and stimulated with 10 μg/ml Gal-lectin, 1 μg/ml ionomycin and 20 μg/ml brefeldin A (Sigma) for 6 hours. Cells were washed in PBS and resuspended at 1 x 10^6 cells/ml in PBS and stained with PE-conjugated anti-CD4 antibody (BD Pharmingen) for 30 min at 4 °C. Cells were then washed 3 times and fixed using Fix & Perm Cell Permeabilization Kit (Caltag Laboratories) and stained with either FITC-conjugated anti-mouse IL-4 mAb or anti-mouse IFN-γ mAb for 30 min at 4 °C. Cells were washed and analyzed by FACScan cytometry and Cell Quest software.

Real-Time PCR

Mesenteric lymph node cells were harvested from immunized or control animals and pelleted at 12,000-x g for 5 minutes at 4 °C. The supernatants were decanted and the pellets vortexed to resuspend the cells. mRNA was extracted with Trizol® (Invitrogen) as per manufacturer's instructions. cDNA was generated from 2.5 μg mRNA using random hexamer primers and M-MLV reverse transcriptase (Invitrogen). Gerbil cytokines were analyzed with Taqman probes as previously described [20], and mouse cytokines were measured using SyBr Green reagent and mouse specific primers [21]. Gerbil cytokines expression was normalized to 18srRNA and mouse cytokines were normalized to GAPDH. All real-time PCR experiments were performed using the Rotor Gene 3000 (Corbett Research) and results were analyzed using the $2^{\Delta\Delta}$CT method [22].

Lymphoproliferation Assay

Mesenteric lymph node cells were harvested and grown at 5×10^5 cell/well in a 96 well plate. Cells were stimulated with either RPMI medium alone, concanavalin A (2.5 μg/ml), Gal-lectin (10 μg/ml) or amebic proteins (20 μg/ml) for 72 hours at 37 °C. To measure proliferation [^{3}H]methyl-thymidine (1 μCi/well) was added for the last 18 hours of incubation. Cells were harvested onto filter paper and [^{3}H]methyl-thymidine incorporation was measured by scintillation counts. Lymphoproliferation is represented as a stimulation index, indicating a fold-increase in proliferation over the proliferation of cells receiving medium alone.

Statistical analysis

All animal experiments were repeated at least twice with similar results. Results are expressed as mean ± SEM of triplicate experiments. Data was analyzed using one – way ANOVA or paired-sample *t* tests. Significance was set at $P< 0.05$ for all tests.

Results

Intranasal vaccination with CpG-ODN and Gal-lectin generates adherence-blocking antibodies

Pre-challenge serum was collected from immunized and control gerbils and C3H mice and tested for the presence of Gal-lectin specific antibodies. Native Gal-lectin was run on 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with serum (1:250) in a western blot. Blots with serum from gerbils and mice immunized with CpG-ODN and Gal-lectin demonstrated a strong band at 170 kDa corresponding to the Gal-lectin heavy sub-unit (Fig.7.1A). This band was also present in the blot with a monoclonal antibody (1G7) against the Gal-lectin, whereas serum from CpG control animals did not recognize the Gal-lectin. In order to test the ability of serum antibodies to block Gal-lectin mediated parasite adherence, we performed a CHO adherence assay. Anti-gal-lectin antibodies in the serum of vaccinated gerbils and C3H mice were capable of significantly ($*P<0.05$) inhibiting parasite adherence by 80 % and 75% respectively (Fig.7.2A). An adherence assay was also performed with stool antibodies and similar inhibition was observed. CpG+Gal-lectin vaccinated gerbils and C3H mice had stool antibodies titers capable

of blocking parasite adherence by 75 % and 65%. (Fig.7.2B). Stool and serum preparations from PBS vaccinated and CpG control animals did not significantly inhibit parasite adherence. To verify the presence of anti-Gal-lectin IgA antibodies in the stool preparations the native Gal-lectin protein was probed in a western with stool preparation (1:250) and detected with anti-mouse IgA antibody. A strong band at 170 kDa appeared in the blots for vaccinated animals but not for control animals (Fig.7.1B). These results suggested that vaccination induced the production of both Gal-lectin specific serum IgG and mucosal IgA antibodies.

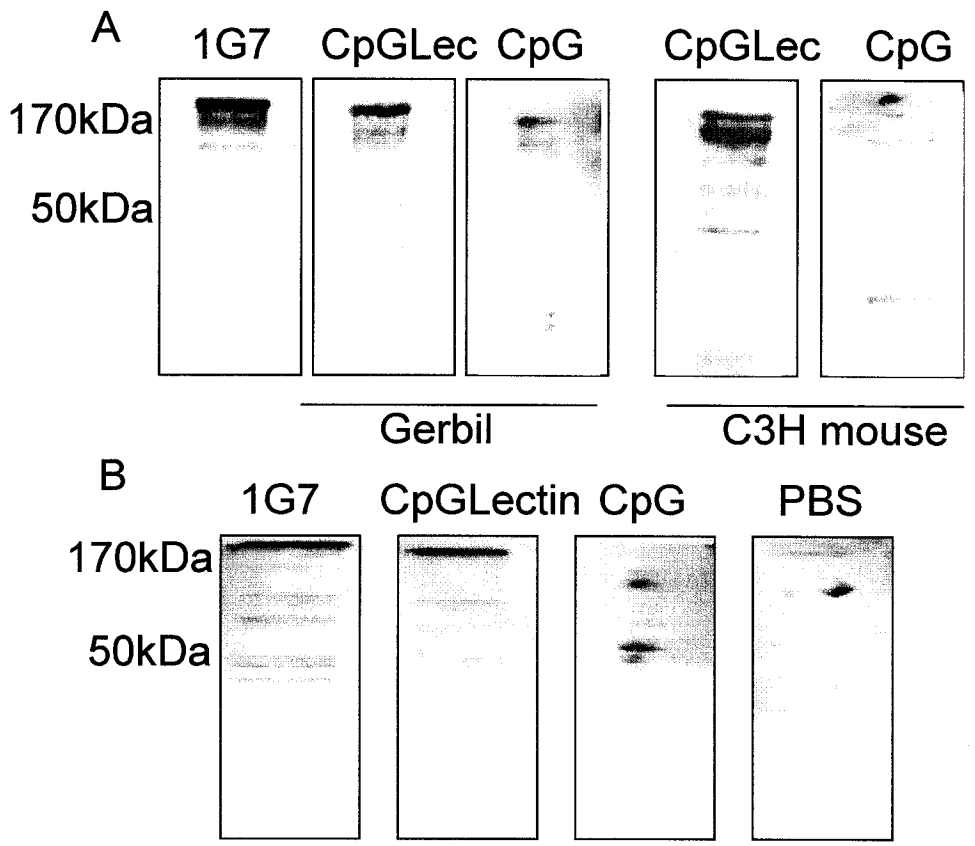

Figure 7.1. Immunoblot with immune or control gerbil and C3H mouse serum against purified Gal-lectin (A). Gal-lectin specific serum IgG (1:250) from immunized gerbils recognize the 170 kDa heavy sub-unit band. (B) Stool IgA from immunized C3H mice recognize the Gal-lectin. The Gal-lectin was probed with stool antigen preparations (1:250) and detected with anti-mouse IgA antibody. Data shown are representative of three independent experiments.

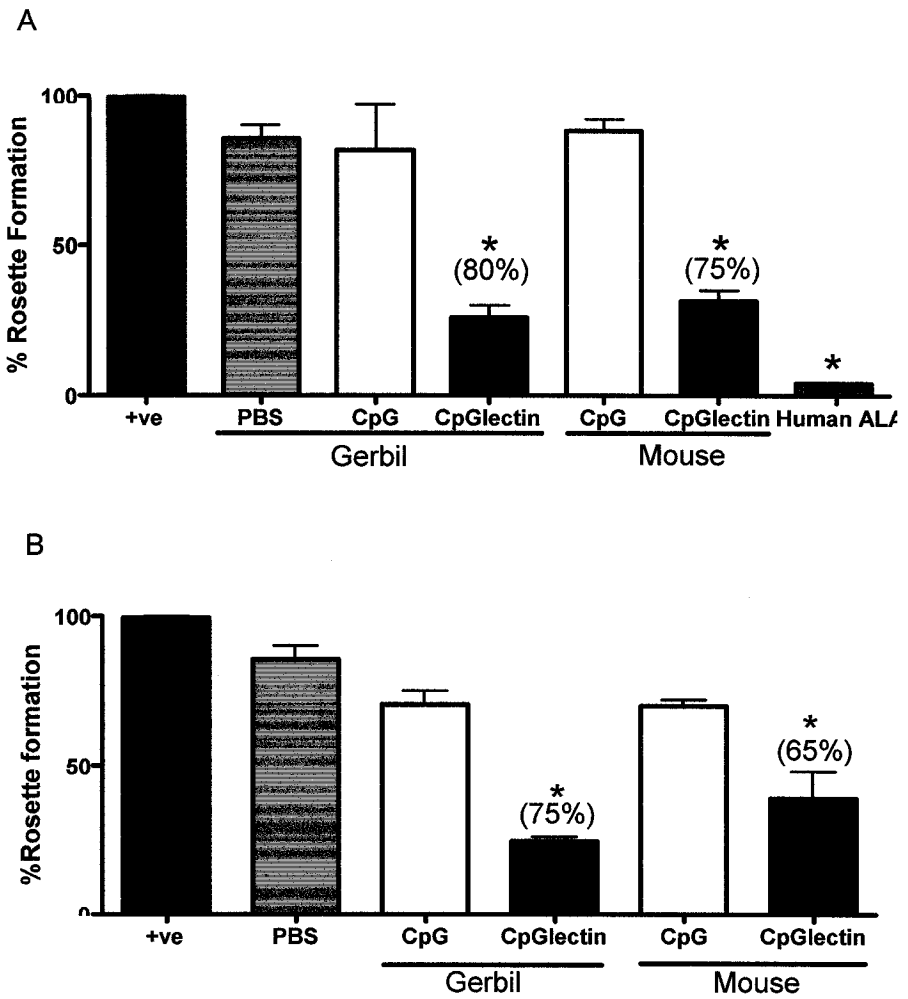

Figure 7.2. CHO Adherence assay.(A) Inhibition of amebic adherence to target CHO cells by immune gerbil and C3H mouse serum. Amebae were preincubated with 1:100 immune or control serum dilutions and subsequent amebic adherence to CHO cells was determined by adherence assay. CpG+Gal-lectin immune serum significantly inhibited amebic adherence (*P<0.05). (B) Inhibition of amebic adherence to CHO cells by gerbil and C3H mouse stool IgA. Compared to controls, immunized animal had Gal-lectin specific stool IgA capable of significantly blocking parasite adherence (*P<0.05). Data shown are from triplicates of three independent experiments. Bars represent the percent amebic adherence to target cells. Numbers in parentheses represent percent inhibition.

CpG-ODN and Gal-lectin vaccination induces a cell-mediated immune response

We evaluated the cellular immune response in both gerbils and C3H mice 30 days after the first immunization. Cell proliferation was measured by [^{3}H]methyl-thymidine incorporation in spleen lymphocytes cultured in the presence of media, con A , native Gal-lectin and whole amebic proteins. Animals immunized with CpG-ODN and Gal-lectin had greater proliferation response to amebic Gal-lectin (*P<0.05) and amebic proteins than control animals (Fig.7.3). Proliferation responses were greater in gerbils than in mice, but both cell types demonstrated viability with strong proliferation in response to the T cell mitogen con A.

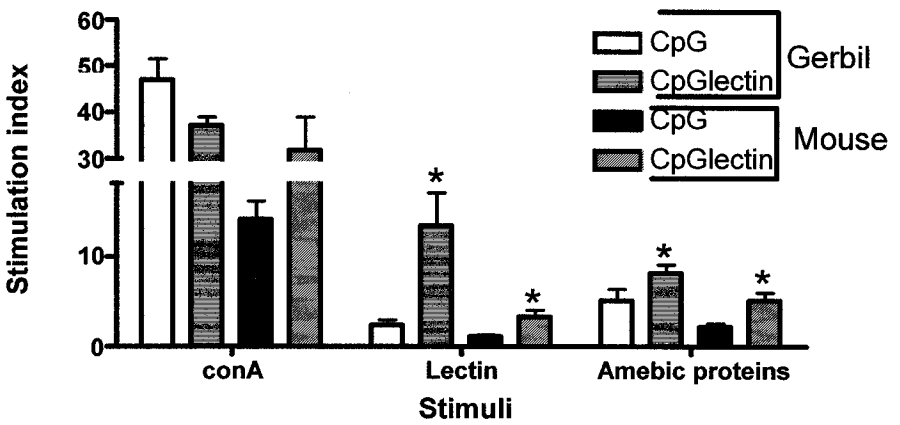

Figure 7.3. Lymphoproliferation of gerbil and C3H mouse splenocytes in response to Gal-lectin stimulation. Immunized or control spleens were collected prechallenge infection and cells were restimulated *in vitro* for 72 h with either ConA (2.5μg), Gal-lectin (10μg) or 50 μg of soluble amebic proteins. Proliferation is represented as a stimulation index (cpms of cells with Ag/cpms of cells without Ag). Asterisks indicate a significantly higher proliferation in response to amebic antigen or Gal-lectin compared to matched controls (*P<0.05).

Induction of Th1 cytokines

Five days after the last immunization, animals were sacrificed and mesenteric lymph nodes were collected. Cell suspensions were prepared from the MLNs and restimulated in vitro with media or native Gal-lectin. Cytokine mRNA levels were measure by Real-time PCR for gerbil and C3H mice using species-specific primers and probes. Compared to cells stimulated with media alone, vaccinated animals showed an increase in IFN-γ mRNA (**P<0.05) when stimulated with Gal-lectin (Fig.7.4). Control animals did not demonstrate a specific response, having similar levels of IL-4 and IFN-γ mRNA. Control animals, however had higher levels of IL-4 then vaccinated animals (*P<0.05).

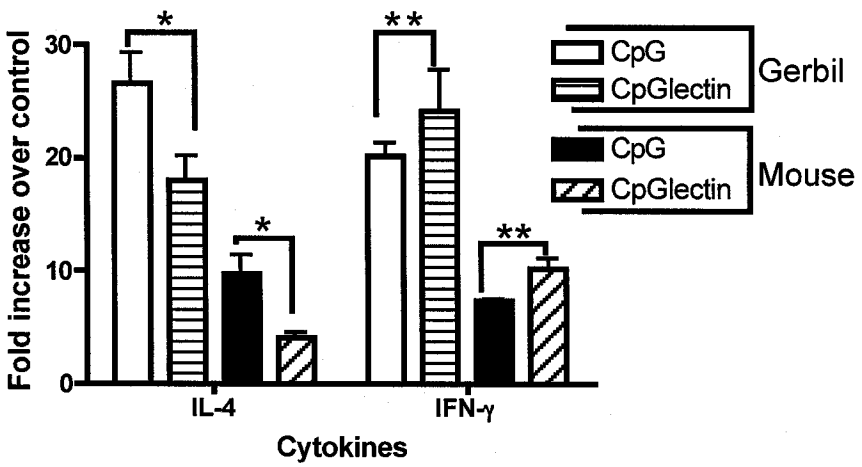

Figure 7.4. Real-time PCR analysis of mesenteric lymph node cytokine gene expression. Gene expression was normalized to house keeping genes and represented as fold increase over normal non-treated mRNA. There was substantially higher IFN-γ levels in the vaccinated animals (**P<0.05), whereas IL-4 was more prevalent in CpG control animals (*P<0.05). Data points represent means ± SEM of results from three independent PCR reactions.

To evaluate the production of Th1 cytokines in vaccinated animals, we performed intracellular staining in restimulated MLN cells from C3H mice. Cells were stained for CD4+ T cells and either IL-4 or IFN-γ protein and analyzed by FACS scan. Consistent with the results of Real-time PCR, vaccinated animals had greater (*$P<0.05$) IFN-γ producing CD4 T-cells (62%) than animals receiving CpG only (26%). The Th2 cytokine IL-4 was detected in both groups but was not significantly higher in vaccinated animals (Fig.7.5). These data clearly suggest that mucosal immunization with CpG-ODN and Gal-lectin specifically induces IFN-γ production.

Mucosal immunization protects against intrahepatic challenge infection

Next, we evaluated the protective effects of mucosal vaccination with CpG-ODN and Gal-lectin on amebic liver abscess development. Five days after the last immunization, gerbils were challenged by direct intrahepatic inoculation of virulent trophozoites. Gerbils were sacrificed at different days post challenge (2,5, 10, and 15) and their livers were excised and examined for ALA formation. At day two post challenge there were no detectable differences in ALA size between vaccinated and control animals (Fig.7.6). However, after 5 days post challenge no abscesses could be observed in vaccinated gerbils, while CpG-ODN control animals developed larger abscesses and greater hepatic damage. These results indicate that intranasal administration of CpG-ODN and Gal-lectin is sufficient to protect animals against amebic liver abscess formation.

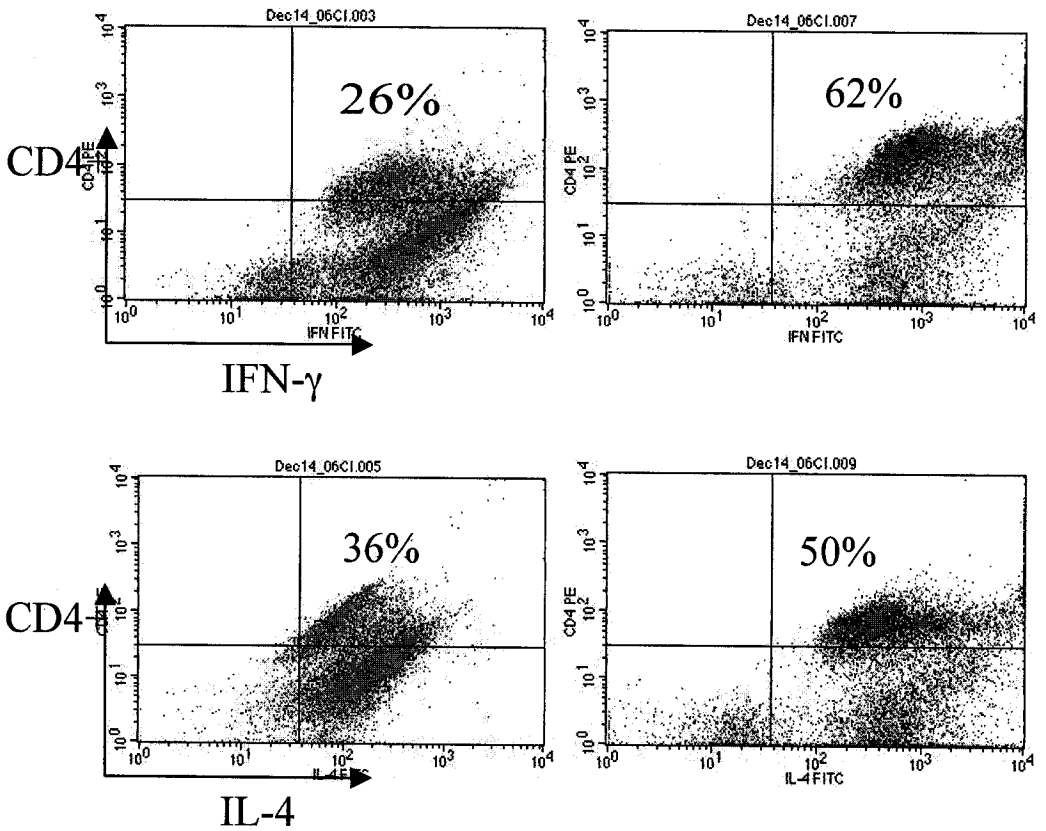

Figure 7.5. Intracellular cytokine staining in MLN cells. Control and vaccinated C3H mice (three mice per group) were sacrificed after the last immunization and MLN cells were restimulated in vitro with Gal-lectin. Cells were stained for CD4+ T cells and either IFN-γ or IL-4. Numbers in the quadrant represent the percent positive cells for CD4+ and the cytokine. The figure shows results obtained from one mouse from each group and is representative of the whole group. Similar results were obtained in two separate experiments.

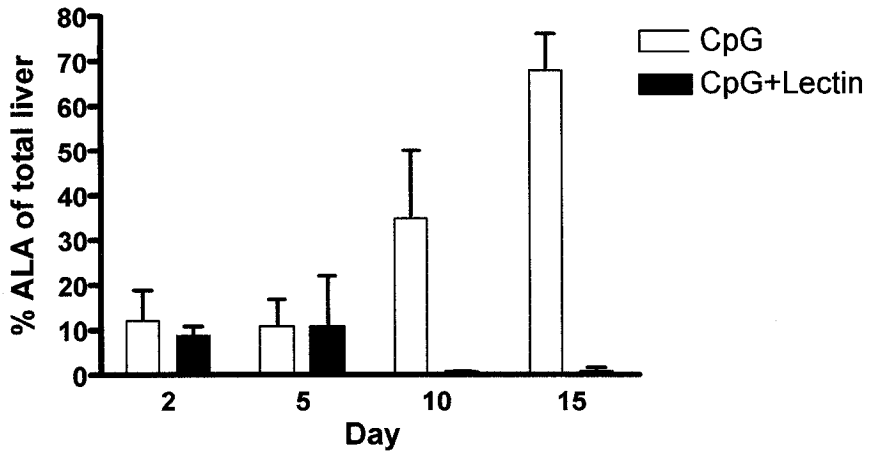

Figure 7.6. Progression of ALA formation. Gerbils were sacrificed at days 2, 5, 10 and 15 post challenge and their livers were examined for ALA formation. Abscess size is represented as precent of total liver weight. CpG+Gal-lectin vaccinated gerbils had no detectable abscesses after day 5 post challenge (N/A). N= 6 per group per day post challenge.

Discussion

In this work we studied the use of CpG-ODN as adjuvant in a mucosal Gal-lectin based vaccine against *Entamoeba histolytica*. Here we report the safe and effective combination of CpG-ODN with native Gal-lectin to induce protective immunity in animal models of amebiasis. Intranasal immunization with this vaccine induced both anti-Gal-lectin IgG and IgA, both capable of blocking parasite adherence *in vitro*. Cellular immunity was mediated by an IFN-γ response and immunization protected against systemic challenge infection in gerbils.

Several properties of CpG-ODN and the Gal-lectin protein contribute to the efficacy of this vaccine. First, CpG-ODNs are recognized by TLR9 and are known to function

as powerful adjuvant by activating antigen presenting cells and B cells. CpG-ODN have been shown to induce B cell proliferation in a non-antigen specific way and to synergize with B cell signaling through the B cell antigen receptor [13,23]. Specially designed ODNs can elicit both innate and acquired immune responses predominantly of the Th1 type, while potentiating the specific immune response to a co-injected antigen. Since the type of immune response induced by a vaccine is crucial to its efficacy, using CpG-ODN adjuvant can direct the response generated towards Th1. However, the hallmark of mucosal vaccination is the production of secretory IgA antibodies, which can be synthesized in the context of Th1 or Th2 milieus [24]. In this study we report the production of both anti-Gal-lectin IgG and IgA following intranasal vaccination with CpG-ODN adjuvant.

Second, the Gal-lectin antigen is also a potent immunogen capable of activating dendritic cells and macrophages [25-27]. The Gal-lectin is known to induce Th1 cytokine production in immune cells and can induce protective immunity in animal models of amebiasis [6-8]. Not only is the Gal-lectin immunogenic, it is also the major surface lectin of the parasite essential for biological processes including: colonization, cytotoxicity, and complement resistance. Combining the effectiveness of CpG-ODN and Gal-lectin to activate innate immune cells and the antigenicity of the Gal-lectin, resulted in a potent vaccine.

We have previously shown that CpG-ODN and the Gal-lectin can protect against amebic liver challenge infection when administered parenterally [20]. In general

parenteral injections induce systemic immunity, whereas mucosal immunizations can induce mucosal immune responses at local and distant sites as well as systemic immunity [28,29]. The majority of infectious diseases are acquired through mucosal surfaces including *Entamoeba histolytica,* therefore mucosal vaccination against the parasite could theoretically prevent colonization and protect against invasive disease. Here we report the production of mucosal IgA and systemic immunity after intranasal administration of a CpG-ODN and Gal-lectin vaccine. Fecal anti-Gal-lectin IgA was detected in immunized mice and could effectively block parasite adherence to target cells *in vitro*. Gerbils were protected from systemic infection with live trophozoites, demonstrating the production of an effective systemic response after mucosal vaccination. Although we did not correlate anti-Gal-lectin IgA with protection in the C3H mouse model in this study, it has been reported that acquired resistance to infection in humans is linked to intestinal IgA against the carbohydrate recognition domain of the Gal-lectin [30]. Protection could be correlated, however, with elevated levels of IFN-γ and high proliferative responses. IFN-γ is a signature Th1 cytokine and has been shown to stimulate immune cell amebicidal activity *in vitro* [31,32]. In fact, recent reports from a Bangladesh study correlated higher IFN-γ production with reduced risk of amebic disease [33]. Antigen specific IFN-γ as well as local release of IFN-γ in both mice and gerbils could account for the protective responses against amebic challenge infection.

In summary, the production of a Th1-type immune response characterized by IFN-γ production and humoral response characterized by IgA anti-bodies is a consequence

of stimulating CpG-ODN adjuvant and a potent vaccine antigen. The choice of adjuvant in this vaccine formulation is an attractive candidate for potential use in a human vaccine against *Entamoeba histolytica.* Intranasal immunization represents a non-invasive, fast and easy method to induce both humoral and systemic immunity in laboratory animals. In humans, however, nasal immunizations seem to result in antibody responses in the upper airway mucosa but not in intestinal responses [34,35]. At present it is difficult to predict the efficacy of vaccines in protecting against amebiasis in humans, as there is a lack of adequate animal models and *E. histolytica* is reported to be able to degrade human IgA *in vitro* [36]. Future studies should therefore examine the efficacy of this vaccine in an intestinal amebiasis model and should elucidate the specific role of anti-Gal-lectin IgA in protection.

Acknowledgements

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CONNECTING STATEMENT III

The vaccination studies demonstrated that using a combination of pathogen derived products that can activate immune cells, enhances the magnitude of Gal-lectin specific immune responses. It is logical to think that other amebic molecules could be immunostimulatory and contribute to the protective effects of the Gal-lectin. We hypothesized that amebic DNA might act in a similar manner to CpG-ODN and amplify immune responses. Hence we sought to study the immune activating potential of *E. histolytica* DNA.

CHAPTER 8: MANUSCRIPT IV

TLR-9 dependent macrophage activation by *Entamoeba histolytica* DNA

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Manuscript submitted for publication

Abstract

Activation of the innate immune system by bacterial DNA and other invertebrate DNA represents a pathogen recognition mechanism. In this study we have investigated macrophage responses to DNA from the intestinal protozoan parasite *Entamoeba histolytica*. *E.histolytica* genomic DNA (E.h DNA) was purified from log phase trophozoites and tested with mouse macrophage cell line, RAW 264.7. RAW cells treated with E.h DNA demonstrated an increase TNF-α mRNA and protein production. TNF-α production was blocked by pre-treatment with chloroquine or monensin. In fact, an NF-κB luciferase reporter assay in HEK cells transfected with human TLR9 demonstrated that E.h DNA signaled through TLR9 similar to CpG-ODN. Immunofluorescence confirmed NF-κB activation in RAW cells, as seen by nuclear translocation of the p65 sub-unit. Western blot analysis demonstrated MAPK activation by E.h DNA. E.h DNA effects were abolished in MYD88-/- mice derived macrophages. In the context of disease, immunization with E.h DNA protected gerbils from *Entamoeba histolytica* challenge infection. Taken together, these results suggest that E.h DNA is recognized by TLR9 to activate macrophages and may provide an innate defense mechanism, characterized by the induction of the inflammatory mediator TNF-α.

Introduction

The vertebrate immune system has evolved to recognize pathogen associated molecular patterns (PAMP) in order to counteract bacterial, viral and parasitic infections [1]. The Toll-like receptor (TLR) family of proteins specifically recognizes these PAMPs and activates anti-pathogen responses characterized by Th1 cytokines [2]. The role of TLRs is to discriminate self from "non-self" molecules, such as unmethylated CpG-DNA. Mammalian DNA is highly methylated at the CG dinucleotide whereas bacteria and other invertebrate DNA is not, and this feature is key to its immunostimulatory activity [3,4]. CpG-DNA must first be endocytosed and upon endosomal acidification it is specifically engaged by TLR9 [5,6]. The activated receptor initiates a signaling cascade starting with the recruitment of MYD88, IRAK and TRAF6, which ultimately leads to the downstream activation of MAPK and NF-KB [7,8]. CpG-DNA demonstrates mitogenic activity to B cells [4,9], and activates dendritic cells and macrophages to produce pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 [10,11].

The intestinal protozoan parasite *E. histolytica* is the etiological agent of amebiasis in humans. Although amebiasis remains a prevalent disease in developing countries, only 10-14% of infected individuals progress to invasive amebiasis, characterized by amebic colitis. The pathological events or immune events leading to amebic invasion are still unknown, however recent epidemiological studies revealed acquired resistance and inherited susceptibility to amebiasis [12-14]. Many studies have also established that cell-mediate or Th1 immunity is of importance in host defense

against amebiasis. Lymphocytes from individuals who have recovered from invasive amebiasis display amebicidal activity and secrete high amounts of IL-2 and IFN-γ [15,16]. Other cells demonstrate amebicidal activity such as activated neutrophils and macrophages [17-19], and TNF-α as well as nitric oxide had a crucial role in initiating this effect.

Th1 polarization in response to amebic infection could be due to innate activation of TLR. It is obvious that NF-KB must be involved in the observed inflammation response, which can lead to tissue damage, however it may also be required for disease resolution. It has been shown that inhibition of NF-KB blocks the inflammatory response to ameba in human intestinal xenograft models [20] whereas patients given corticosteroids have increased disease severity [21]. Several parasite molecules could be involved in early immune activation events. *E. histolytica* lipopeptidophosphoglycan has been shown to signal through TLR2 and TLR4 inducing Th1 cytokines in monocytes [22]. The parasite cell surface lectin, galactose/N-acetyl-D-galactosamine inhibitable lectin (Gal-lectin), is well characterized and known to induce direct dendritic cell and macrophage activation [17,18,23]. The role of other parasite molecules in innate immunity and Th1 polarization remains unclear. Given the immunogenicity of foreign DNA and its role in Th1 induction via TLR9, there is a potential role for *E. histolytica* DNA in the activation of innate immunity.

In this study, we determined whether *E. histolytica* DNA (E.h DNA) could activate naïve murine macrophages. Herein we demonstrate that E.h DNA activates macrophages to produce TNF-α and that this activation is mediated through TLR9. Blocking endosomal maturation greatly reduced TNF-α expression and furthermore this expression was lost in MYD88-/- bone marrow derived macrophages. Repeated injection of E.h DNA in gerbils conferred protection from amebic challenge infection and was characterized by IFN-γ expression. Thus we identified another parasite molecule capable of activating an innate immune response, which may contribute to host defense against amebiasis.

Materials and Methods

Reagents and parasites

E. histolytica strain HM1: IMSS trophozoites were grown in axenic culture in TYI-S-33 medium. Parasites were grown for 72 hours (log-phase) for use in all experiments. The native Gal-lectin was purified from amoebae on an immunoaffinity column as previously described [24]. E.h DNA was extracted from trophozoites as previously described [25]. Briefly, the parasite pellet was frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. The pellet was resuspended in lysis buffer containing 1 mg/ml proteinase K (Invitrogen) and incubated for 30 min at 65 °C. The lysate was subjected to a series of phenol:chloroform extractions and DNA was precipitated with ethanol. DNA was treated with RNAse A (Qiagen) for 30 minutes at 37 °C before use in experiments.

LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich, St. Louis, MO) was used at 100 ng/ml or 1 μg/ml as indicated. CpG-ODN 10103 (TCG TCG TTT CGT CGT TTT GTC GTT) with a full phosphorothioate backbone was purchased from Coley Pharmaceutical Group (Kanata, Canada) and used at 2 μM. Calf thymus DNA (Sigma) was used as negative control at 30 μg/ml. Salmonella genomic DNA was purified as previously described [26] and used as positive control at 30 μg/ml. Chloroquine and monensin (Sigma) were used for blocking experiments and DNAse I (Qiagen) was used to digest DNA overnight. Polymyxin B sulfate was purchased from Sigma.

Cell culture

The mouse macrophage cell line, RAW 264.7, and HEK 293 cells were cultivated in DMEM (Invitrogen) supplemented with 10% FBS, 10mM HEPES and 5 mg/ml penicillin/streptomycin. L929 mouse fibroblast cell line was cultivated in MEM (Invitrogen) supplemented as above. Bone marrow derived macrophages were propagated from wild type (C57BL/6) or MYD88-/- mice as previously described [23]. Briefly, femurs and tibias were removed and the bone marrow flushed using a 27gauge syringe. Red blood cells were lysed with Red Blood Cell Lysing Buffer (Sigma) and washed three times in RPMI-1640 (Invitrogen). Cells were cultured in 6 well plates (2.5×10^5 cells/ml) in RPMI-1640 media supplemented with 5% FBS, 10mM HEPES, 50 μM 2-ME, 2mM glutamine, 5 mg/ml penicillin/streptomycin sulfate and 10 ng/ml rmGM-CSF (R&D Systems, Minneapolis, MN). Non-adherent

cells were removed after 5 days and remaining cells were cultured another 48hrs then sub-cultured into new plates to enrich the macrophage population.

Real Time PCR

RAW cells or bone marrow derived macrophages were stimulated with the indicated antigens or media alone for 3 hours. Total mRNA was extracted using Trizol ® (Invitrogen) following the manufacturer's instructions. 1-2μg of DNase treated total mRNA was reverse transcribed with MMLV enzyme and random hexamer primers (Invitrogen). Real-time PCR analysis using SyBrGreen (Qiagen) and mouse specific primers [27] was used to determine relative cytokine gene expression. Cytokine gene expression is represented as fold increase over RPMI stimulated control corrected for GAPDH house keeping gene, as determine by the $2^{\Delta\Delta ct}$ method [32]. Real-time DNA amplification assays were conducted in triplicates with the Rotor-Gene 3000 (Corbett Research). For gerbil cytokine expression, spleen cells were stimulated *in vitro* for 18 hours and mRNA was extracted and cDNA prepared as above. Real time PCR was performed as above but with gerbil specific Taqman probes [28].

TNF-α bioassay and western blot

The mouse fibroblast cell line, L929, was used in a TNF-α bioassay with treated RAW cell supernatants. L929 cells were trypsinized and resuspended at cell density of 3.5×10^5 cell/ml. 100 μl of cell suspension was added per well in a 96 well plate and cells were grown overnight. The following day the media was removed and replaced with MEM containing 50 μg/ml cycloheximide and either TNF-α standards

or 24 hour stimulated RAW cell supernatants. Plates were incubated for 18 hours at 37 °C then analyzed for cell viability with crystal violet stain. Cell supernatants were also concentrated and separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose and probed for TNF-α protein (Santa Cruz).

Griess Reaction

NO production in RAW cell culture supernatants (96 hour stimulation) was measured as nitrite accumulation in the Griess Reaction assay. Cell supernatants (50 μl/well) were transferred to a 96 well plate and mixed with equal volumes of 0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride (Sigma) and 1% sulfanilamide (Sigma) in 2.5 % H_3PO_4. Absorbance was read at 540 nm and compared to a sodium nitrite ($NaNO_2$) standard curve.

MAPK Western blotting

RAW cells were stimulated with the indicated antigens for 30 minutes. The cells were washed and the cell pellet was collected. Cytoplasmic extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) following the manufacturers' instructions. Cytoplasmic proteins (30 μg) were resolved by 10 % SDS PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with a 5% milk solution, washed with T-TBS and incubated with primary antibody against phosphorylated p38, JNK, ERK (SantaCruz). Bound antibody was visualized using secondary HRP-conjugated antibody and enhanced chemiluminescence (ECL plus Reagent, Amersham).

Immunofluorescence

RAW cells (1.5 x 10^6 cell/ml) were grown on glass coverslips overnight for use in an immunofluoresence assay. The following day, cells were washed and treated with LPS (1 μg/ml), E.h DNA (30 μg/ml) or control DNA (30 μg/ml) for 45 minutes at 37 °C. After incubation, cells were washed and fixed with 100% methanol at -20 °C for 5 minutes. Cells were washed again and permeabilized with 2 % Igepal (Sigma) for 5 minutes at room temperature. Cells were washed again, then blocked with 1% BSA-PBS for 30 minutes at room temperature and washed thoroughly. To visualize NF-KB translocation, cells were stained with p65 antibody (Santa Cruz 8008) diluted 1:200 in PBS-0.1% BSA for 1 hour at room temperature in a humidified chamber. Cells were washed and stained with anti-mouse FITC conjugated secondary antibody (Chemicon) for 30 minutes as above. After incubation the cells were washed and mounted with VectaShield® onto glass slides. Slides were visualized with the Nikon Eclipse E400 fluorescence microscope.

Transient transfection and reporter assay

HEK 293 cells were seeded at 6.0 x 10^5 cell/well and transfected overnight in 6 well/plates by $CaCl_2$ precipitation method with 1 μg pNFKB-Luc (Stratagene) and 1 μg pcDNA human TLR9 or control plasmid. After overnight culture the transfection media was removed and cells were stimulated with various stimuli for 12 hours. Cell lysates were assayed for luciferase activity using the Luciferase Report Assay System (Promega). Luciferase activity was measured with a luminometer (Victor II, Wallac)

and was normalized to control transfections and expressed as fold increase over matched controls.

Animal immunizations

Male gerbils (*Meriones unguiculatus*) 6-9 weeks old (Charles River, St.Constant, Canada) were injected intramuscularly in the hind legs with, either 50 μg E. h DNA only, 10 μg of Gal-lectin and 50 μg E.h DNA or 10 μg Gal-lectin only in 100 μl PBS. Gerbils received identical booster injections at 7 and 14 days post initial injection. At day 20, gerbils were anesthetized and challenged via intrahepatic injection of 10^6 *E. histolytica* amebae (HM1:IMSS) into the left liver lobe as previously described [29]. Gerbils were sacrificed at 10 days post challenge and their spleens and sera were collected. Livers were removed and ALA weight relative to total liver weight was measured. All protocols in this study were carried out with the approval of the University of Calgary Animal Care Committee.

Statistical analysis

Differences in the mean values between experimental groups were compared using Student's unpaired *t*-test. *P* values <0.05 are considered significant.

Results

Induction of TNF-α by E.h DNA

CpG-DNA is known to induce a range of cytokines in macrophages including IL-12, TNF-α, IL-1β and IL-6 [11]. We first evaluated the ability of E.h DNA to activate macrophages by determining TNF-α induction. RAW mouse macrophages were

stimulated with increasing amounts of E.h DNA and TNF-α mRNA expression was measured by real time PCR. In Figure 8.1(A), we show that E.h DNA induces increase TNF-α mRNA expression in a dose dependent manner. Treatment of E.h DNA with polymyxin B did not alter the TNF-α response in macrophages, indicating that the activation was not due to LPS contamination (data not shown). We also compared TNF-α induction by E.h DNA to CpG-ODN and LPS, both PAMPs known to activate macrophages. E.h DNA induced TNF-α expression was not as high as LPS and CpG-ODN positive controls, however it was significantly higher than the non-stimulatory control GpC-ODN (Fig.8.1B). Additionally, E.h DNA was able to stimulate IL-1β and IL-6 mRNA expression (data not shown).

In order to confirm that the observed increase in TNF-α mRNA corresponds to an increase in cytokine release from macrophages we performed a bioassay with mouse L929 fibroblasts which are sensitive to TNF-α. RAW cells were treated for 24 hours and cell culture supernatants added to the responder cells for 18 hours. In agreement with the real time PCR data, LPS and E.h DNA were both positive ($P<0.05$) for the production of TNF-α protein as was *Salmonella typhimurium* DNA (Fig.8.2). In contrast, calf thymus DNA and DNAse treated E.h DNA did not induce significant amounts of TNF-α secretion. These data implied that macrophages were activated by E.h DNA and thus focused our attention on the characterization of this response.

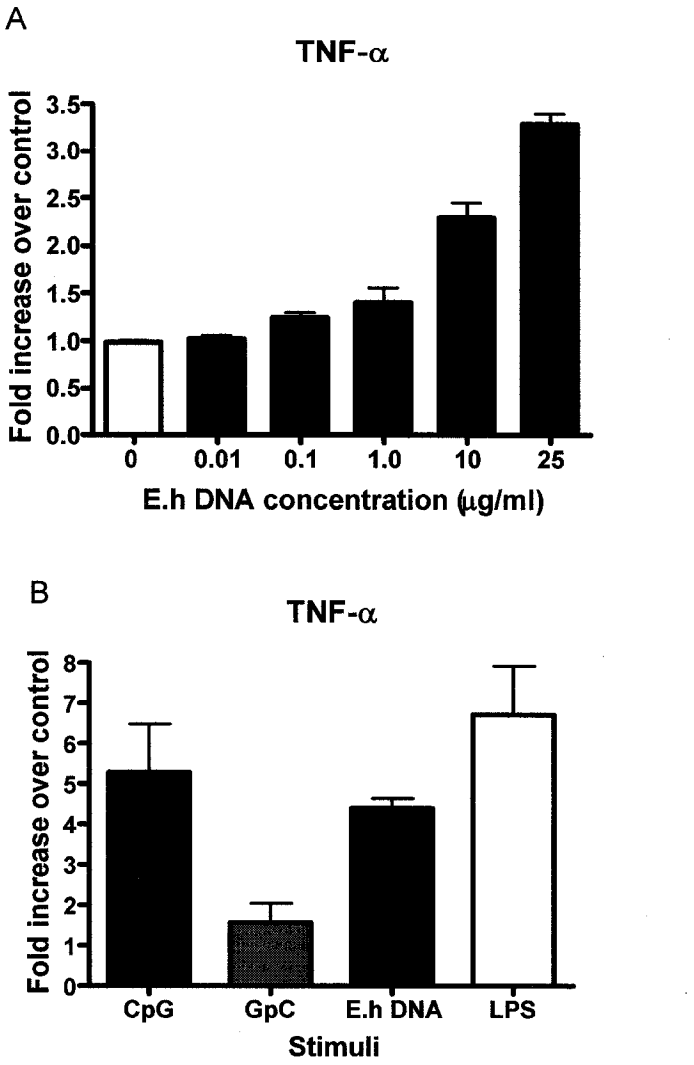

Figure 8.1. *E. histolytica* DNA activates macrophage TNF-α production. A) Real-time PCR analysis was performed on RAW 264.7 macrophage mRNA collected after 3 hours of *in vitro* incubation with increasing amounts of E.h DNA (1-25 μg/ml). Data are represented as fold increase over medium only control for mRNA expression. B) TNF-α mRNA levels of RAW 264.7 macrophages treated with CpG-ODN or GpC-ODN (2 μM), E.h DNA (30 μg/ml) or LPS (1 μg/ml) for 3 hours. Data points represent mean ± SEM from three experiments.

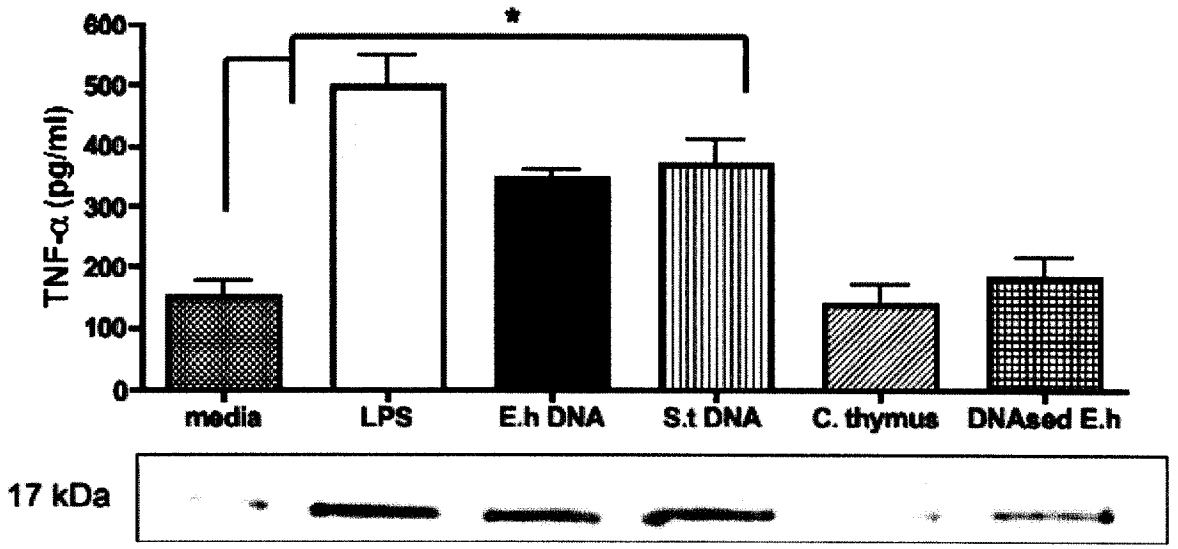

Figure 8.2. *E. histolytica* DNA induces TNF-α protein production. RAW 264.7 macrophages were treated *in vitro* for 24 hours with media only, LPS (1 μg/ml), E.h DNA (30 μg/ml), S.t DNA (30 μg/ml), calf thymus DNA (30 μg/ml) or DNAse treated E.h DNA (30 μg/ml). Culture supernatants were used in an L929 TNF-α bioassay. Data points represent mean ± SEM from three experiments. An asterisk indicates a significant increase in TNF-α protein compared to cells treated with media only ($P<0.05$). The blot represents the corresponding band for TNF-α protein (17 kDa) in culture supernatants.

Effect on nitric oxide production

Activated macrophages are a source of nitric oxide, which helps generate effective innate immunity against viruses, bacteria and parasites. Nitric oxide is also the main cytotoxic molecule produced by activated macrophages for cytotoxicity against *E. histolytica.* We evaluated the effect of various stimuli on macrophage nitric oxide production. In the experiment shown in Figure 8.3(A), we measured iNOS mRNA expression in control and treated macrophages. The data show that E.h DNA could increase iNOS mRNA to a similar extent as CpG-ODN, while LPS and S.t DNA induced higher mRNA expression. Calf thymus DNA, however could not induce an

increase in iNOS mRNA. We used 96-hour culture supernatants in a Griess reaction to indirectly measure nitric oxide production by stimulated macrophages. E.h DNA was able to induce nitric oxide production significantly more then control and calf thymus DNA ($P<0.05$). The amount of NO measured, however, was less than LPS, CpG-ODN or S.t DNA stimulation.

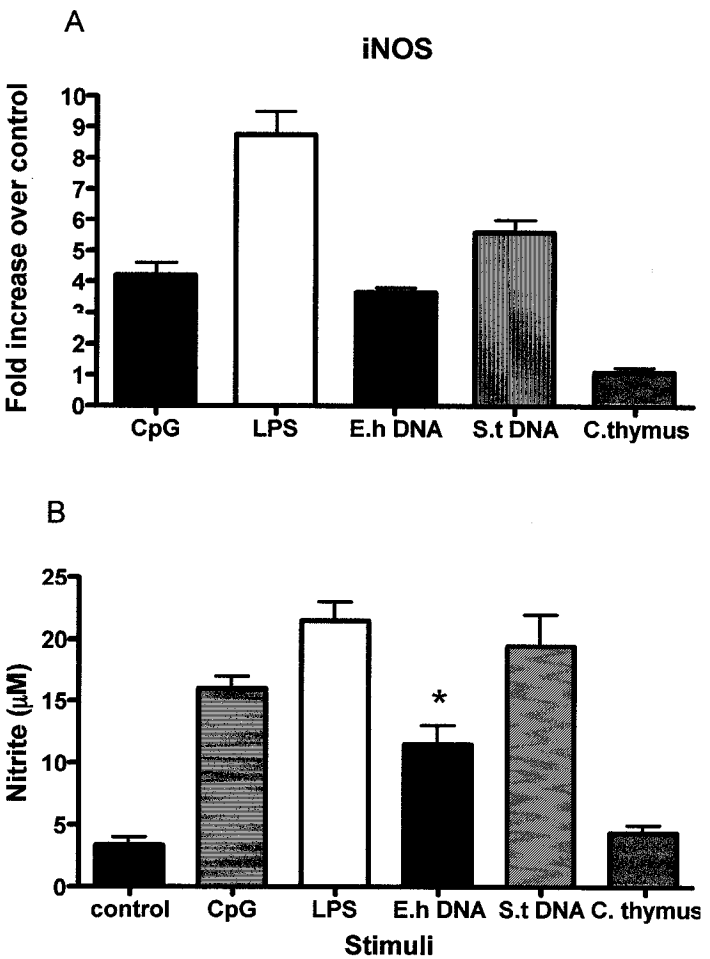

Figure 8.3. *E. histolytica* DNA activates macrophage iNOS production. A) Real-time PCR analysis was performed on IFN-γ (100 U/ml) primed RAW 264.7 macrophage mRNA collected after 6 hours of *in vitro* incubation with CpG-ODN (2 μM), LPS (1 μg/ml), E.h DNA (30 μg/ml), S.t DNA (30 μg/ml), and calf thymus DNA (30 μg/ml). Data are represented as fold increase over medium only control for mRNA expression. B) Macrophages were stimulated as above for 24 hours and culture supernatants were used in the Griess reaction to measure nitrite levels. Data points represent mean ± SEM from three experiments. An asterisk indicates a significant increase in nitrite compared to cells treated with media only ($P<0.05$).

Inhibition of acidification

TLR9 is activated intracellularly by bacterial DNA and CpG-ODNs, by translocating from the ER to endosomes containing the CpG DNA [6]. Endosomal acidification and maturation seem to be required for proper TLR9 signaling. We tested the effects of chloroquine (5 μg/ml) and monensin (20 μM), both inhibitors of endosomal acidification, on macrophage TNF-α expression after stimulation with various stimuli. Pre-treatment of RAW macrophages with choloroquine or monensin reduced the potency of both E.h DNA and S.t DNA ($P<0.05$) but not LPS (Fig.8.4). In fact, retained activity with LPS demonstrated specificity for TLR9 signaling and also demonstrated that the concentration of inhibitors used did not cause cytotoxicity, which could have accounted for reduced mRNA levels.

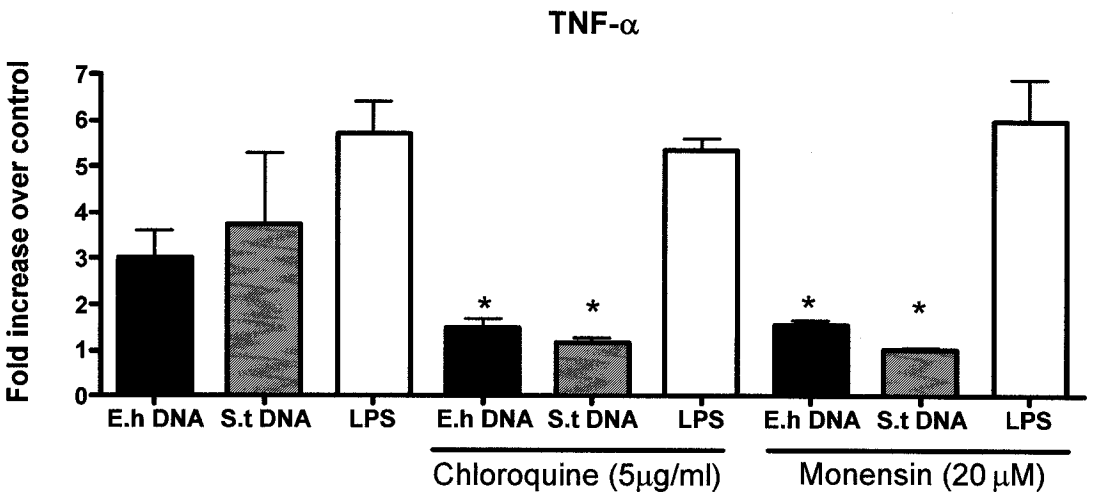

Figure 8.4. TNF-α expression by E.h DNA stimulated macrophages requires endosomal acidification. RAW 264.7 macrophages were pre-treated for 30 minutes with or without chloroquine (5 μg/ml) or monensin (20 μM) and subsequently stimulated for 3 hours with E.h DNA (30 μg/ml), S.t DNA (30 μg/ml) or LPS (1 μg/ml). mRNA was collected and the level of TNF-α expression was measured by real time PCR. Data points represent mean ± SEM from three experiments. An asterisk indicates a significant decrease compared to cells not treated with inhibitors ($P<0.05$).

HEK-TLR9 NF-KB reporter assay

We suspected that TLR9 might be involved in E.h DNA macrophage activation, in which case NF-KB would be involved for pro-inflammatory cytokine production. HEK 293 cells were transiently transfected with human TLR9 and an NF-KB-luciferase reporter plasmid and treated with various stimuli. The most effective NF-KB activators were CpG-ODN followed by S.t DNA and E.h DNA (Fig.8.5). In contrast, DNAse treated E.h DNA had reduced NF-KB activity while calf thymus DNA did not significantly induce any activation.

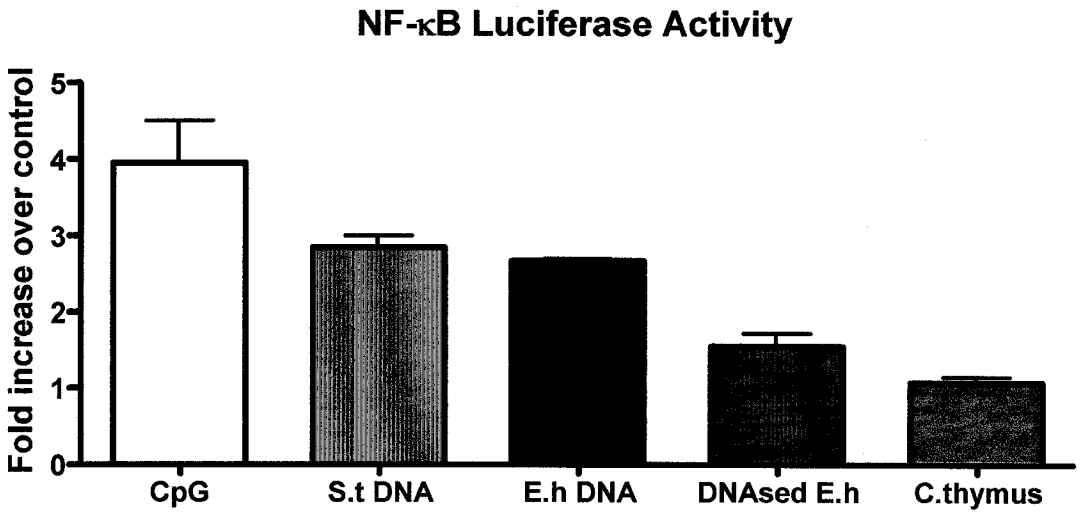

Figure 8.5. TLR-9 dependent NF-KB activation by E.h DNA. HEK 293 cells were transiently transfected with pcDNA-hTLR9 and NF-KB luciferase reporter plasmid, and treated with CpG-ODN (2 μM), S.t DNA (30 μg/ml), E.h DNA (30 μg/ml), DNAsed treated E.h DNA (30 μg/ml) or calf thymus DNA (30 μg/ml). Luciferase induction was measured and reported as fold increase over cells transfected with empty pcDNA plasmid. Data points represent mean ± SEM from three experiments.

Impaired response to E.h DNA in MYD88-/- cells

MYD88 is an adaptor protein in the signal transduction pathway mediated by toll receptors and it is essential to mount an innate immune response. We propagated bone marrow derived macrophages from wild type and MYD88-/- mice to assess the biological function of TLR signaling in response to E.h DNA. In wild type macrophages both LPS and E.h DNA induced high TNF-α mRNA expression (Fig.8.6 A and B). In contrast, MYD88-/- macrophages showed significantly reduced TNF-α expression in response to E.h DNA. LPS also had reduced potency in the MYD88-/- macrophages but to a lesser extent than E.h DNA. Thus E.h DNA mediated signal transduction is dependent on MYD88, a key adaptor protein in TLR9 signaling.

MAPK and NF-KB activation by E.h DNA

MYD88 is known to be involved in the activation of all three MAPK pathways in TLR signaling. Therefore we analyzed the MAPK activation in response to various stimuli known to require TLR signaling. Cells were treated for 30 minutes and cellular extracts were probed in a western blot for phosphorylated MAPK. CpG-ODN, LPS and S.t DNA as well as E.h DNA all induced phosphorylation of p38, ERK and JNK while calf thymus DNA and media alone did not (Fig.8.7). Next we confirmed the activation of NF-KB by detecting p65 sub-unit translocation into the nucleus by immunofluorescence. Treated and untreated cells were stained with a p65 antibody and visualized for fluorescence. RAW macrophages that were not treated or

treated with calf thymus DNA showed p65 staining in the cytoplasm, indicating no NF-KB activation (Fig.8.8 a,b). However, macrophages treated with S.t DNA, LPS or E.h DNA exhibited strong p65 staining in the nucleus, indicative of p65 translocation and NF-KB activation (Fig.8.8 c,d,e). Thus E.h DNA activates NF-KB in a similar manner to LPS and bacterial DNA.

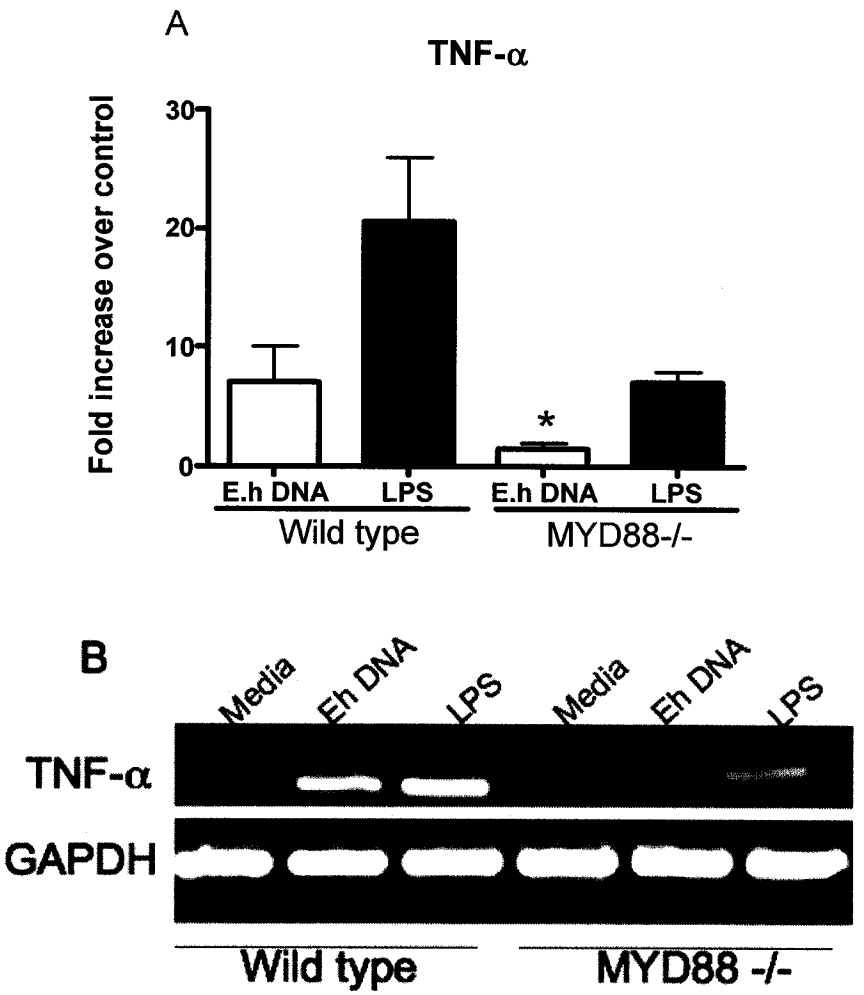

Figure 8.6. MYD88 is required for E.h DNA induced TNF-α expression. Bone marrow derived macrophages were propagated from wild type and MYD88-/- mice and stimulated *in vitro* for 3 hours with media, LPS (1 μg/ml) or E.h DNA (30 μg/ml). TNF-α mRNA expression was measured by real time PCR (A) and PCR products were run on 1.5% agarose gel (B). Data points represent mean ± SEM from three experiments. An asterisk indicates a significant decrease in TNF-α expression compared to wild type macrophages ($P<0.05$).

Interplay between LPS and E.h DNA

Desensitization, or tolerance, can occur *in vitro* with sub-maximal doses of LPS that reduce macrophage effector functions to subsequent stimulation with other activators. Similarly, activation through MYD88-dependent pathways can prime cells to respond to LPS in a MYD88-independent way [30]. We determined the sub-maximal dose of LPS and E.h DNA (data not shown) and used those concentrations to evaluate the interplay between LPS and E.h DNA macrophage activation. Sub-maximal doses of LPS and E.h DNA alone did not induce TNF-α expression (Fig.8.9A); neither did treatment with both sub-maximal doses simultaneously. Pre-treatment of cells with E.h DNA, however, increased the subsequent response to sub-maximal LPS stimulation. On the other hand, treatment with LPS tolerized the response to E.h DNA, as this order of stimulation induced less TNF-α expression. In fact, western blot analysis demonstrated that sub-maximal LPS doses do not induce p38 phosphorylation, while pre-treatment with E.h DNA could increase this phosphorylation event (Fig.8.9B). We compared the priming effects of sub-maximal E.h DNA to those of CpG-ODN and found that TNF-α expression patterns were similar with both treatments (Fig.8.9C). In both cases, pre-treatment with CpG-DNA (ODN or E.h) increase the response to LPS ($P<0.05$), whereas LPS reduced the response to CpG-DNA.

In vivo pro-inflammatory effect of E.h DNA

Finally we addressed the *in vivo* response to E.h DNA in the gerbil model of amebiasis. We evaluated the protective potential of E.h DNA in comparison to a

known parasite immunogen, the Gal-lectin. Animals were immunized with 50 μg E.h DNA or 10 μg Gal-lectin or both E.h DNA and Gal-lectin. Upon challenge infection with live amebic trophozoites, E.h DNA and Gal-lectin alone demonstrated equal protective immunity against amebic liver abscess formation (Table 8.1). Immunization with both E.h DNA and Gal-lectin, however, conferred complete protection. Spleen cells were isolated from immunized animals and cytokine expression was analyzed by real time PCR. Figure 8.10 showed that both E.h DNA and Gal-lectin induced the expression of IL-4 and IFN-γ, but that animals immunized with both antigens had significantly higher cytokine expression, specifically IFN-γ ($P<0.05$).

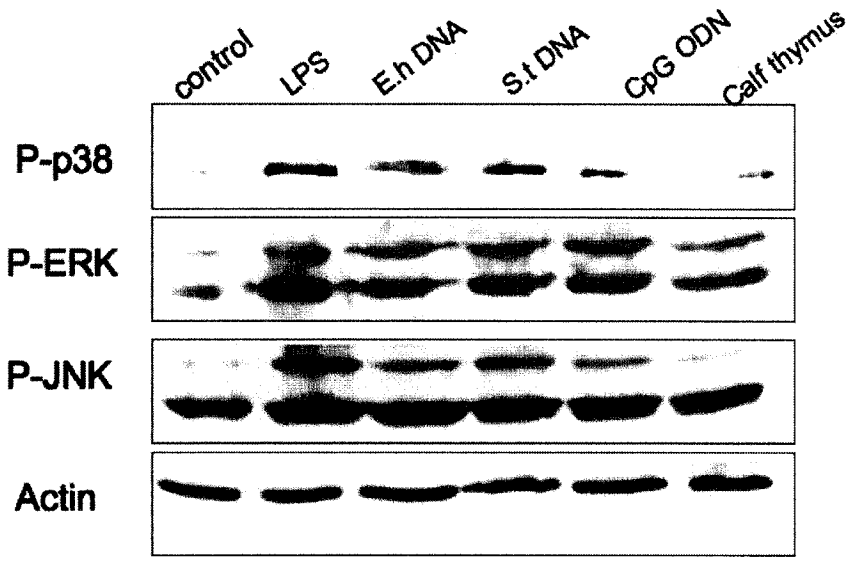

Figure 8.7. Macrophage activation by E.h DNA is mediated by MAPK. RAW 264.7 macrophages were stimulated for 30 minutes with medium only, LPS (1 μg/ml), E.h DNA (30 μg/ml), S.t DNA (30 μg/ml), CpG-ODN (2 μM), and calf thymus DNA (30 μg/ml). Cellular extracts were separated by electrophoresis, blotted and probed with antibodies specific for phosphorylated ERK, JNK or p38. Total actin antibody was used as loading control. Data shown are from one experiment and are representative of three independent experiments.

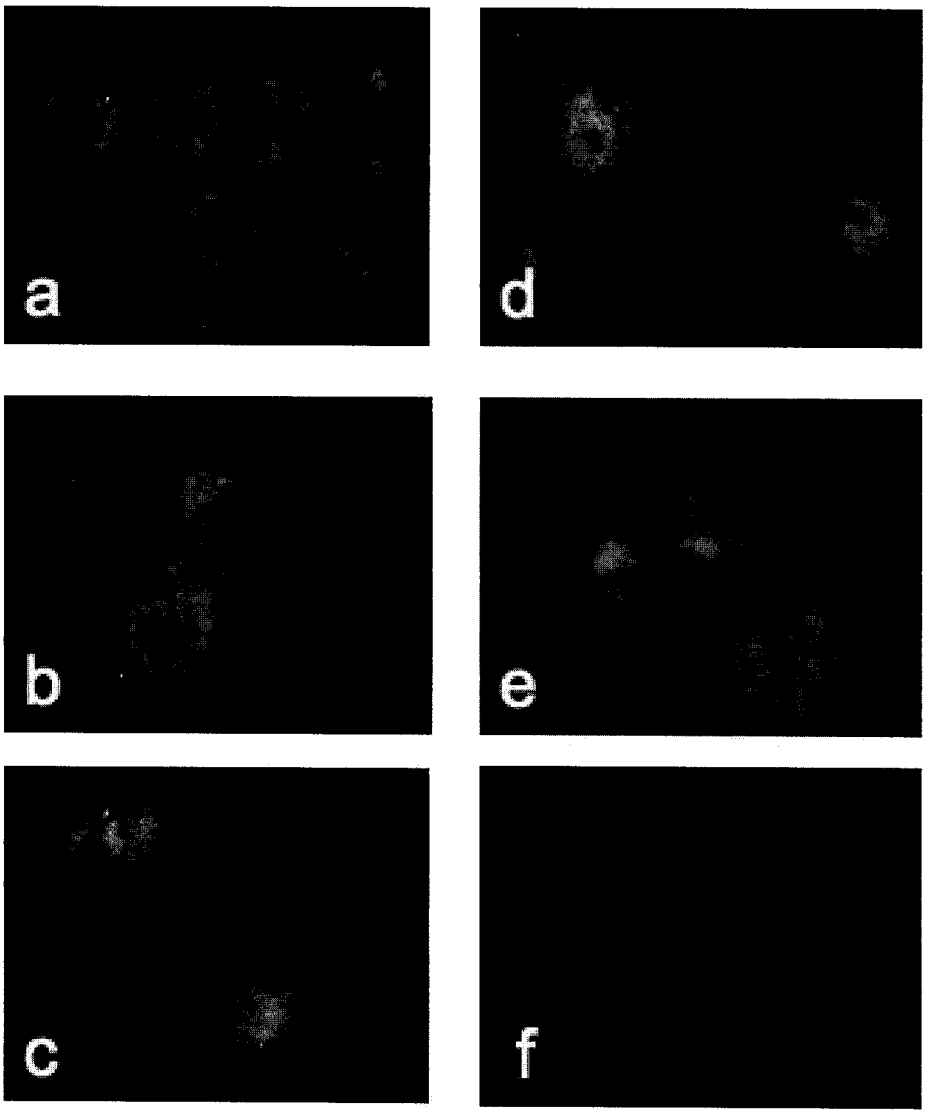

Figure 8.8. Immunofluorescence analysis of NF-KB activation. RAW 264.7 cells were treated *in vitro* for 45 minutes with (a) medium (b) calf thymus DNA, (c) S.t DNA, (d) LPS, or (e) E.h DNA and assessed for p65 sub-unit localization. Isotype controls were stimulated with LPS and probed with normal mouse IgG (f). Images are representative of three independent experiments.

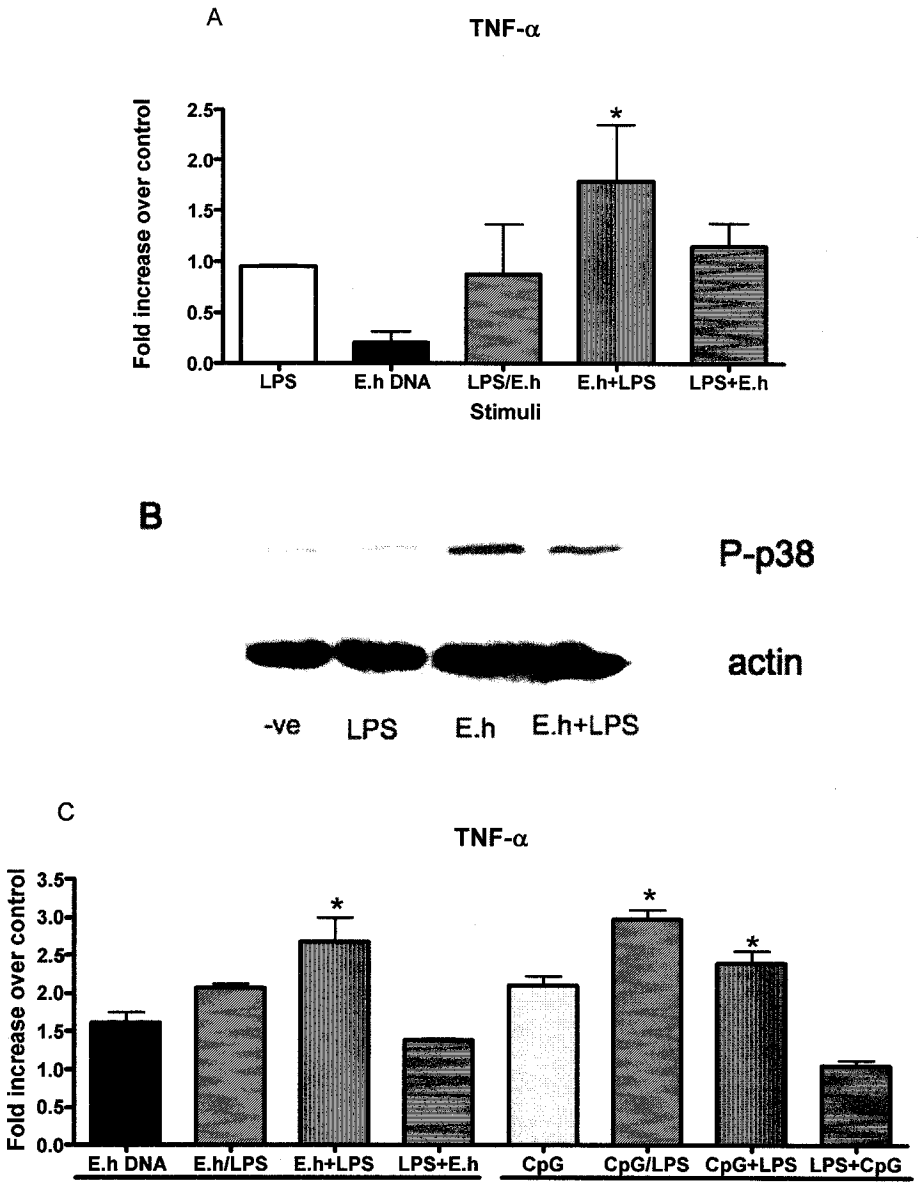

Figure 8.9. E.h DNA pre-treatment enhances subsequent activation of macrophages by sub-maximal LPS. A) RAW 264.7 cells were left untreated or pre-treated for 6 hours with sub-maximal LPS (0.1 ng/ml) or E.h DNA (1 μg/ml). After pre-treatment cells were washed and treated for 3 hours with media, LPS, E.h DNA or both simultaneously (LPS/Eh) at the concentrations above. TNF-α mRNA expression was measured by real time PCR. B) Western blot analysis of p38 phosphorylation in cells treated with medium, LPS (0.1 ng/ml), E.h DNA (1 μg/ml), or pre-treated with E.h DNA as above and subsequently stimulated with LPS. C) TNF-α mRNA expression in cells treated as above, or treated with CpG-ODN (2 μM) instead of E.h DNA. Data points represent mean ± SEM from three experiments. An asterisk indicates a significant increase in TNF-α expression compared to cells treated with sub-maximal doses alone ($P<0.05$).

Table 8.1. Prevention of ALA in gerbils by immunization with E.h DNA

Immunization group	%ALA of total liver weight	Viable parasites[a] ±	% Efficacy[b]
E.h DNA	26%±5	+	33
Gal-lectin	3% ±3	+	25
E.h DNA+Gal-lectin	0%	-	100*

[a] ALA contents were aspirated and viable trophozoites determined by cultured *in vitro*.
[b] Treatment efficacy to protect against ALA= 100 x (protected gerbils/infected gerbils)
(*P<0.05)

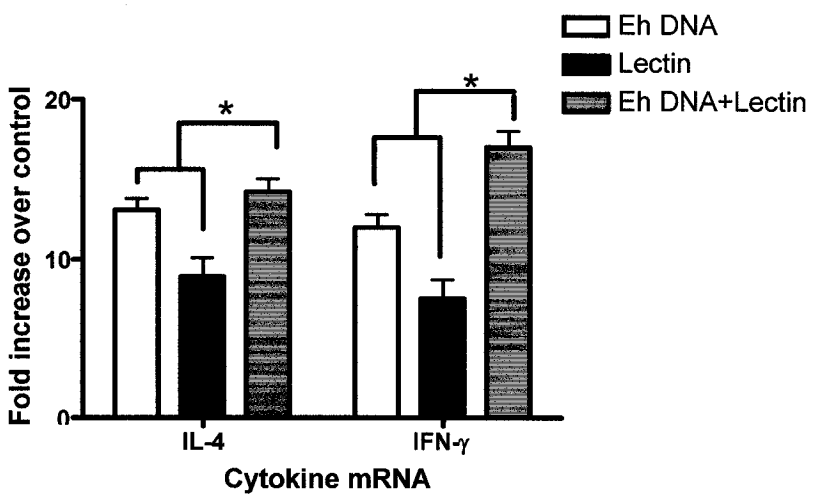

Figure 8.10. Taqman Real-time PCR analysis of spleen cytokine gene expression. Gene expression was normalized to 18srRNA and represented as fold increase over normal non-treated gerbil mRNA. There were higher levels of IFN-γ and IL-4 in the protected gerbils (*P<0.05). Data points represent means ± SEM of results from three independent PCR reactions.

Discussion

The mammalian immune system recognizes foreign DNA through TLR-9 expressed on immune cells. This recognition is ascribed to unmethylated CpG motifs, which are present at higher frequency in bacterial and other pathogen DNA. In the event of an *E. histolytica* infection, the lysis of trophozoites may allow the intestinal immune system to sample amebic DNA and induce an inflammatory response. In this report we show that *E. histolytica* DNA stimulates macrophage activation in a TLR-9 dependent manner, similar to CpG-DNA. Our studies clearly demonstrate the E.h DNA is a stimulus for macrophage activation. Consistent with the activation potential of CpG-DNA, E.h DNA induced TNF-α expression in murine macrophages. This cytokine expression was blocked by chloroquine and monensin, indicating signaling via TLR9. Moreover, we found that E.h DNA could induce NF-KB activation in HEK-TLR9 transfected cells. TLR signaling was confirmed by the requirement for MYD88 for E.h DNA activity. Taken together, these results demonstrate that E.h DNA can trigger innate immune responses.

Monocyte-macrophages, dendritic cells and B cells recognize unmethylated CpG dinucleotides through TLR9 [6]. It is well established that bacterial DNA is a potent immune stimulator due to the high frequency of these unmethylated CpG motifs. In contrast to bacterial DNA, *E. histolytica* DNA has an AT rich genome (75.3%) and CG dinucleotides are underrepresented [31]. It has been reported that *E. histolytica* has a DNA methyltransferase of the DNMT2 family, which targets ribosomal RNA [32]. Methylation seems to occur mostly at AT dinucleotides and not at the common

CpG site like in vertebrate systems [33]. It is interesting to note that despite the differences in DNA composition between bacteria and this parasite, E.h DNA was still able to induce some activation in macrophages. However, the variation in the magnitude of responses could be attributed to these innate differences in DNA composition, mainly the degree of CpG frequency methylation patterns. Consistent with this theory was the observation that *Salmonella typhimurium* DNA and CpG-ODN had greater activation potential than E.h DNA. Other protozoan parasite DNA like *Trypanosoma brucei*, have also been reported to activate immune cells, however at lower extents than bacterial DNA due to differential unmethylated CpG frequencies [34].

Prominent inflammation is a major component of amebiais and NF-KB activation is known to be required for initiation of this response to amebic infection [20]. NF-KB controls the gene expression of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. Here we report that E.h DNA can activate NF-KB via TLR9 signaling and induce the production of the pro-inflammatory cytokine TNF-α as well as nitric oxide in macrophages. It has been shown that activation of macrophages with Th1 cytokines results in amebicial activity mediated by nitric oxide production, and that this activation may be essential to innate immunity against the parasite [17,18,35]. Since macrophages have an important role in disease protection, it was important to identify a parasite molecule that was able to stimulate a cell-mediated response. Consistent with the crucial role of cellular responses, our immunization studies demonstrated resistance to amebic infection mediated by IFN-γ production. In

humans, higher IFN-γ production has been correlated to a reduced risk of amebic disease [36]. IFN-γ is a key Th1 cytokine, which could prime macrophages at the site of infection to respond amebic DNA and become cytotoxic against the invading parasite. Previous studies have demonstrated that repeated administration of CpG-ODN in mice could protect against lethal bacterial or parasite challenge infections and that this protection was associated with high IL-6 and IFN-γ production [37,38]. In this way, E.h DNA injections could have elicited immune activation sufficient enough to provide some protection against parasite challenge infections. However, combining E.h DNA with another strong Th1 immunogen, Gal-lectin, conferred complete protection mediated by higher Th1 responses.

The majority of cases of *E. histolytica* infection do not result in invasive disease however, the initiation of the invasion process remains unknown. Evidence suggests that modulation of host response may result in the establishment and persistence of amebic infection. It is possible that amebic colitis may be a result of immune responses to direct parasite insult but it could also be due to a combination of gut immune events that could facilitate invasion. It is increasingly observed that different TLR agonists can have an impact on the immune response to other TLR ligands resulting in altered inflammatory responses [39,40]. During infection, the immune system is exposed to various stimuli and the interplay between them could potentiate disease or attenuate it. In this study we have evaluated the priming and tolerance effects of LPS and E.h DNA. LPS is recognized by TLR4, which can signal via a MYD88-dependent and MYD88-indepent pathway [30], while CpG-DNA can only

signal in a MYD88-dependent manner [8]. Our results demonstrated that E.h DNA could prime macrophages to be responsive to otherwise unstimulating LPS doses. On the other hand, LPS rendered macrophages less responsive to E.h DNA, causing a tolerance effect. This effect could be due to the MYD88 pathways involved in TLR4 signaling, whereby pretreatment with LPS would down-regulate the potential to subsequently signal through TLR9, as MYD88 would have been activated. The reverse however, allows subsequent stimulation with LPS to signal through an alternative MYD88-independent pathway. Susceptibility to amebic infection is unknown, however one could postulate that previous, concurrent and subsequent intestinal infections with other pathogens could alter the outcome of amebic infection.

In conclusion, the results reported herein indicate that *E. histolytica* DNA could be a pathogen associated molecular pattern that is recognized by the innate immune system through TLR9. This finding further elucidates the initial pro-inflammatory host response to amebic infection and identifies another parasite molecule involved in the multifactorial process of amebiasis. Future studies will need to evaluate the activation potential of E.h DNA on other cell types and identify its role in pathogenesis and or protection from disease.

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SECTION III: GENERAL DISCUSSION

In this work, I address some of the important issues in the development of an amebiasis vaccine. *E. histolytica* Gal-lectin is a key molecule in parasite colonization and pathogenesis. The importance and relevance of this surface adhesin has made the Gal-lectin an extensively studied *E. histolytica* molecule. Its immunological properties are well documented and continue to be the focus of immunization trials. The Gal-lectin is highly antigenic and has been selected as a candidate molecule for an amebiasis vaccine. Vaccination studies to date have consistently reported the production of anti-Gal-lectin antibodies upon immunization; however, serum antibodies have not been correlated with protection from disease. At the intestinal level, stool anti-Gal-lectin IgA has been associated with resistance to disease in both humans and mice [1, 2]. However, in the mice model of amebiasis, vaccination prevented colonization but not disease, as the unprotected immunized animals developed cecitis. Therefore the mechanisms involved in the protection the Gal-lectin confers are undefined.

Dendritic cells (DCs) act as nature's adjuvant for regulating antigen-specific immunity. The recent discovery of Toll-like receptors (TLRs) has been a major advancement in our understanding of the mechanisms of innate immunity. In fact, DCs express TLRs that recognize foreign antigens, which in turn activate the cell and lead to immune responses against pathogens [3]. Pathogen-mediated activation can induce DCs to mature and migrate to secondary lymphoid organs where they prime T

cells. Depending on the pattern of cytokines induced by activation, the T cells will polarize toward either Th1 or Th2 response. *In vitro* studies have shown that re-stimulation of lymphocytes from drug-cured amebiasis patients with the Gal-lectin induces a specific Th1 profile, characterized by the secretion of INF-γ [4]. Our observation that the Gal-lectin could activate immature DCs and specifically induce Th1 cytokines both *in vitro* and *in vivo*, is consistent with the activity of TLR ligands. Although, we did not identify the TLR involved in Gal-lectin recognition, we demonstrated activation levels similar to those seen with a prototypical TLR ligand LPS. The results from our DCs studies help elucidate the mechanism by which the Gal-lectin can induce protective immunity. Upon vaccination, the Gal-lectin antigen is captured by sentinel immature DCs. The Gal-lectin is able to mature the DCs, as reported by upregulation of co-stimulatory molecules. This maturation initiates the migration to lymphoid organs where the Gal-lectin loaded DCs interact with naive T cells. The DCs secrete IL-12 and express the correct cell surface markers for T cell activation, which leads to Th1 polarization and the production of IFN-γ. In fact, we observed this phenomenon with the adoptive transfer of Gal-lectin pulsed DCs and it mirrored the effects of direct Gal-lectin immunizations. Thus, our work demonstrated that the Gal-lectin has the capacity to initiate the complex process of DC maturation, which is essential to convey pathogen-associated signals to the adaptive branch of the immune system.

We demonstrated that the Gal-lectin itself can induce IFN-γ production in vaccinated animals. Since cell-mediated responses, including the amebicidal activity of

macrophages are thought to be responsible to clear tissue dwelling parasites, INF-γ may be the key to protection against disease. Recent epidemiological evidence has demonstrated an association with higher INF-γ levels and resistance to symptomatic amebiasis [5]. To enhance the natural Gal-lectin immune response we set out to further modulate innate immunity by favoring a Th1 response with CpG-ODN adjuvant. CpG-ONDs directly activate DCs and B cells through TLR9, inducing a cascade of Th1 responses. These responses are non-specific, however, upon injection with an antigen, CpG-ODN increase the magnitude of the immune response to that antigen. Our observation that CpG-ODN and Gal-lectin immunization had a 100% vaccine efficacy, was attributed to increased Th1 responses. Consistent with our DCs studies, Gal-lectin alone provided some degree of protection, presumably by its innate Th1 driving properties. When this feature was enhanced with CpG-ODN we observed significant increases in IFN-γ , IL-12, and IL-2 at the time of infection compared to controls. We correlated increased Th1 responses with protection from amebic liver abscess development in the gerbil model of amebiasis.

E. histolytica is an intestinal pathogen and it is logical to direct vaccination research to increase mucosal immunity. CpG-ODNs are potent adjuvants at the mucosal level, significantly increasing antigen-specific IgA. Mucosal administration of CpG-ODN is known to induce both mucosal and systemic immunity [6]. The major limitation in the development of human mucosal vaccines is the lack of safe mucosal adjuvants. CpG-ODNs have shown promising clinical results when administered parenterally or mucosally. We tested our vaccine formulation via intranasal immunizations and

observed the induction of both Gal-lectin specific IgA and systemic responses. We showed for the first time that immunization with a novel adjuvant, with potential use in human, could induce stool IgA against the Gal-lectin but also provide systemic immunity, which conferred protection against amebic liver abscess challenge. The stool IgA against the Gal-lectin was capable of blocking parasite adherence to target cells *in vitro*. We did not evaluate the colonization blocking potential of stool anti-Gal-lectin IgA, but future studies will determine if CpG-ODN and Gal-lectin can effectively prevent intestinal amebiasis. Protection from amebic liver abscess formation was associated with higher pre-challenge INF-γ levels, indicating that mucosal immunization was sufficient to induce similar protective immunity as observed with parenteral vaccine administration. In light of the need to concentrate amebiasis vaccine research on mucosal immunizations, our study has identified an appropriate adjuvant capable of inducing protective systemic immunity and inducing potential colonization blocking anti-Gal-lectin IgA.

In the context of using TLR ligands to skew the immune response towards cell-mediated Th1 immunity, we identified *E. histolytica* DNA as an amebic molecule able to activate macrophages through TLR9. Our observation that *E.histolytica* DNA could effectively induce macrophage TNF-α production was surprising. *E. histolytica* is known to have an under representation of CpG dinucleotides in its genome and also has an active DNA methyltransferase [7, 8]. These features would indicate that the parasite has evolved to avoid immune recognition, but our results demonstrate the activation potential of *E. histolytica* DNA. This could be due to a several factors: first

the parasite's DNA methyltransferase does not seem to mediate the methylation of genomic DNA, which would leave the CpG dinucleotide unmethylated and recognizable; second the paucity of CpG dinucleotides does not preclude their immunostimulatory activity. We observed that the magnitude *E. histolytica* DNA activation was not as impressive as with LPS or *Salmonella* DNA, however, it was significantly higher than mammalian DNA controls. Amebic DNA induced macrophage TNF-α secretion and nitric oxide production, which could potentiate macrophage amebicidal activity. These *E. histolytica* DNA effects were mediated by TLR9, as shown by blocking endosomal acidification and by loss of activation in MYD88 deficient dendritic cells. This identifies *E. histolytica* DNA as a pathogen associated molecular pattern which like Gal-lectin has the capacity to activate innate immune system cells. When gerbils were immunized with *E. histolytica* DNA some protective immunity was observed, however combining the DNA with the Gal-lectin conferred 100% protection against amebic liver abscess challenge. This confirmed that by further enhancing the activation of immune cells with *E. histolytica* DNA towards a Th1 response, the protective properties of the Gal-lectin were also magnified. Furthermore *E. histolytica* DNA could have increased cell-mediated macrophage responses required to clear the parasite.

Putting these observations together, we envisage a vaccination system (see Figure III) whereby the Gal-lectin and another innate system stimulator (CpG-ODN or *E. histolytica* DNA) work together to produce Gal-lectin specific immunity. The Gal-lectin can activate DCs and stimulate the production of IL-12 which directs a IFN-γ

mediated response. CpG-ODN or *E. histolytica* DNA further enhance immune activation of DCs, B cells and macrophages. The production of INF-γ primes macrophages for nitric oxide production upon TNF-α activation. Gal-lectin specific IgG and IgA are produced that can block parasite adherence. Memory Gal-lectin specific T and B cells are produce and upon subsequent challenge infection a strong Th1 and IgA response is activated that can efficiently eliminate the parasite. In this way the Gal-lectin confers protective immunity, by harnessing the activation power of TLRs in innate immunity with CpG-ODNs and *E. histolytica* DNA.

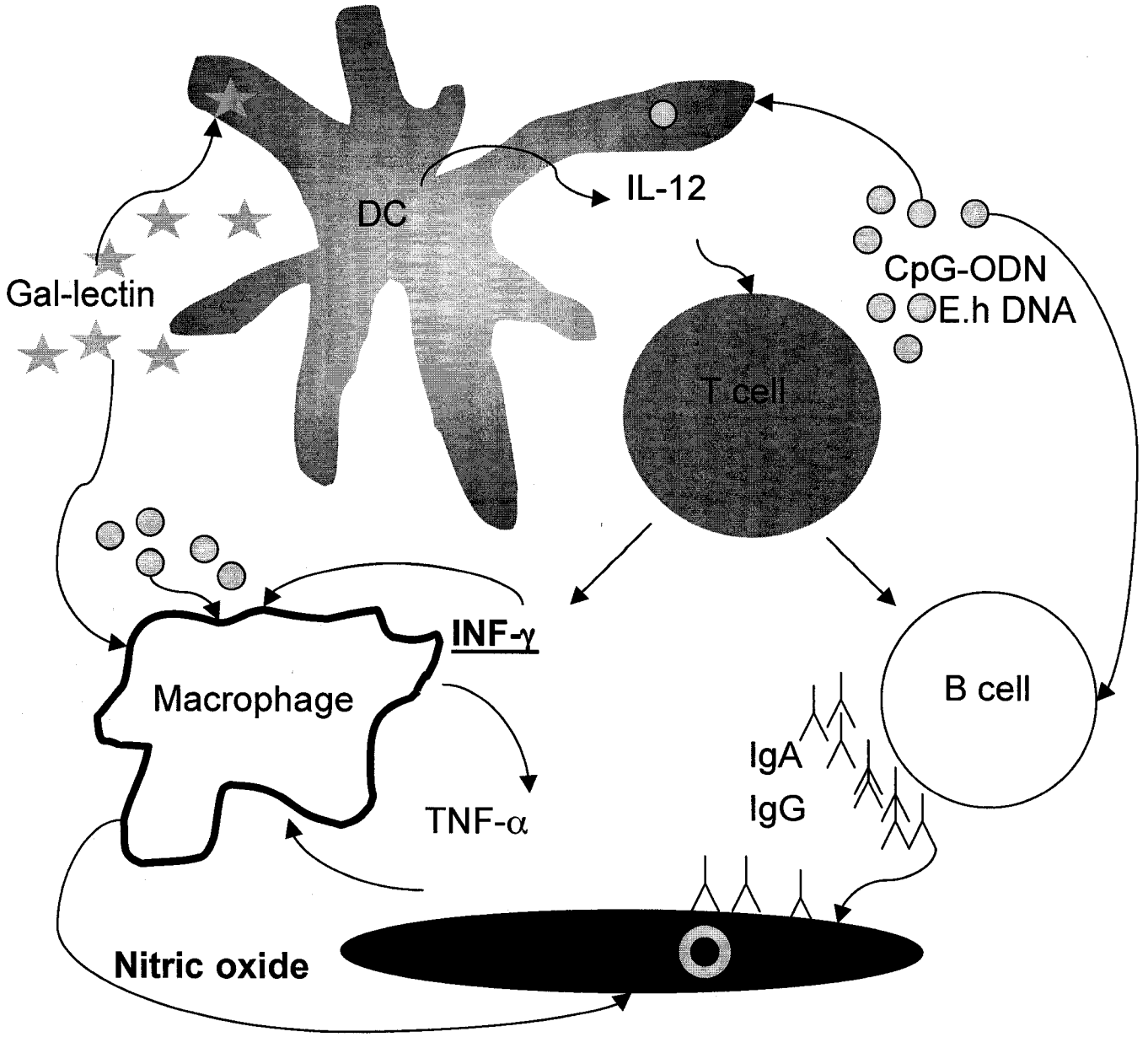

Figure III: Model of protective immunity conferred by a Gal-lectin and CpG-ODN vaccine. Upon immunization with Gal-lectin and CpG-OND, dendritic cells (DCs) capture the antigen and are activated to mature and migrate to T cell rich lymphoid organs. Gal-lectin loaded DC produce IL-12 and signal the differentiation of Gal-lection specific Th1 T cells. Immunization with CpG-ODN adjuvant further activates DCs, B cells and macrophages. Gal-lectin specific IgA (mucosal) and IgG (systemic) are produced. Upon challenge infection with *E. histolytica,* Gal-lectin specific antibodies can block parasite colonization and T cells respond with INF-γ production, which primes macrophages for activation with Gal-lectin or *E. histolytica* DNA to become amebicidal.

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Appendix

Thesis guidelines state that: " Theses involving human participants, animal subjects, microorganisms, living cells, other biohazards, and/or radioactive materials, shall include the appropriate compliance certification."

The required documents are appended in the following pages. It should be noted that certification was obtained both at McGill University and the University of Calgary. Also included in this section are the copyright waiver forms for published manuscripts.

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07

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PATHOGENS/PIMS: Human Cell Lines

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