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The Role of Cytokines in Host Defence Against *Entamoeba histolytica*

John Darren Campbell

Institute of Parasitology

McGill University, Montréal

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Doctor of Philosophy

ABSTRACT

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis. While infection is associated with suppression of cell-mediated immunity, drug-cured patients are resistant to reinvasion by amoebae. Macrophages are the principal effector cells involved in host defence against *E. histolytica* via production of nitric oxide which is cytotoxic for the parasite. The objective of this study was to determine the T cell cytokine responses associated with host defence against *E. histolytica*. A mixed Th1/Th2 (Th0) response predominated at days 5-10 of amoebic liver abscess development in gerbils, as indicated by spleen and hepatic lymph node cell IL-2 (Th1 marker) and IL-4 (Th2 marker) production. However, T cell responses were profoundly suppressed at day 20 of infection. Serum collected at day 20, but not at other times postinfection, markedly suppressed T cell proliferative responses by inhibiting IL-2 production. A switch to a Th1 response occurred after day 20 of infection. Following drug-abbreviation of infection at day 20, animals were completely resistant to challenge infection in the liver and demonstrated a Th1 response. The Gal-lectin 170-kDa heavy subunit of *E. histolytica* is a protective antigen in gerbils and a potential subunit vaccine candidate. We determined which region of the Gal-lectin stimulates IL-12 production, as IL-12 is key to inducing Th1 cytokine responses. Native Gal-lectin plus interferon-γ stimulated IL-12 p40 and p35 gene transcription and IL-12 p70 protein production in human macrophages. Using a panel of anti-170-kDa subunit monoclonal antibodies in inhibition studies, aa 596-998 was identified as the IL-12-inducing domain. These results suggest that this portion of the Gal-lectin has potential for use as a subunit vaccine to induce Th1-mediated immunity against *E. histolytica*.

ABRÉGÉ

Entamoeba histolytica est le parasite protozoaire qui cause l'amibiase. Bien que l'infection est associée à une suppression de l'immunité cellulaire, les patients qui ont été guéris à l'aide de médicaments deviennent résistants à la réinvasion. Les macrophages sont les principales cellules effectrices impliquées dans la défense de l'hôte contre *E. histolytica,* en vertu de leur production d'oxyde nitrique, laquelle est cytotoxique pour le parasite. L'objectif de cette étude était d'identifier les cytokines de lymphocyte T qui sont associées à la défense de l'hôte contre *E. histolytica.* Une réponse mixte Th1/Th2 (Th0) dominait aux jours 5-10 du développement d'abcès hépatiques chez les gerboises infectées, puisque les cellules de la rate et du ganglion lymphatique hépatique produisaient de l'IL-2 (marqueur de Th1) et de l'IL-4 (marqueur de Th2). Toutefois, la réponse des lymphocytes T était sévèrement supprimée au jour 20 de l'infection. Seul le sérum prélevé à ce jour a supprimé de façon marquée la prolifération des lymphocytes T en empêchant leur production d'IL-2. Les cellules ont ensuite exprimé une réponse Th1 aux jours subséquents. Suite au traitement de l'infection à l'aide de médicaments au jour 20, tous les animaux étaient résistants à une réinfection dans le foie, et leurs lymphocytes T exhibaient une réponse Th1. L'unité de 170 kDa de la Gal-lectine d'*E. histolytica* est un antigène protecteur chez les gerboises et un vaccin potentiel. Nous avons identifié la portion de la Gal-lectine qui est responsable de la stimulation de la production d'IL-12, une cytokine importante pour l'induction d'une réponse Th1. La Gal-lectine purifiée, en présence d'interféron-, a stimulé la transcription des gènes d'IL-12p40 et p35, et la production de la protéine IL-12p70 par des macrophages humains. En se servant d'une panoplie d'anticorps monoclonaux qui reconnaissent l'unité de 170 kDa, les acides aminés 596-998 ont été identifiés comme étant responsables de l'induction d'IL-12. Ces résultats suggèrent que cette portion de la Gal-lectine est un vaccin potentiel pour l'induction d'une immunité cellulaire contre *E. histolytica.*

ACKNOWLEDGEMENTS

Firstly, I should like to thank my research supervisor, Dr. Kris Chadee, whose support, guidance, patience and encouragement made this work possible and are most appreciated.

I'd also like to express my thanks to all the other professors at the Institute of Parasitology for their advice and help throughout my Ph.D. programme. Additional thanks go to Dr. Barbara Mann at the University of Virginia for providing the Gal-lectin and anti-lectin antibodies used in this work.

A special thanks is extended to Kathy Keller, the research technician in our laboratory. Kathy's continual help, resourcefulness and friendship were crucial to the completion of the experiments in this thesis. Thanks also to Farzaneh Jalili for blood collection and Gordon Bingham for taking care of the gerbils.

My fellow lab mates, past and present, made our laboratory a truly enjoyable place in which to work. A big thanks to these individuals for their support and companionship: Dr. Jian Lin, Rosanne Séguin, Adam Belley, Dr. Markus Göettke, Dr. Sergio Arias, Rosita Garcia, Miriam Borrios-Rodiles, Denis Gaucher, Yi Yu, and Darcy Moncada. Also, thanks to all the students and staff at the Institute for their friendship.

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THESIS OFFICE STATEMENT

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

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Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (*eg.* in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

STATEMENT OF ORIGINALITY

The following aspects described in this thesis are considered contributions to original knowledge:

MANUSCRIPT I

Th1 cytokines promote macrophage activation, which is important for anti-amoebic defence. Th2 cytokines have suppressive effects on macrophages. However, the relative production of Th1 and Th2 cytokines during *Entamoeba histolytica* infection and in humans or animals resistant to infection has not been examined. This work demonstrated that a mixed Th1/Th2 (Th0) response occurs during early amoebic liver abscess development in gerbils, followed by suppression of T cell responses and a switch to a Th1 response during late infection. Drug-cured gerbils also demonstrated a Th 1 response and were resistant to challenge infection. Therefore, the data in this manuscript indicate that a Th1 cytokine response is associated with resistance to *E. histolytica* infection.

MANUSCRIPT II

This is the first report to identify a mechanism involved in suppression of T cell proliferative responses during amoebiasis. Serum collected from gerbils at day 20 of infection suppressed T cell proliferative responses *in vitro*. The inhibitory mechanism was independent of nitric oxide and prostaglandin but was associated with reduced interleukin-2 (IL-2) production. T cell proliferation was partially restored by the addition of exogenous IL-2. These findings indicate that serum suppression of T cell proliferative responses during acute amoebiasis is mediated by inhibition of IL-2 production.

MANUSCRIPT III

The *E. histolytica* galactose-adherence lectin (Gal-lectin) is a protective antigen in

the gerbil model of amoebiasis. This is the first report to demonstrate that the Gal-lectin promotes IL-12 production from macrophages. Furthermore, the region of the molecule mediating IL-12 induction in human macrophages was identified and shown to be immunogenic. IL-12 drives the differentiation of antigen-stimulated T cells into the Th1 phenotype which is associated with resistance to *E. histolytica* (Manuscript 1). Hence, this work reveals a mechanism by which immunization with the Gal-lectin may act to induce a protective Th1 response. The IL-12-inducing region of the Gal-lectin has strong potential for use as a subunit vaccine against amoebiasis.

STATEMENT OF AUTHORSHIP

The experiments described in Manuscripts I-III were designed and carried out by myself. I am also responsible for the data analysis and manuscript preparation. Dr. Kris Chadee, co-author of all three manuscripts, acted as thesis supervisor. Dr. Chadee provided financial resources for the laboratory work and advice on experimental design, data analysis and preparation of the manuscripts and thesis. Mr. Denis Gaucher, a co-author of Manuscript II, provided some of the gerbil serum samples used in the study and critical review of the manuscript. Dr. Barbara Mann, a co-author of Manuscript III, prepared and provided the purified *Entamoeba histolytica* galactose-adherence lectin and anti-lectin monoclonal antibodies used in the study.

SECTION I: LITERATURE REVIEW

INTRODUCTION

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis. Intestinal and extra-intestinal amoebiasis is estimated to cause 40,000 to 110,000 deaths every year (1, 2). Among parasitic diseases, only malaria and schistosomiasis cause more morbidity and mortality on a global scale (1). Invasive *E. histolytica* infections can be controlled, in most cases, by metronidazole (3). However, extensive tissue pathology may occur by the time of diagnosis and treatment which emphasizes the importance of developing effective immunotherapy. Immunization of gerbils with amoebic molecules such as the galactose-adherence lectin (Gal-lectin) protects against *E. histolytica* infection (4, 5). The Gal-lectin is a 260-kDa surface molecule composed of 170-kDa and 35-kDa subunits (6). The Gal-lectin mediates amoebic adherence to colonic mucins and mammalian cells and is the most promising *E. histolytica* vaccine candidate molecule (7).

Patients with amoebic liver abscess have progressive disease in the presence of high titres of anti-*E. histolytica* antibodies (8). Indeed, amoebae have innate resistance to antibody-mediated defences (9-11). In contrast, the parasite is sensitive to cell-mediated immunity (CMI). Antigen-stimulated T cells from cured amoebiasis patients killed *E. histolytica in vitro* (12). In addition, supernatants from these T cells activated normal macrophages for contact-dependent amoebicidal activity. Murine macrophages activated *in vitro* with interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) or IFN-γ and lipopolysaccharide (LPS) killed amoebae by a nitric oxide (NO)-dependent mechanism (13, 14). However, T cell and macrophage functions are suppressed during amoebic infections. Macrophages in the vicinity of the amoebic abscess are hyporesponsive for cytokine and NO production as well as amoebicidal activity (15-17). T cells from amoebiasis patients

are also hyporesponsive to mitogen-induced proliferation (12, 18). The association of progressive infection and suppressed T cell and macrophage responses provides corroborating evidence for the importance of functional CMI in host defence.

Although T cell cytokine responses in amoebiasis remain largely uncharacterized, available data suggest that resistance to *E. histolytica* is mediated by Th1 responses *in vivo*. T cell responses have been divided into Th1 and Th2 phenotypes based on the pattern of cytokines produced following antigen-stimulated T cell activation. Th1 responses are characterized by interleukin-2 (IL-2), IFN-γ and TNF-ß cytokine production and promote activation of CMI. Th2 responses are characterized by IL-4, -5, -6, -10 and -13 production and promote humoral immunity (19). Mixed Th1/Th2 (Th0) responses may also develop. The development of Th1 and Th2 responses is profoundly influenced by IL-4 and IL-12 produced by cells of the innate immune system early in infection. Basophils produce IL-4 which drives Th2 development, whereas macrophages produce IL-12 which directs differentiation to Th1 cells (20, 21). Resistance or susceptibility to infectious diseases is often critically dependent on the development of the appropriate Th cell response (22). We hypothesized that immunization with the Gal-lectin stimulates IL-12 production and a protective Th1 response. In support of this possibility, the Gal-lectin has already been shown to stimulate murine macrophage TNF-α production (23). In addition, T cells from cured amoebiasis patients produce the Th1 cytokines IFN-γ and IL-2 when stimulated with Gal-lectin *in vitro* (24).

The objective of this study was to determine the role of Th cytokine responses in host defence against *E. histolytica*. The specific aims were: (1) to determine Th1/Th2 cytokine responses associated with infection and resistance to *E. histolytica* in gerbils, (2) to elucidate mechanisms involved in suppression of T cell responses during infection, and (3) to determine whether the Gal-lectin could potentially influence Th cell responses by inducing macrophages to produce IL-12. Contingent on this, a further goal was to identify the region of the Gal-lectin responsible for stimulating IL-12 production. The long-term

goal of this research is to develop a Gal-lectin-based subunit vaccine to prevent amoebiasis. In this respect, it is essential to understand the nature of the immune response(s) to *E. histolytica* and to identify the region of the Gal-lectin that is both immunogenic and key to inducing the desired Th cell response.

REFERENCES

1. Walsh J.A. (1986) Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev. Infect. Dis.* 8: 228-238.

2. World Health Organization (1995) *The World Health Report.* World Health Organization, Geneva.

3. Biagi F. (1981) Amebiasis. *Antibiot. Chemother.* 30: 20-27.

4. Petri W.A. Jr., and Ravdin J.I. (1991) Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of *Entamoeba histoyltica. Infect. Immun.* 59: 97-101.

5. Soong C-J. G., Kain K.C., Abd-Alla M., Jackson T.F.H.G., and Ravdin J.I. (1995) A recombinant cysteine-rich section of the *Entamoeba histolytica* galactose-inhibitable lectin is efficacious as a subunit vaccine in the gerbil model of amebic liver abscess. *J. Infect. Dis.* 171: 645-651.

6. Petri W.A. Jr., Chapman M.D., Snodgrass T.L., Mann B.J., Broman J., and Ravdin J.I. (1989) Subunit structure of the galactose and N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica. J. Biol. Chem.* 264: 3007-3012.

7. Petri W.A. Jr., Smith R.D., Schlesinger P.H., and Ravdin J.I. (1987) Isolation of the galactose-binding lectin which mediates the *in vitro* adherence of *Entamoeba histolytica. J. Clin.Invest.* 80:1238-1244.

8. Krupp I. (1970) Antibody response in intestinal and extraintestinal amebiasis. *Am. J.*

Trop. Med. Hyg. 19: 57-62.

9. Espinosa-Cantellano M., and Martínez-Palomo A. (1994) *Entamoeba histolytica*: Mechanism of surface receptor capping. *Exp. Parasitol.* 79: 424-435.

10. Tran V.Q., Herdman D.S., Torian B.E., and Reed S.L. (1998) The neutral cysteine proteinase of *Entamoeba histolytica* degrades IgG and prevents its binding. *J. Infect. Dis.* 177: 508-511.

11. Braga L.L., Ninomiya H., McCoy J.J., Eacker S., Wiedmer T., Pham C., Wood S., Sims P.J., and Petri W.A. Jr. (1992) Inhibition of the complement membrane attack complex by the galactose-specific adhesin of *Entamoeba histolytica*. *J. Clin. Invest.* 90: 1131-1137.

12. Salata R.A., Martinez-Palomo A., Murray H.W., Conales L., Trevino N., Segovia E., Murphy C.F., and Ravdin J.I. (1986) Patients treated for amebic liver abscess develop cell-mediated immune responses effective in vitro against *Entamoeba histolytica. J. Immunol.* 136: 2633-2639.

13. Lin J-Y., and Chadee K. (1992) Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. *J. Immunol.* 148: 3999-4005.

14. Lin J-Y., Séguin R., Keller K., and Chadee K. (1994) Tumor necrosis factor alpha augments nitric oxide-dependent macrophage cytotoxicity against *Entamoeba histolytica* by enhanced expression of the nitric oxide synthase gene. *Infect. Immun.* 62: 1534-1541.

15. Denis M., and Chadee K. (1988) *In vitro* and *in vivo* studies of macrophage functions in amebiasis. *Infect. Immun.* 56: 3126-3131.

16. Wang W., Keller K., and Chadee K. (1992) Modulation of tumor necrosis factor production by macrophages in *Entamoeba histolytica* infection. *Infect. Immun.* 60: 3169-3174.

17. Wang W., Keller K., and Chadee K. (1994) *Entamoeba histolytica* modulates the

nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxoicity against amoebae and tumour cells. *Immunology* 83: 601-610.

18. Simjee A.E., Gathiram V., Coovadia H.M., Jackson T.F.G.H., Kiepiela P., Pudifin D.J., and Stretton M. (1985) Cell-mediated immunity amoebiasis.*Trans. R. Soc.Trop. Med.Hyg.* 79: 165-168.

19. Mosmann T.R., and Sad S. (1998) The expanding universe of T cell subsets: Th1, Th2 and more. Immunol. Today 17: 138-146.

20. Scott P. (1993) IL-12: Initiation cytokine for cell-mediated immunity. *Science* 260: 496-497.

21. Swain S.L. (1993) IL-4 dictates T-cell differentiation. *Res. Immunol.* 144: 567-651.

22. Powrie F., and Coffman R.L. (1993) Cytokine regulation of T-cell function: potential for therapeutic intervention. *Immunol. Today* 14: 270-274.

23. Séguin R., Mann B.J., Keller K., and Chadee K. (1995) Identification of the galactose-adherence lectin epitopes of *Entamoeba histolytica* that stimulate tumor necrosis factor-alpha production by macrophages. *Proc. Natl. Acad. Sci. USA* 92: 12175-12179.

24. Schain D.C., Salata R.A., and Ravdin J.I. (1992) Human T-lymphocyte proliferation, lymphokine production, and amebicidal activity elicited by the galactose-inhibitable adherence protein of *Entamoeba histolytica*. *Infect. Immun.* 60: 2143-2146.

CHAPTER 1

Entamoeba histolytica and Amoebiasis[1]

1.1 *E. histolytica* Infection

E. histolytica is the causative agent of intestinal and extra-intestinal amoebiasis. The infection is initiated by the ingestion of *E. histolytica* cysts in faecally-contaminated food or water. The life cycle has been well documented (1). Following ingestion, the cysts excyst in either the small or large intestine. Eight amoebic trophozoites arise from one cyst and colonize the mucosal layer of the colon. Trophozoites continue to multiply by binary fission and form cysts that pass out with the stool. Infected individuals may secrete up to 45 million cysts per day (1). Previously, it was thought that *E. histolytica* organisms could be either pathogenic or non-pathogenic as not all gut infections were associated with disease. It is now recognized that there are two morphologically similar but distinct species of *Entamoeba*; *E. histolytica* and *E. dispar* (2). *E.histolytica* is the pathogen which causes invasive amoebiasis, whereas *E. dispar* is a gut commensal. The two species of *Entamoeba* can be clearly differentiated by isoenzyme analysis, typing by monoclonal antibodies and DNA analysis (2).

Invasion of the colonic epithelium by *E. histolytica* causes intestinal amoebiasis (amoebic dysentery). Patients with amoebic dysentery typically present with lower abdominal pain and bloody diarrhoea. The resulting dehydration can be fatal in untreated

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[1]Some sections of Chapter 1 have been adapted from the following publications;

Campbell D. and Chadee K. (1999). Mechanisms of Host Resistance to *Entamoeba histolytica*, in *Tropical Medicine: Science and Practice, vol.1:Amebiasis* (S. Hoffman and G. Pasvol, eds.). Imperial College Press, London (in press)

Campbell D. and Chadee K. (1997) Survival strategies of *Entamoeba histolytica*: Modulation of cell-mediated immune responses. *Parasitol.Today* 13: 184-190.

individuals. Following disruption of the colonic epithelium, amoebae may initiate extra-intestinal amoebiasis by disseminating via blood and lymph to other organs, most commonly the liver. Patients who develop amoebic liver abscess generally experience right upper abdominal pain and fever (3). The abscess appears as a well-defined fluid-filled lesion surrounded by a thin wall of connective tissue (4). The natural tendency for the amoebic liver abscess is to enlarge over time. Intraperitoneal rupture of the abscess is a serious and potentially fatal complication. Intestinal colonization by *E. histolytica* can be effectively treated with luminal agents such as paromomycin, which is well tolerated (5). The drug of choice for invasive amoebiasis is metronidazole in combination with a luminal agent. Metronidazole is usually effective, but can have debilitating side effects and a few patients fail to respond to the drug (6). While drug resistance does not appear to be a serious problem at present, a multidrug resistance phenotype of *E. histolytica* has been generated *in vitro* (7). This suggests the possibility of drug resistance *in vivo* and highlights the importance of developing an effective vaccine to prevent infection.

1.2 Epidemiology of Amoebiasis

There are between 40 and 50 million cases of intestinal and extra-intestinal disease resulting from *E. histolytica* infection every year. The disease claims 40,000 to 110,000 lives annually, making it the third leading parasitic cause of death behind malaria and schistosomiasis (8, 9). Amoebiasis has a global distribution, but the highest levels of *E. histolytica* infection and endemicity occur in developing countries in Central/South America, Africa and Asia (10-12). Poor sanitary conditions, inadequate personal hygiene and malnutrition favour the spread of amoebiasis (13). Amoebic liver abscess appears to be about ten times more common in men compared to women (14). However, young children, the elderly and pregnant women experience the most severe cases of amoebiasis (8, 15). In the developed world, the principal risk factor is travel to an endemic area (16).

In addition, sexually active male homosexuals, those infected with HIV and institutionalized individuals are also at higher than average risk of contracting *E. histolytica* and developing amoebiasis (16-18).

1.3 Pathogenesis of Amoebiasis

Invasion of the intestinal epithelium and liver by *E. histolytica* causes extensive tissue pathology. Amoebic trophozoites establish in the gut by adherence to the intestinal mucus layer. *E. histolytica* initiates the process of invasion by producing a potent mucus secretagogue which can cause hypersecretion and the eventual depletion of mucin stores (19). This exposes the epithelial layer, permitting amoebic adherence to and lysis of intestinal cells followed by tissue invasion.

Adherence is an absolute requirement for *E. histolytica* gut colonization, lysis of target cells and tissue invasion. Using Chinese hamster ovary (CHO) cells as targets, it was found that amoebic adherence to and subsequent lysis of cells was inhibited by galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) but not by other monosaccharides (20). Galactose-inhibition of adherence was confirmed in studies of amoebic adherence to human neutrophils (21), monocytes (22) and human and rat colonic mucins (23). The Gal/GalNAc-inhibitable amoebic adherence molecule is a heterodimeric 260-kDa surface lectin (Gal-lectin) composed of a 170-kDa heavy subunit and a 31-35-kDa light subunit, linked by disulphide bonds (24). Monoclonal antibody (mAb)-inhibition studies have demonstrated that the 170-kDa heavy subunit contains the carbohydrate-binding domain (25). The cytoplasmic domain of the 170-kDa subunit has amino acid sequence identity to ß2 integrin cytoplasmic tails, including the residues involved in activating adhesion by transduction of signals to the extracellular integrin domains (26). Intracellular expression of a fusion protein containing the cytoplasmic domain of the Gal-lectin heavy subunit had a dominant negative effect, inhibiting amoebic adhesion to target cells and amoebic liver

abscess formation in gerbils (26). These results provide direct *in vivo* evidence of the importance of Gal-lectin-mediated adherence to amoebic virulence.

The gut environment may influence the virulence of *E. histolytica* and its potential to invade. Adherence and phagocytosis of live bacteria increased amoebic virulence as measured by ability to destroy cell monolayers *in vitro* (27, 28). Ingested bacteria may act as scavengers of oxidized molecules and stimulate the electron transport system of amoebae (27). Upon invasion of the epithelium, amoebae diffuse laterally and lyse neighbouring cells causing extensive tissue damage and ulceration in the process. Amoebae may then spread via the hepatic portal vein to the liver and initiate amoebic liver abscesses.

Both host and parasite mechanisms are involved in the pathology of amoebiasis. Soluble products from *E. histolytica* stimulate Interleukin-8 (IL-8) production from human colonic cells (29). IL-8 is a potent neutrophil chemoattractant; an activator which can initiate an inflammatory response and tissue injury of the intestinal epithelium prior to amoebic invasion (29). Trophozoites produce neutral cysteine proteases which are capable of causing pathology through a cytopathic effect on cells and degradation of cell-anchoring proteins, such as fibronectin and laminin (30, 31). The exact contribution of cysteine proteases to amoebic liver abscess development remains to be clarified. Treatment of amoebic trophozoites with the protease inhibitor E64 suppressed liver abscess formation in severe combined immunodeficient (SCID) mice (32). However, antisense inhibition of *E. histolytica* cysteine protease expression affects phagocytosis but not cytopathic activity (33).

The development of amoebic liver abscesses in gerbils is characterized by infiltration of inflammatory cells such as neutrophils, macrophages and lymphocytes (34). *E. histolytica* promotes inflammatory cell infiltration by producing a chemoattractant signal for neutrophils (35). Much of the tissue pathology of amoebic liver abscess may be caused by products released by infiltrating cells after they are lysed by amoebae. Lysis of human neutrophils by *E. histolytica* has been shown to damage hepatocytes in an *in vitro* model

(36). In addition, studies in animal models demonstrated that toxic products released from dying neutrophils (neutral proteases, cathepsins and lysozymes) are important in the initial development of hepatic lesions (34, 37). Target cells are killed within 5-15 min after amoebae adhere via the Gal-lectin (38). Amoebic calcium-dependent and calcium-independent phospholipase A have been implicated in the cytolysis (39). Phospholipase A may disrupt cell membranes, rendering the target cell vulnerable to attack by amoebic enzymes or toxins. Amoebae also secrete an ion channel pore-forming protein (amoebapore) that spontaneously incorporates itself in target cell membranes making them permeable to ion influx and consequent depolarization (40, 41). Whether the target cells undergo a necrotic death or apoptosis is unclear. Human myeloid cell lines exposed to purified amoebapores were killed by necrosis, rather than apoptosis, as revealed by morphological criteria and the absence of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) (42). In contrast, murine myeloid cells incubated with *E. histolytica* died by apoptosis as revealed by DNA ladder fragmentation (43). Also, TUNEL staining and DNA laddering demonstrated that apoptosis occurred in inflammatory cells and hepatocytes isolated from the amoebic liver abscess (44).

1.4 Host Defence in Amoebiasis

Both humoral and cell-mediated immune responses to *E. histolytica* develop in natural human infections and in experimentally-induced animal infections. However, cell-mediated immunity is suppressed during infection and amoebae have innate defences which protect against antibody responses. Accordingly, the host immune response is often ineffective in controlling initial *E. histolytica* infections (45). In contrast, evidence from clinical studies and animal models suggests that immunological resistance to re-infection with *E. histolytica* develops. Patients drug-cured of amoebic liver abscess rarely have recurrent invasive amoebiasis. A recurrence rate of only 0.29% was reported in a 5-year

study of amoebic liver abscess patients in Mexico (46), a level much lower than would have been expected in an endemic area. In addition, it has been repeatedly demonstrated that various immunization regimes with defined amoebic antigens generate protective immunity in animal models of the disease (47). These findings speak directly to the feasibility of future vaccine development for control of amoebiasis.

1.4.1 Innate Defences

Amoebae encounter natural "barriers" to infection in both the gut and systemic circulation following tissue invasion. These innate defences may prevent or partially inhibit invasive amoebiasis, although the level of protection afforded by innate resistance is difficult to quantify precisely. In the gut, factors such as competition by intestinal bacteria for attachment to mucins may inhibit amoebic colonization (48). Binding to colonic mucins inhibits amoebic adherence to and cytolysis of target cells (23). Therefore, mucins serve as a first line of host defence in the gut by inhibiting amoebic attachment to the underlying mucosal epithelial cells.

In the systemic circulation, *E. histolytica* is exposed to complement, another innate resistance mechanism that should act to decrease the numbers of invading amoebae. *E. histolytica* activates both the alternative and classical pathways of complement but is resistant to complement-mediated lysis (49). This resistance is due to the Gal-lectin which has sequence homology and antigenic cross-reactivity with CD59, a membrane inhibitor of C5b-9 in human erythrocytes (50). The Gal-lectin binds C8 and C9, effectively preventing assembly of the C5b-C9 complement membrane attack complex and subsequent lysis (50). Treatment with a monoclonal antibody (mAb) to the Gal-lectin abrogates complement resistance (50).

1.4.2 Humoral Responses

Seroepidemiologic studies indicate that 81% to 100% of patients with invasive

amoebiasis develop specific circulatory antibodies against *E. histolytica* (51). High titre serum antibody responses to *E. histolytica* are detectable within 7 days of infection and a wide variety of amoeba antigens react with antibodies (52, 53). Major amoebic antigens recognized by patient serum samples include the serine-rich *E. histolytica* protein (SREHP) (54) and the Gal-lectin (55, 56).

E. histolytica initiates infection at the intestinal mucosal surface and first encounters humoral responses at this site. Secretory anti-amoebic IgA (sIgA) has been detected in the bile of intra-caecally-immunized rats (57) and in the faeces of amoebiasis patients (58). sIgA responses against amoebae are present at other mucosal sites as well. Anti-amoebic sIgA has been detected in human milk (59) and saliva (58, 60). Apart from serum IgA (58), the dominant class of serum antibodies produced against *E. histolytica* appears to be IgG (51). Both the Gal-lectin and SREHP, for example, stimulate strong serum IgG responses in immunized animals (55, 61). Serum IgM has been detected in human cases of intestinal and extra-intestinal disease (62, 63), whereas IgE may be produced during amoebiasis but definitive reports are rare (64). The frequencies of different antibody subclasses in amoebiasis remain to be investigated.

Recent reports have shown various levels of protection against amoebic liver abscess following passive immunization of SCID mice with anti-*E. histolytica* serum or purified antibodies or with anti-serum against the Gal-lectin or SREHP (65-68). In contrast, passive immunization with various concentrations of ammonium sulphate-precipitated anti-amoebic antibodies from rabbits or hamsters failed to protect immunologically competent hamsters from amoebic liver abscess (69). Whereas key mechanisms used by macrophages in killing amoebae have been identified (70, 71), how passive transfer of antibodies protects SCID mice from subsequent intra-hepatic challenge with *E. histolytica* remains to be demonstrated. Several reports of immunization in animals have suggested a link between protection and the presence of antibodies shown to have adherence-inhibitory effects *in vitro* (72-74). Human anti-amoebic antibodies have also

been shown to inhibit adherence of *E. histolytica* to target cells *in vitro* (75). However, these *in vitro* adherence assays were performed at 4° C (67, 72). At 37° C amoebae cap and release or ingest antibodies bound to their surface (76, 77). *E. histolytica* also produces a neutral cysteine protease which degrades human serum and secretory IgA (78). Purified amoebic neutral cysteine protease cleaves IgG as well, reducing binding of IgG to amoebae by 83% in one study (79). Therefore, antibodies bound to the surface of *E. histolytica* may not last long enough to have significant effects.

Possibly, antibodies contribute to resistance through transient agglutination of trophozoites which are subsequently dealt with by other mechanisms. SCID mice resolve amoebic liver infections spontaneously in the absence of antibodies (80). Therefore, other protective mechanisms must be present. Studies in nude mice confirm this. Nude mice fail to develop antibody responses to injected amoebae whereas their thymus-intact littermates do (81). Nonetheless, nude mice are resistant to amoebic liver abscess unless depleted of macrophages by silica treatment (81). In SCID mice, which have functional natural killer (NK) cells and macrophages, NK cell activation of macrophages has been identified as an important mechanism controlling microbial infections (82) and may play a role in limiting amoebic liver abscess in these mice.

In general, antibody responses have not been considered protective in amoebiasis (51, 83). Antibody titres do not correlate with resistance to infection in either clinical disease or animal models (60, 67, 72, 84). In patients, intestinal colonization with *E. histolytica* and progressive amoebiasis appear to be uninhibited by high antibody titres (52, 85). Antibody responses may merely be an indication of current or recent invasive disease (58).

1.4.3 T Cell Responses

A role for antigen-specific T cells in controlling invasive amoebiasis is evident from studies demonstrating that thymectomy or treatment with anti-T cell serum results in

exacerbation of infection in animal models of the disease (86-88). Conversely, it has been demonstrated that passive transfer of immunity against hepatic amoebiasis in hamsters using spleen cells was dependent on T cells (89). Subsequent to drug-cure of infection, peripheral blood mononuclear cells (PBMC) from patients retain the ability to proliferate and produce cytokines when stimulated with amoebic antigen, demonstrating the development of antigen-specific memory (90, 91).

Antigen-activated $CD8^+$ T cells have been shown to directly lyse *E. histolytica in vitro* (92). T cells from amoebic liver abscess patients killed amoebic trophozoites in a contact-dependent and antibody-independent fashion following 5 days stimulation with crude amoebic proteins (i.e. antigen) *in vitro* (91). In contrast, unstimulated patient T cells or control T cells stimulated with amoebic proteins failed to kill *E. histolytica*. The Gal-lectin stimulates T cell amoebicidal activity. In Gal-lectin-immunized gerbils, it was demonstrated that co-incubation of amoebae with Gal-lectin-stimulated spleen cells resulted in amoebic viability being reduced to 51.6% compared to controls (93). Under similar conditions, Gal-lectin-stimulated PBMC from amoebiasis patients reduced *E. histolytica* viability to 61% compared to controls (90). While the precise killing mechanism has not been defined, it occurs in a major histocompatibility complex (MHC) unrestricted fashion and possibly involves tumour necrosis factor-α (TNF-α) (94). T cells have also been reported to kill fungal, bacterial and other parasite species *in vitro* in a MHC unrestricted manner (95).

There is considerable experimental evidence indicating that the major mechanism of T cell-mediated defence in amoebiasis involves activation of macrophages for amoebicidal activity. Delayed-type hypersensitivity (DTH) reactions to amoebic antigen have been detected in both humans and animals (96, 97), indicating that cell-mediated immunity is activated in response to *E. histolytica in vivo*. In addition, several *in vitro* studies indicate that T cell-derived cytokines activate macrophages to kill amoebae. A study using macrophages and T cells from uninfected humans demonstrated that supernatants from

Concanavalin A (Con A) or phytohaemagglutinin (PHA)-stimulated PBMC activated macrophages to kill 55% of the amoebae within 3 hr (22). Supernatants of amoebic antigen-stimulated PBMC from cured amoebiasis patients also activate normal monocyte-derived macrophages to kill amoebae (91).

The identity of the T cell cytokine(s) involved in activating macrophage amoebicidal activity was addressed in a study of PBMC from patients with a history of amoebiasis (98). It was demonstrated that normal macrophages activated with 300 U/ml of recombinant Interferon-γ (IFN-γ) killed 47% of co-incubated amoebae in 6 hr. Stimulation of patient PBMC and normal PBMC with Con A resulted in equal levels of IFN-γ, but patient PBMC produced significantly more IFN-γ than normal PBMC when stimulated with amoebic antigen (1,862 U/ml vs. 174 U/ml). Activation of naive macrophages with amoebic antigen-stimulated patient PBMC supernatants resulted in killing of 48% of co-incubated *E. histolytica*. This number was reduced to 18% killed in the presence of anti-IFN-γ antibodies, indicating that T cell-derived IFN-γ plays a major, but not exclusive, role in activating macrophages for amoebicidal activity. Other T cell-derived cytokines may be co-released with IFN-γ and participate in activating macrophages. Tumour necrosis factor-α/ß (TNF-α/ß) and Interleukin-2 (IL-2), for example, both have macrophage activating capacity (99, 100). IFN-γ and IL-2 were co-released from patient PBMC and from splenocytes of immune gerbils when cells were stimulated with the Gal-lectin *in vitro* (90, 93).

Taken together, the data suggest that a Th1 cytokine response would favour control of *E. histolytica* infection. Th1 cytokine responses are characterized by T cell release of IL-2, IFN-γ and TNF-ß, whereas Th2 cytokine responses are characterized by IL-4, -5, -6, -10 and -13 production and Th0 responses consist of a mix of Th1 and Th2 cytokines (101). Th1 responses tend to promote predominately cell-mediated immunity (CMI) and IgG2a responses in mice (IgG1 in humans) while Th2 responses are largely associated with humoral immunity (101). The type of T cell cytokine response controls susceptibility or resistance to infection in a number of infectious diseases. However, the balance of

Th1/Th2 responses during amoebiasis and in animals resistant to infection remains to be analysed.

1.4.4 Macrophage Responses

Evidence from *in vivo* and *in vitro* studies suggest that macrophage-mediated amoebicidal activity is the principal mode of host defence against invasive *E. histolytica* infections. *In vivo* treatments such as silica injection and anti-macrophage serum effectively knockout macrophages and render animals susceptible to amoebic liver abscess or increased severity of infection, indicating the requirement for these cells in anti-amoebic defence (81, 102, 103). In addition, the precise mechanisms of macrophage amoebicidal activity have been extensively characterized.

As well as activation of macrophages by human T cell supernatants (98), recombinant cytokines have been shown to activate macrophages to kill *E. histolytica* trophozoites *in vitro*. IFN-γ alone or in combination with bacterial lipopolysaccharide (LPS) activated murine bone marrow-derived macrophages (BMM) to kill 24-60% of amoebae in a 6 hr assay (104). Macrophages isolated from mouse livers (Kupffer cells) responded to IFN-γ and LPS stimulation by killing amoebic trophozoites (105), suggesting that these tissue macrophages may be responsive during liver infections. Other cytokines such as colony-stimulating factor-1 and TNF-α, alone or in synergy with IFN-γ, also stimulate murine macrophage amoebicidal activity (104). *In vivo*, both TNF-ß (produced by T cells) and TNF-α (produced by T cells and macrophages) may promote macrophage activation. LPS and the cytokines IFN-γ and TNF-α have no direct cytotoxic effects on *E. histolytica* (104).

IFN-γ acts to prime macrophages for enhanced responsiveness to triggering signals such as TNF-α (106). Under certain conditions, transforming growth factor-ß (TGF-ß) further primes macrophages for amoebicidal activity (107). Pretreatment of murine bone

marrow macrophages for 4 hr with TGF-ß primed these cells for elevated amoebic killing in response to IFN-γ and TNF-α or IFN-γ and LPS (15% and 23% increases, respectively). However, this priming effect was highly dependent on the LPS (>100ng) or TNF-α (>100 U) triggering dose in the presence of IFN-γ. At lower concentrations of LPS or TNF-α, TGF-ß had a suppressive effect.

Following cytokine activation *in vitro*, macrophages kill *E. histolytica* trophozoites in a contact-dependent and antibody-independent manner (22, 104, 105). There is no evidence to support a role for macrophages in antibody-dependent cellular cytotoxicity in amoebiasis (22, 108). In contrast, early reports demonstrated that macrophage-mediated killing was partially inhibited by catalase but not superoxide dismutase, suggesting a role for H_2O_2 but not O_2^- in amoebicidal activity (22, 104). More recently, a correlation between murine macrophage nitric oxide (NO) production (measured as a stable end product; NO_2^-) and amoebicidal activity was found (71). Activation of murine macrophages with IFN-γ and LPS resulted in high NO production and reduction of amoebic viability from 97 to 26% (71). Macrophage-mediated amoebicidal activity was inhibited in a dose dependent fashion by arginase and N^G-monomethyl L-arginine, which are inhibitors of L-arginine, the substrate for inducible nitric oxide synthase (iNOS). Catalase was found to inhibit NO_2^- levels even at low concentrations (71). This suggests that catalase-inhibitable killing attributed to H_2O_2 in previous studies may also have involved NO. However, it is possible that H_2O_2 acts as a co-factor or synergizes with NO in amoebicidal activity.

Macrophage amoebicidal activity mediated by NO has been confirmed in subsequent studies (70, 109) and further investigation has revealed a key autocrine role for TNF-α in the upregulation of macrophage NO production. Anti-serum to TNF-α suppressed IFN-γ and LPS-stimulated TNF-α release, NO_2^- production and amoebicidal activity by 93, 53 and 86%, respectively (70). Exogenous TNF-α, in combination with

IFN-γ, also stimulated NO_2^- production and amoebicidal activity. Macrophages stimulated with IFN-γ and TNF-α or LPS demonstrated enhanced levels of iNOS mRNA, peaking at 24 hr, and rapid expression of TNF-α mRNA, stable from 4 to 48 hr, concomitant with increased NO_2^- and TNF-α levels (70). Taken together, these data argue that TNF-α is central for NO-dependent amoebicidal activity *via* elevating levels of iNOS mRNA expression, which may be associated with accumulation of TNF-α mRNA.

The Gal-lectin is, thus far, the only specific amoebic molecule shown to directly stimulate macrophage functions. Gal-lectin stimulates TNF-α mRNA expression and TNF-α protein production from mouse BMM (110). A polyclonal anti-Gal-lectin serum inhibited TNF-α mRNA induction in response to Gal-lectin but not LPS. More specifically, anti-Gal-lectin mAbs 8C12, H85 and 1G7, which mapped to amino acids (aa) 596-1082 of the cysteine-rich region of the Gal-lectin 170 kDa subunit (25), inhibited TNF-α mRNA expression by 28, 82 and 84% respectively (110). Given that TNF-α was important in inducing macrophage NO production (70), a subsequent study examined the potential of Gal-lectin to stimulate macrophage iNOS mRNA expression and NO production (111). IFN-γ-primed mouse BMM responded to Gal-lectin with increased levels of iNOS mRNA and NO_2^- production which was specifically inhibited by mAb 1G7. In addition, IFN-γ and Gal-lectin stimulated macrophage amoebicidal activity (53% amoebae killed) (111).

1.4.5 Neutrophil and Eosinophil Responses

Despite the contribution of *E. histolytica*-lysed neutrophils to tissue pathology, once activated, these effector cells develop anti-amoebic activity that may contribute to defence against amoebiasis. Activation of human neutrophils with IFN-γ or IFN-γ and TNF-α generated resistance to lysis by amoebae and stimulated killing of 30-60% amoebae in a 6 hr *in vitro* assay (112). Physical separation of amoebae and neutrophils by transwell

membranes (0.45 μm) further augmented IFN-γ and TNF-α–stimulated neutrophil amoebicidal activity (up to 97% of the amoebae were killed). Killing was inhibited 73% in the presence of catalase, suggesting that H_2O_2 was the principal cytotoxic molecule. As human neutrophils also produce NO (113), which is inhibited by catalase (71), it is possible that neutrophil-derived NO could contribute to amoebicidal activity. In SCID mice, depletion of neutrophils resulted in larger amoebic liver abscesses at day 2 of infection (46.3% compared to 12.6% of liver abscessed) (80). The effect of neutrophil depletion was less marked by the seventh day of infection, suggesting that neutrophils may act early to inhibit abscess development.

Limited evidence suggests a possible role for eosinophils in resistance to *E. histolytica*. Eosinophils are activated for amoebicidal activity *in vitro* by treatment with f-Met-Leu-Phe (114). Eosinophils may be stimulated in response to amoebae *in vivo*. Eosinophilic infiltration of rat gut tissues occurred in response to intragastric immunization with glutaraldehyde-fixed amoebic trophozoites (64). In addition, one study showed that gerbils made eosinophilic through *Toxocara canis* antigen inoculation experience smaller amoebic liver abscesses than control gerbils (115).

1.4.6 Immunosuppression in Amoebiasis

A complex interaction occurs between host cells and amoebae during hepatic infections. *E. histolytica* rapidly kills naïve macrophages, T cells and neutrophils on contact (22). Amoebae are also capable of modulating several T cell and macrophage responses during active infection. Specifically, the acute phase of hepatic amoebiasis in humans and in animal models is associated with a state of immunosuppression, favouring amoebic survival (116-118). Overcoming amoebic immunomodulatory effects is probably key to controlling the infection. In contrast, prolonged infection may represent an inability to overcome the suppression.

Active infection is associated with systemic T cell hyporesponsiveness as quantified

by *in vitro* proliferative responses of patient PBMC to mitogens Con A and PHA (91, 119). In animal infections, antigen-specific proliferative responses are lower in the early or acute stage of infection (118). Hence, invasive amoebiasis is associated with reduced T cell numbers (120). Depressed DTH reactions during acute disease indicate inhibited antigen-specific T cell responses (116). The suppression may be mediated by an amoebic component(s) or host factor(s) produced in response to the infection. Injection of mice with *E. histolytica* proteins resulted in lower *ex vivo* proliferative responses to mitogen, for example (121). Involvement of a serum soluble factor, of either host or parasite origin, is suggested by the inhibitory effect of amoebiasis patient serum on antigen-specific T cell proliferation and IFN-γ production (122). T cells from patients treated for amoebic liver abscess showed a 63% reduction in amoebic antigen-induced proliferation and a 93% reduction in IFN-γ production in the presence of patient serum as compared to control serum (122). IL-2 (T cell growth factor) production was not examined in this study. The inhibitory effect of amoebiasis patient serum decreased as time between therapy and serum collection increased (122), suggesting that the effect predominated at acute disease. The mechanism(s) involved in suppression of T cell responses remains to be identified. In addition, B cell responsiveness during amoebiasis has not been examined.

During hepatic amoebiasis, macrophages in the vicinity of the amoebic abscess are profoundly suppressed in several key accessory and effector functions while those distal from the abscess are in a heightened state of activation (117). This suggests that macrophage suppression in amoebiasis is a local event mediated by direct exposure to amoebae or amoebic products. The *in vitro* modulatory effects of amoebic proteins on naïve macrophages support this hypothesis. Liver-abscess-derived macrophages produce low basal levels of TNF-α that may contribute to abscess development (123). However, pretreatment of naïve macrophages with amoebic proteins *in vitro* inhibited LPS- or IFN-γ and LPS-stimulated TNF-α gene expression and protein production partially *via* induction of macrophage prostaglandin-E_2 (PGE_2) production (123, 124). *E. histolytica* also

suppressed macrophage IL-1 production by an unknown mechanism (117). Significantly, amoebic liver abscess is associated with modulation of macrophage effector molecule production and amoebicidal activity. Abscess-derived macrophages were found to be hyporesponsive to PMA-triggered H_2O_2 production (117), and IFN-γ and LPS stimulation for NO production and amoebicidal activity (124). *In vitro*, pretreatment of naïve macrophages with amoebic proteins suppressed iNOS mRNA expression, NO production and amoebicidal activity *via* a PGE_2-independent mechanism (124).

In vivo, macrophages may be inhibited by suppressed ability to respond to activating signals for amoebic killing and by low production of activating cytokines from T cells. Macrophages may be partially responsible for weak T cell activation. Pretreatment of naïve macrophages *in vitro* with soluble amoebic proteins or secreted products from trophozoites downregulated macrophage I-Aß mRNA accumulation and surface Ia (MHC class II) molecule expression in response to IFN-γ (125). The effect of amoebic proteins on Ia expression was partially inhibited by indomethacin, indicating a role for PGE_2 in suppression of macrophage Ia expression (125). Reduced Ia expression could inhibit macrophage antigen-presenting cell capability resulting in suppressed antigen-specific activation of T cells. The nature of amoebic antigens recognized by T cells may also play a role in the inhibition of macrophage activation in *E. histolytica* infections. A 220-kDa amoebic surface protein stimulated a Th2-like response in mice characterized by IL-4 and IL-10 production (126). Both IL-4 and IL-10 act to suppress macrophage anti-microbial functions (127).

Sensitization of the immune system by immunization or as a consequence of a drug-terminated initial infection appears to be sufficient for the host to surmount the suppressive effects of *E. histolytica*. The suppression of cell-mediated immunity associated with acute infection supports the concept that macrophage-mediated resistance is central to controlling invasive amoebiasis.

1.5 The *E. histolytica* Galactose/N-Acetyl-D-Galactosamine Adherence Lectin (Gal-lectin)

The 260-kDa Gal-lectin is the most characterized of all purified or cloned *E. histolytica* proteins. The Gal-lectin is a heteroduplex consisting of a 170-kDa transmembrane heavy subunit and a 31-35-kDa glycosylphosphatidylinositol-anchored light subunit (128). The heavy subunit is encoded by a family of five *hgl* genes that share >89% amino acid sequence identity. Similarly, the light subunit is encoded by a family of at least seven *lgl* genes which share 79-85% amino acid sequence identity (129). The *hgl* genes encode amino-termini signal sequences, indicating that the Gal-lectin is a cell surface molecule.

The extracellular amino terminus (aa 1-187) of the 170-kDa subunit is cysteine (3.2%) and tryptophan (2.1%) rich. This is followed by a cysteine-free stretch (aa 188-378) and an extremely cysteine-rich (10.8%) region (aa 379-1209) that forms the largest extracellular section of the Gal-lectin heavy subunit. The putative transmembrane domain (aa 1210-1235) precedes the 41 carboxyl-terminal amino acids that are believed to form the cytoplasmic domain (130-132). The position and number of cysteine residues in the cysteine-rich region is completely conserved amongst the *hgl* genes (129), suggesting that these residues may be critical to the function of the Gal-lectin. Indeed, mAb-inhibition studies revealed that adherence, cytotoxicity and complement resistance as well as activation of macrophages for TNF-α and NO production were mediated by the cysteine-rich region of the Gal-lectin 170-kDa subunit (25, 110, 111). To date, no known functions have been ascribed to the Gal-lectin light subunit.

The Gal-lectin is an immunodominant amoebic antigen recognized by serum antibodies from 95% of amoebiasis patients worldwide (56). Compared to SREHP, the other major amoebic vaccine candidate molecule, the Gal-lectin induces a stronger and longer-lasting immune response in drug-treated amoebic liver abscess patients (133).

Immunization studies in gerbils indicate that the Gal-lectin is a promising candidate for vaccination against amoebiasis. Intraperitoneal immunization of gerbils with native Gal-lectin in Freund's adjuvant protected 67% of the animals from amoebic liver abscess formation (72). However, liver abscesses were larger in the unprotected gerbils as compared to controls. A later study demonstrated that immunization with the 170-kDa subunit NH_2-terminal domain (aa 1-436) was responsible for the exacerbating effect (67). The 170-kDa cysteine-rich region has been demonstrated to contain B and T cell epitopes recognized by human sera and PBMCs (134, 135). Recombinant cysteine-rich region proteins containing aa 649-1202 and aa 758-1134 protected gerbils from amoebic liver abscess with 81% and 71% efficacy, respectively (136, 55). In addition, oral immunization with *Salmonella dublin* expressing aa 482-1138 of the cysteine rich region resulted in a significant decrease in amoebic liver abscess size in gerbils (137). In no case did immunization with recombinant portions of the cysteine-rich region exacerbate disease (55, 136, 137) Taken together, these results demonstrate that the cysteine-rich region of the Gal-lectin 170-kDa subunit shows strong potential as a vaccine against *E. histolytica*. However, the precise mechanisms involved in generating protection following immunization with the Gal-lectin remain to be identified.

1.6 References

1. Barker D.C., and Swales L.S. (1972) Characteristics of ribosomes during differentiation from trophozoite to cyst in axenic *Entamoeba histolytica* sp. *Cell. Differ.* 1: 297-306.

2. Jackson T.F.H.G. (1998) *Entamoeba histolytica* and *Entamoeba dispar* are distinct species; clinical, epidemiological and serological evidence. *Int. J. Parasitol.* 28: 181-186.

3. Petri W.A. Jr. (1996) Recent advances in amebiasis. *Crit. Rev. Clin. Lab. Sci.* 33: 1-

37.

4. Perez-Tamayo R. (1986) Pathology of amebiasis. In *Amebiasis.* (Martinez-Palomo A., ed.). Elsevier, New York pp. 45-94.

5. McAuley J.B., and Juranek D.D. (1992) Paromomycin in the treatment of mild-to-moderate intestinal amebiasis. *Clin. Infect. Dis.* 15: 551-552.

6. Biagi F. (1981) Amebiasis. *Antibiot. Chemother.* 30: 20-27.

7. Orozco E., Pérez D.G., Gómez M.C., and Ayala P. (1995) Multidrug resistance in *Entamoeba histolytica. Parasitol. Today* 11: 473-475.

8. Walsh J.A. (1986) Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev. Infect. Dis.* 8: 228-238.

9. World Health Organization (1995) *The World Health Report.* World Health Organization, Geneva.

10. Caballero-Salcedo A., Viveros-Rogel M., Salvatierra B., Tapia-Conyer R., Sepulveda-Amor J., Gutierrez G., and Ortiz-Ortiz L. (1994) Seroepidemiology of amebiasis in Mexico. *Am. J. Trop. Med. Hyg.* 50: 412-419.

11. Magambo J.K., Zeyhle E., and Wachira T.M. (1998) Prevalence of intestinal parasites among children in southern Sudan. *East Afr. Med. J.* 75: 288-290.

12. Hossain M.M., Ljungstrom I., Glass R.I., Lundin L., Stoll B.J., and Huldt G. (1983) Amoebiasis and giardiasis in Bangladesh: parasitological and serological studies. *Trans. R. Soc. Trop. Med. Hyg.* 77: 552-554.

13. Sepúlveda B. (1982) Amoebiasis: host-pathogen biology. *Rev. Infect. Dis.* 4: 836-842.

14. Brandt H., and Pérez-Tamayo R. (1970) Pathology of human amebiasis. *Hum. Pathol.* 1: 351-385.

15. Armon P.J. (1978) Amoebiasis in pregnancy and the puerperium. *B. J. Ob. Gynecol.* 85: 264-269.

16. Weinke T., Friedrich-Janicke B., Hopp P., and Janitschke K. (1990) Prevalence and clinical importance of *Entamoeba histolytica* in two high risk groups: travelers returning from the tropics and male homosexuals. *J. Infect. Dis.* 161: 1029-1031.

17. Smith P.D., Lane H.C., Gill V.J., Manischewitz J.F., Quinnan G.V., Fauci A.S., and Masur H. (1988) Intestinal infections in patients with the acquired immunodefiency syndrome (AIDS). Etiology and response to therapy. *Ann. Intern. Med.* 108: 328-333.

18. Nagakura K., Tachibana H., Tanaka T., Kaneda Y., Tokunaga M., Sasao M., and Takeuchi T. (1989) An outbreak of amebiasis in an institution for the mentally retarded in Japan. *Jpn. J. Med. Sci. Biol.* 42: 63-76.

19. Chadee K., Keller K., Forstner J., Innes D.J., and Ravdin J.I. (1991) Mucin and nonmucin secretagogue activity of *Entamoeba histolytica* and cholera toxin in rat colon. *Gastroenterology* 100: 986-997.

20. Ravdin J.I., and Guerrant R.L. (1981) Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*: Study with mammalian tissue culture cells and human erythrocytes. *J. Clin. Invest.* 68: 1305-1313.

21. Ravdin J.I., Murphy C.F., Salata R.A., Guerrant R.L., and Hewlett E.L. (1985) N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. I. Partial purification and relation to amebic virulence *in vitro*. *J. Infect. Dis.* 151: 804-815.

22. Salata R.A., Pearson R.D., and Ravdin J.I. (1985) Interaction of human leukocytes and *Entamoeba histolytica*: killing of virulent amebae by the activated macrophage. *J. Clin. Invest.* 76: 491-499.

23. Chadee K., Petri W.A., Innes D.J., and Ravdin J.I. (1987) Rat and human colonic mucins bind to and inhibit the adherence lectin of *Entamoeba histolytica*. *J. Clin. Invest.* 80: 1245-1254.

24. Petri W.A. Jr., Smith R.D., Schlesinger P.H., and Ravdin J.I. (1987) Isolation of the galactose-binding lectin which mediates the *in vitro* adherence of *Entamoeba histolytica. J. Clin.Invest.* 80:1238-1244.

25. Mann B.J., Chung C.Y., Dodson L.S., Ashley L.S., Braga L.L., and Snodgrass T.L. (1993) Neutralizing monoclonal antibody epitopes of the *Entamoeba histolytica* galactose adhesin map to the cysteine-rich extracellular domain of the 170-kilodalton subunit. *Infect. Immun.* 61: 1772-1778.

26. Vines R.R., Ramakrishnan G., Rogers J.B., Lockhart L.A., Mann B.J., and Petri W.A. Jr. (1998) Regulation of adherence and virulence by the *Entamoeba histolytica* lectin cytoplasmic domain, which contains a ß2 integrin motif. *Mol. Biol. Cell* 9: 2069-2079.

27. Bracha R., and Mirelman D. (1984) Virulence of *Entamoeba histolytica* trophozoites: effects of bacteria, microaerobic conditions, and metronidazole. *J. Exp. Med.* 160: 353-368.

28. Leroy A., De Bruyne G., Verspeelt A., Lauwaet T., Nelis H., and Mareel M. (1997) Bacterium-assisted invasion of *Entamoeba histolytica* through human enteric epithelia in two-compartment chambers. *Invasion and Metastasis* 17: 138-148.

29. Yu Y., and Chadee K. (1997) *Entamoeba histolytica* stimulates interleukin-8 from human colonic epithelial cells without parasite-enterocyte contact. *Gastroenterology* 112: 1536-1547.

30. Keene W.E., Petitt M.G., Allen S., and McKerrow J.H. (1986) The major neutral proteinase of *Entamoeba histolytica. J. Exp.Med.* 163: 536-549.

31. Que X., and Reed S.L. (1997) The role of extracellular cysteine proteinases in pathogenesis of *Entamoeba histolytica* invasion. *Parasitol. Today* 13: 190-194.

32. Stanley S.L. Jr., Zhang T., Rubin D., and Li E. (1995) Role of *Entamoeba histolytica* cysteine protease in amebic liver abscess formation in severe combined immunodeficient mice. *Infect. Immun.* 63: 1587-1590.

33. Ankri S., Stolarsky T., and Mirelman D. (1998) Antisense inhibition of expression of cysteine proteinase does not affect *Entamoeba histolytica* cytopathic or haemolytic activity but inhibits phagocytosis. *Mol. Microbiol.* 28: 777-785.

34. Chadee K., and Meerovitch E. (1984) The pathogenesis of experimentally induced amebic liver abscess in the gerbil (*Meriones unguiculatus*). *Am. J. Pathol.* 117: 71-80.

35. Chadee K., Moreau F., and Meerovitch E. (1987) *Entamoeba histolytica*: Chemoattractant activity for gerbil neutrophils *in vivo* and *in vitro*. *Exp. Parasitol.* 64: 12-23.

36. Salata R.A., Pearson R.D., and Ravdin J.I. (1986) The interaction of human neutrophils and *Entamoeba histolytica* increases cytopathogenicity for liver cell monolayers. *J. Infect. Dis.* 154: 19-26.

37. Tsutsumi V., Mena-Lopez R., Anaya-Velazguez F., and Martinez-Palomo A. (1984) Cellular bases of experimental amebic liver abscess formation. *Am. J. Pathol.* 117: 81-91.

38. Ravdin J.I., Moreau R., Sullivan J.A., Petri W.A. Jr., and Mandell G.L. (1988) Relationship of free intracellular calcium to the cytolytic activity of *Entamoeba histolytica*. *Infect. Immun.* 56: 1505-1512.

39. Long-Krug S.A., Fischer K.G., Hysmith R.M., and Ravdin J.I. (1985) Phospholipase A enzymes of *Entamoeba histolytica*: description and subcellular localization. *J. Infect. Dis.* 152: 536-541.

40. Lynch E.C., Rosenberg I.M., and Gitler C. (1982) An ion-channel forming protein produced by *Entamoeba histolytica*. *EMBO J.* 7: 801-806.

41. Rosenberg I., Bach D., Loew L.M., and Gitler C. (1989) Isolation, characterization and partial purification of a transferable membrane channel (amoebapore) produced by *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* 33:237-248.

42. Berninghausen O., and Leippe M. (1997) Necrosis versus apoptosis as the mechanism of target cell death induced by *Entamoeba histolytica. Infect. Immun.* 65: 3615-3621.

43. Ragland B.D., Ashley L.S., Vaux D.L., and Petri W.A. Jr. (1994) *Entamoeba histolytica*: Target cells killed by trophozoites undergo DNA fragmentation which is not blocked by Bcl-2. *Exp. Parasitol.* 79: 460-467.

44. Seydel K. B., and Stanley S.L. Jr. (1998) *Entamoeba histolytica* induces host cell death in amebic liver abscess by a non-fas-dependent, non-tumor necrosis factor alpha-dependent pathway of apoptosis. *Infect. Immun.* 66: 2980-2983.

45. Adams E.B., and MacLeod I.N. (1977) Invasive amebiasis. II. Amebic liver abscess and its complications. *Medicine* 56: 325-334.

46. Deleon A. (1970) Pronostico tardio en el abscesso hepatico amibiano. *Arch. Invest. Med. (Mex., Suppl.)* 1: 205-206.

47. Stanley S.L. Jr. (1996) Progress towards an amoebiasis vaccine. *Parasitol. Today* 12: 7-14.

48. Ravdin J.I. (1989) Immunobiology of human infection by *Entamoeba histolytica. Pathol. Immunopathol. Res.* 8: 179-205.

49. Reed S.L., Curd J.G., Gigli I., Gillin F.D., and Braude A.I. (1986) Activation of complement by pathogenic and nonpathogenic *Entamoeba histolytica*. *J. Immunol.* 136: 2265-2270.

50. Braga L.L., Ninomiya H., McCoy J.J., Eacker S., Wiedmer T., Pham C., Wood S., Sims P.J., and Petri W.A. Jr. (1992) Inhibition of the complement membrane attack complex by the galactose-specific adhesin of *Entamoeba histolytica*. *J. Clin. Invest.* 90: 1131-1137.

51. Salata R.A., and Ravdin J.I. (1986) Review of the human immune mechanisms directed against *Entamoeba histolytica. Rev. Infect. Dis.* 8: 261-272.

52. Katzenstein D., Rickerson V., and Braude A. (1982) New concepts of amebic liver abscess derived from hepatic imaging, serodiagnosis, and hepatic enzymes in 67 consecutive cases in San Diego. *Medicine* 61: 237-246.

53. Ximénez C., Sosa O., Leyva P., Morán P., Melendro E.I., and Ramiro M. (1992) Western blot of *Entamoeba histolytica* antigenic fractions: reactivity analysis with sera from intestinal amoebiasis patients. *Ann. Trop. Med. Parasitol.* 86: 121-127.

54. Stanley S.L. Jr., Jackson T.F.H.G., Reed S.L., Calderon J., Kunz-Jenkins C., Gathiram V., and Li. E. (1991) Serodiagnosis of invasive amebiasis using a recombinant *Entamoeba histolytica* protein. *JAMA* 266: 1984-1986.

55. Soong C-J. G., Kain K.C., Abd-Alla M., Jackson T.F.H.G., and Ravdin J.I. (1995) A recombinant cysteine-rich section of the *Entamoeba histolytica* galactose-inhibitable lectin is efficacious as a subunit vaccine in the gerbil model of amebic liver abscess. *J. Infect. Dis.* 171: 645-651.

56. Petri W.A. Jr., Broman J., Healy G., Quinn T., and Ravdin J.I. (1989) Antigenic stability and immunodominance of the Gal/GalNAc adherence lectin of *Entamoeba histolytica. Am. J. Med. Sci.* 296: 163-165.

57. Acosta G., Campos R., Barranco C., Isibasi A., and Kumate J. (1983) Secretory IgA antibodies from bile of immunized rats reactive with trophozoites of *Entamoeba histolytica. Ann. N. Y. Acad. Sci.* 409: 760-765.

58. Abou-El-Magd I., Soong C-J. G., El-Hawey A.M., and Ravdin J.I. (1996) Humoral and mucosal IgA antibody reponse to a recombinant 52-kDa cysteine-rich portion of the *Entamoeba histolytica* galactose-inhibitable lectin correlates with dectection of native 170 k-Da lectin antigen in serum of patients with amebic colitis. *J. Infect. Dis.* 174: 157-162.

59. Grundy M.S., Cartwright-Taylor L., Lundin L., Thors C., and Huldt G. (1983) Antibodies against *Entamoeba histolytica* in human milk and serum in Kenya. *J. Clin. Microbiol.* 17: 753-758.

60. Kelsall B.L., Jackson T.G.F.H., Gathiram V., Salig S.B., Vaithilingum M.,

Pearson R.D., and Ravdin J.I. (1994) Secretory immunoglobulin A antibodies to the galactose-inhibitable adherence protein in the saliva of patients with amebic liver disease. *Am. J. Trop. Med. Hyg.* 51: 454-459.

61. Zhang T., Li E., and Stanley S.L. Jr. (1995) Oral immunization with the dodecapeptide repeat of the serine rich *Entamoeba histolytica* protein (SREHP) fused to the cholera toxin B subunit induces a mucosal and systemic anti-SREHP antibody response. *Infect. Immun.* 63: 1349-1355.

62. Harris W.G., and Bray R.S. (1976) Cellular sensitivity in amoebiasis. Preliminary results of lymphocytic transformation in response to specific antigen and to mitogen in carrier and disease states. *Trans. R. Trop. Med. Hyg.* 70: 340-343.

63. Schulz T.F., Kollaritsch H., Hengster P., Stemberger H., Scheiner O., Wiedemann G., and Dierich M.P. (1987) Molecular weight analysis of *Entamoeba histolytica* antigens recognized by IgG and IgM antibodies in the sera of patients with amoebiasis. *Trop. Med. Parasitol.* 38: 149-152.

64. Navarro-Garcia F., Lopez-Revilla R., Vega-Lopez M.A., Dominguez-Robles M.C., Enriquez-Rincon F., and Tsutsumi V. (1997) Intragastric immunization of rats with *Entamoeba histolytica* trophozoites induces cecal mucosal IgE, eosinophilic infiltration, and type 1 hypersensitivity. *Clin. Immunol. Immunopath.* 82: 221-229.

65. Cieslak P.R., Virgin H.W., and Stanley S.L. (1992) A severe combined immunodeficient (SCID) mouse model for infection with *Entamoeba histolytica*. *J. Exp. Med.* 176: 1605-1609.

66. Seydel K.B., Braun K.L., Zhong T., Jackson T.F.H.G., and Stanley S.L. Jr. (1996) Protection against amebic liver abscess formation in the severe combined immunodeficient mouse by human anti-amebic antibodies. *Am. J. Trop. Med. Hyg.* 55: 330-332.

67. Lotter H., Zhang T., Seydel K.B., Stanley S.L. Jr., and Tannich E. (1997) Identification of an epitope on the *Entamoeba histolytica* 170-kDa lectin conferring antibody-mediated protection against invasive amebiasis. *J. Exp. Med.* 185: 1793-1801.

68. Zhang T., Cieslak P.R., Foster L., Kunz-Jenkins C., and Stanley S.L. Jr. (1994) Antibodies to the serine rich *Entamoeba histolytica* protein (SREHP) prevent amoebic liver abscess in severe combined immunodeficient (SCID) mice. *Parasite Immunol.* 16: 225-230.

69. Campos-Rodríguez R., Shibayama-Salas M., Pacheco-Yépez J., Martínez-Palomo A., and Tsutsumi V. (1995) Passive immunization during experimental amebic liver-abscess development. *Parasitol. Res.* 81: 86-88.

70. Lin J-Y., Séguin R., Keller K., and Chadee K. (1994) Tumor necrosis factor alpha augments nitric oxide-dependent macrophage cytotoxicity against *Entamoeba histolytica* by enhanced expression of the nitric oxide synthase gene. *Infect. Immun.* 62: 1534-1541.

71. Lin J-Y., and Chadee K. (1992) Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. *J. Immunol.* 148: 3999-4005.

72. Petri W.A. Jr., and Ravdin J.I. (1991) Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of *Entamoeba histoyltica*. *Infect. Immun.* 59: 97-101.

73. Beving D.E., Soong C-J. G., and Ravdin J.I. (1996) Oral immunization with a recombinant cysteine-rich section of the *Entamoeba histolytica* galactose-inhibitable lectin elicits an intestinal secretory immunoglobulin A response that has *in vitro* adherence inhibition activity. *Infect. Immun.* 64: 1473-1476.

74. Kelsall B.L., and Ravdin J.I. (1995) Immunization of rats with the 260-kilodalton *Entamoeba histolytica* galactose-inhibitable lectin elicits an intestinal secretory immunoglobulin A response that has *in vitro* adherence-inhibitory activity. *Infect. Immun.* 63: 686-689.

75. Petri W.A. Jr., Joyce M.P., Broman J., Smith R.D., Murphy C.F., and Ravdin J.I. (1987) Recognition of the galactose- or N-acetylgalactosamine-binding lectin of *Entamoeba histolytica* by human immune sera. *Infect. Immun.* 55: 2327-2331.

76. Calderón J., Muñoz M.L., and Acosta H.M. (1980) Surface redistribution and release of antibody-induced caps in Entamoebae. *J. Exp. Med.* 151: 184-193.

77. Espinosa-Cantellano M., and Martínez-Palomo A. (1994) *Entamoeba histolytica*: Mechanism of surface receptor capping. *Exp. Parasitol.* 79: 424-435.

78. Kelsall B.L., and Ravdin J.I. (1993) Degradation of human IgA by *Entamoeba histolytica*. *J. Infect. Dis.* 168: 1319-1322.

79. Tran V.Q., Herdman D.S., Torian B.E., and Reed S.L. (1998) The neutral cysteine proteinase of *Entamoeba histolytica* degrades IgG and prevents its binding. *J. Infect. Dis.* 177: 508-511.

80. Seydel K.B., Zhang T., and Stanley S.L.Jr. (1997) Neutrophils play a critical role in early resistance to amebic liver abscesses in severe combined immunodeficient mice. *Infect. Immun.* 65: 3951-3953.

81. Stern J.J., Graybill J.R., and Drutz D.J. (1984) Murine amebiasis: the role of the macrophage in host defense. Am. J. Trop. Med. Hyg. 33: 372-380.

82. Bancroft G.J., Schreiber R.D., and Unanue E.R. (1991) Natural immunity: A T-cell-independent pathway of macrophage activation, defined in the scid mouse. *Immunological Rev.* 124: 5-24.

83. Jackson T.F.H.G., Gathiram V., and Simjee A.E. (1985) Seropidemiological study of antibody responses to the zymodemes of *Entamoeba histolytica*. *Lancet* 2: 716-719.

84. Krupp I. (1970) Antibody response in intestinal and extraintestinal amebiasis. *Am. J. Trop. Med. Hyg.* 19: 57-62.

85. Irusen E.M., Jackson T.F., and Simjee A.E. (1992) Asymptomatic intestinal colonization by pathogenic *Entamoeba histolytica* in amebic liver abscess: prevalence, response to therapy and pathogenic potential. *Clin. Infect. Dis.* 15: 559.

86. Desai A.C., and Bhide M.B. (1977) Effect of depressed cell mediated immunity on hepatic amebiasis in hamsters. *Bull. Haffkine Inst.* 5: 35-37.

87. Ghadirian E., and Meerovitch E. (1981) Effect of immunosuppression on the size and metastasis of amoebic liver abscesses in hamsters. *Parasite Immunol.* 3: 329-338.

88. Vinayak V.K., Sawhney S., Jain P., Chugh S., and Chakravarti R.N. (1982) Immunosuppression and experimental amoebiasis in guinea pigs. *Ann. Trop. Med. Parasitol.* 76: 309-316.

89. Ghadirian E., and Meerovitch E. (1983) Passive transfer of immunity against hepatic amebiasis in the hamster by cells. *Parasite Immunol.* 5:369-376.

90. Schain D.C., Salata R.A., and Ravdin J.I. (1992) Human T-lymphocyte proliferation, lymphokine production, and amebicidal activity elicited by the galactose-inhibitable adherence protein of *Entamoeba histolytica. Infect. Immun.* 60: 2143-2146.

91. Salata R.A., Martinez-Palomo A., Murray H.W., Conales L., Trevino N., Segovia E., Murphy C.F., and Ravdin J.I. (1986) Patients treated for amebic liver abscess develop cell-mediated immune responses effective in vitro against *Entamoeba histolytica. J. Immunol.* 136: 2633-2639.

92. Salata R.A., Cox J.G., and Ravdin J.I. (1987) The interaction of human T-lymphocytes and *Entamoeba histolytica*: killing of virulent amoebae by lectin-dependent lymphocytes. *Parasite Immunol.* 9: 249-261.

93. Schain D.C., Salata R.A., and Ravdin J.I. (1995) Development of amebicidal cell-mediated immunity in gerbils (*Meriones unguiculatus*) immunized with the galactose-inhibitable adherence lectin of *Entamoeba histolytica. J. Parasitol.* 81: 563-568.

94. Denis M., and Chadee K. (1989) Murine T-cell clones against *Entamoeba histolytica*: *in vivo* and *in vitro* characterization. *Immunology* 66: 76-82.

95. Levitz S.M., Mathews H.L., and Murphy J.W. (1995) Direct antimicrobial activity

of T cells. *Immunol. Today* 16: 387-391.

96. Kretschmer R.R., Sepulveda B., Almazan A., and Gamboe F. (1972) Intradermal reactions to an antigen obtained from axenically cultivated *Entamoeba histolytica*. *Trop. Geogr. Med.* 24: 275-281.

97. Gold D. (1989) Delayed-type hypersensitivity to *Entamoeba histolytica* in mice and hamsters: a comparison. *Parasitol. Res.* 75: 335-342.

98. Salata R.A., Murray H.W., Rubin B.Y., and Ravdin J.I. (1987) The role of gamma interferon in the generation of human macrophages cytotoxic for *Entamoeba histolytica* trophozoites. *Am. J. Trop. Med. Hyg.* 37: 72-78.

99. Nacy C.A., Meierovics A.I., Belosevic M., and Green S.J. (1991) Tumor necrosis factor-alpha: central regulatory cytokine in the induction of macrophage antimicrobial activities. *Pathobiology* 59: 182-184.

100. Espinoza-Delgado I., Bosco M.C., Musso T., Gusella G.L., Longo D.L., and Varesio L. (1995) Interleukin-2 and human monocyte activation. *J. Leukoc. Biol.* 57: 13-19.

101. Mosmann T.R., and Sad S. (1998) The expanding universe of T cell subsets: Th1, Th2 and more. *Immunol. Today* 17: 138-146.

102. Ghadirian E., and Meerovitch E. (1982) Macrophage requirement for host defence against experimental hepatic amoebiasis in the hamster. *Parasite Immunol.* 4: 219-225.

103. Ghadirian E., Meerovitch E., and Kongshavn P.A.L. (1983) Role of macrophages in host defense against hepatic amebiasis in hamsters. *Infect. Immun.* 42: 1017-1019.

104. Denis M., and Chadee K. (1989) Cytokine activation of murine macrophages for *in vitro* killing of *Entamoeba histolytica* trophozoites. *Infect. Immun.* 57: 1750-1756.

105. Ghadirian E., and Salimi A. (1993) *In vitro* effect of recombinant interferon gamma in combination with LPS on amoebicidal activity of murine Kupffer cells.

Immunobiol. 188: 203-219.

106. Krammer P.H., Kubelka C.F., Falk W., and Rappel A. (1985) Priming and triggering of tumoricidal and schistosomulicidal macrophages by two sequential lymphokine signals: interferon-gamma and macrophage inducing factor 2. *J. Immunol.* 135: 3258-3263.

107. Lin J-Y., Séguin R., Keller K., and Chadee K. (1995) Transforming growth factor-β_1 primes macrophages for enhanced expression of the nitric oxide synthase gene for nitric oxide-dependent cytotoxicity against *Entamoeba histolytica. Immunology* 85: 400-407.

108. Saxena A., and Vinayak V.K. (1987) Low susceptibility of trophozoites of virulent *Entamoeba histolytica* to cellular and antibody-dependent cellular cytotoxicity by guinea pig effector cells. *Trans. R. Soc. Trop. Med. Hyg* 81: 933-937.

109. Denis M., and Ghadirian E. (1992) Activated mouse macrophages kill *Entamoeba histolytica* trophozoites by releasing reactive nitrogen intermediates. *Microbial. Pathogenesis* 12: 193-198.

110. Séguin R., Mann B.J., Keller K., and Chadee K. (1995) Identification of the galactose-adherence lectin epitopes of *Entamoeba histolytica* that stimulate tumor necrosis factor-alpha production by macrophages. *Proc. Natl. Acad. Sci. USA* 92: 12175-12179.

111. Séguin R., Mann B.J., Keller K., and Chadee K. (1997) The tumor necrosis factor alpha-stimulating region of galactose-inhibitable lectin of *Entamoeba histolytica* activates gamma interferon-primed macrophages for amebicidal activity by nitric oxide. *Infect. Immun.* 65: 2522-2527.

112. Denis M., and Chadee K. (1989) Human neutrophils activated by Interferon-γ and tumour necrosis factor-α kill *Entamoeba histolytica* trophozoites *in vitro. J. Leukoc. Biol.* 46: 270-274.

113. Evans T.J., Buttery L.D., Carpenter A., Springall D.R., Polak J.M., and Cohen J. (1996) Cytokine-treated human neutrophils contain inducible nitric oxide synthase

that produces nitration of ingested bacteria. *Proc. Natl. Acad. Sci. USA* 93: 9553-9558.

114. López-Osuna M., Arellano J., and Kretschmer R.R. (1992) The destruction of virluent *Entamoeba histolytica* by activated human eosinophils. *Parasite Immunol.* 14: 579-586.

115. López-Osuna M., Velazquez J.R., and Kretschmer R.R. (1997) Does the eosinophil have a protective role in amebiasis? *Memorias do Instituto Oswaldo Cruz* 92 (Suppl. S): 237-240.

116. Ortiz-Ortiz L., Zamacona-Ravelo G., Sepulveda E., and Capin N.R. (1975) Cell-mediated immunity in patients with amoebic liver abscess. *Clin. Immunol. Immunopathol.* 4: 127-134.

117. Denis M., and Chadee K. (1988) *In vitro* and *in vivo* studies of macrophage functions in amebiasis. *Infect. Immun.* 56: 3126-3131.

118. Gill N.J., Ganguly N.K., Dilawari J.B., and Mahajan R.C. (1985) Lymphocyte subpopulations transformation studies in an experimental model of intestinal and hepatic amoebiasis. *Trans. R. Soc.Trop. Med.Hyg.* 79: 618-623.

119. Simjee A.E., Gathiram V., Coovadia H.M., Jackson T.F.G.H., Kiepiela P., Pudifin D.J., and Stretton M. (1985) Cell-mediated immunity amoebiasis.*Trans. R. Soc.Trop. Med.Hyg.* 79: 165-168.

120. Gandhi B.M., Gupta H., Irshad M., Chawala T.C., and Tandon B.N. (1986) Cell-mediated immunity in amoebic liver abscess. *J. Trop. Med. Hyg.* 89: 163-166.

121. Chadee K., Denis M., and Keller K. (1991) Down-regulation of murine lymphocyte responsiveness to mitogens after treatment with antigens of *Entamoeba histolytica*. *Parasitol. Res.* 77: 572-576.

122. Salata R.A., Martinez-Palomo A., Canales L., Murray H.W., Trevino N., and Ravdin J.I. (1990) Suppression of T-lymphocyte responses to *Entamoeba histolytica* antigen by immune sera. *Infect. Immun.* 58: 3941-3946.

123. Wang W., Keller K., and Chadee K. (1992) Modulation of tumor necrosis factor production by macrophages in *Entamoeba histolytica* infection. *Infect. Immun.* 60: 3169-3174.

124. Wang W., Keller K., and Chadee K. (1994) *Entamoeba histolytica* modulates the nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxoicity against amoebae and tumour cells. *Immunology* 83: 601-610.

125. Wang W., and Chadee K. (1995) *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatibility complex Ia molecule and I-Aß mRNA expression by a prostaglandin E_2-dependent mechanism. *Infect. Immun.* 63: 1089-1094.

126. Talamás-Rohana P., Schlie-Guzman M.A., Hernández-Ramírez V.I., and Rosales-Encina J.L. (1995) T-cell suppression and selective in vivo activation of TH2 subpopulation by the *Entamoeba histolytica* 220-kilodalton lectin. *Infect. Immun.* 63: 3953-3958.

127. Taub D.D., and Cox G.W. (1995) Murine Th1 and Th2 cell clones differentially regulate macrophage nitric oxide production. *J. Leukoc. Biol.* 58: 80-90.

128. Petri W.A. Jr., Chapman M.D., Snodgrass T.L., Mann B.J., Broman J., and Ravdin J.I. (1989) Subunit structure of the galactose and N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica. J. Biol. Chem.* 264: 3007-3012.

129. Ramakrishnan G., Ragland B.D., Purdy J.E., and Mann B.J. (1996) Physical mapping and expression of gene families encoding the N-acetyl D-galactosamine adherence lectin of *Entamoeba histolytica. Mol. Microbiol.* 19: 91-100.

130. Mann B.J., Torian B.E., Vedvick T.S., and Petri W.A. Jr. (1991) Sequence of the cysteine-rich heavy subunit of the galactose lectin of *Entamoeba histolytica. Proc. Natl. Acad. Sci. USA* 88: 3248-3252.

131. Purdy J.E., Mann B.J., Shugart E.C., and Petri W.A. Jr. (1993) Analysis of the

gene family encoding the *Entamoeba histolytica* galactose-specific adhesin 170-kDa subunit. *Mol. Biochem. Parasitol.* 62:53-60.

132. Tannich E., Ebert F., and Horstmann R.D. (1991) Primary structure of the 170-kDa surface lectin of pathogenic *Entamoeba histolytica. Proc. Natl. Acad. Sci. USA* 88: 1849-1853.

133. Stanley S.L. Jr., Jackson T.F.H.G., Foster L., and Singh S. (1998) Longitudinal study of the antibody response to recombinant *Entamoeba histolytica* antigens in patients with amebic liver abscess. *Am. J. Trop. Med. Hyg.* 58: 414-416.

134. Zhang Y., Li E., Jackson T.F.G.H., Zhang T., Gathiram V., and Stanley S.L. (1992) Use of recombinant 170-kilodalton surface antigen of *Entamoeba histolytica* for serodiagnosis of amebiasis and identification of immunodominant domains of the native molecule. *J. Clin. Microbiol.* 30: 2788-2792.

135. Velazquez C., Valette I., Cruz M., Labra M-L., Montes J., Stanley S.L. Jr., and Calderon J. (1995) Identification of immunogenic epitopes of the 170-kDa subunit adhesin of *Entamoeba histolytica* in patients with invasive amebiasis. *J. Euk. Microbiol.* 42: 636-641.

136. Zhang T., and Stanley S.L. Jr. (1994) Protection of gerbils from amebic liver abscess by immunization with a recombinant protein derived from the 170-kilodalton surface adhesin of *Entamoeba histolytica. Infect. Immun.* 62: 2605-2608.

137. Mann B.J., Burkholder B.V., and Lockhart L.A. (1997) Protection in a gerbil model of amebiasis by oral immunization with *Salmonella* expressing the galactose/N-acetyl D-galactosamine inhibitable lectin of *Entamoeba histolytica. Vaccine* 15: 695-663.

CHAPTER 2

Interleukin-12: Structure, Induction and Function

2.1 Introduction

Interleukin-12 (IL-12) p70 is a heterodimeric cytokine composed of the disulphide-linked products of IL-12 p40 and p35 genes. IL-12 is most noted for its ability to stimulate differentiation of antigen-activated T cells into the Th1 phenotype: IL-2, tumour necrosis factor-ß (TNF-ß), and interferon-γ (IFN-γ) secreting cells (1). Although IL-12 was first described in 1989 as a product of EBV-transformed human B cells (2, 3), normal B cells do not produce IL-12 (4). Macrophages, dendritic cells and neutrophils are the principal cell sources of IL-12 (5, 6, 7). IL-12 p40 and p35 proteins are encoded by two unrelated genes (8). IL-12 p35 mRNA is constitutively expressed on most cell types but may be up-regulated in response to stimuli. In contrast, IL-12 p40 mRNA is only expressed in response to stimuli and is restricted to cells that produce IL-12 p70 (5, 8). Typically, production of bioactive IL-12 p70 is accompanied by a 5-500 fold excess production of inactive p40 protein (5). IL-12 p35 protein is not secreted on its own. This suggests that IL-12 p40 and p35 genes are differentially regulated. Some of the excess IL-12 p40 form homodimers which bind the human IL-12 receptor (IL-12R) *in vitro* and have been proposed to act as IL-12 antagonists *in vivo* (9).

IL-12R are primarily expressed on T and natural killer (NK) cells (10). High-affinity binding of IL-12 requires the co-expression of two ß-type receptor subunits (ß1 and ß2) that individually exhibit low affinity for IL-12 (9, 11). Resting NK cells express IL-12R but T cells must be activated to express IL-12R (9, 12). Continuous expression of both ß1 and ß2 subunits occurs in Th1 cells while IL-12R ß2 expression and IL-12

responsiveness is lost in Th2 cells: IL-4, -5, -6, -10, and -13 secreting cells (13, 14).

2.2 Stimulation of IL-12 production

2.2.1 Microbial Stimulation

Microbial components can directly activate macrophages, dendritic cells and neutrophils to produce IL-12 at the onset of infection. Human and murine IL-12 production is stimulated *in vivo* and *in vitro* by a variety of components from bacteria, protozoa, viruses and fungi (15). The extent of IL-12 induction *in vivo* and whether it plays a role in infection depends on a variety of factors that vary between different diseases.

Induction of IL-12 production in response to whole bacteria and bacterial products has been extensively documented. Lipopolysaccharide (LPS) from cell walls of gram-negative bacteria such as *Brucella abortus* stimulates IL-12 production by binding the CD14 receptor on the surface of macrophages (16). Lipoteichoic acid, a predominant surface glycolipid of gram-positive bacteria (e.g. *Listeria monocytogenes*), also stimulates IL-12 production through a CD14-dependent pathway (17). Lipoproteins from spirochaetal bacteria are potent IL-12 inducers but likely act through a CD14-independent pathway (18). Clearly, a variety of mechanisms are involved in bacterial-stimulation of IL-12. Both intracellular and extracellular bacteria stimulate IL-12 production (19, 20). Bacterial viability may or may not be required for IL-12 stimulation, depending on the bacterial species (19, 20). Recent studies have also demonstrated that bacterial DNA contain unmethylated CpG motifs which are strong IL-12 inducers (21, 22).

Protozoan parasites can be potent stimulators of macrophage and dendritic cell IL-12 production. Interestingly, this function is associated with life cycle stages derived from vertebrate hosts (23). *Leishmania major* promastigotes selectively inhibit IL-12 p40 mRNA induction in murine bone marrow macrophages (BMM) *in vitro* (24). In contrast,

amastigotes stimulate IL-12 p70 production from mouse dendritic cells (25). Similarly, trypomastigotes but not epimastigotes of *Trypanosoma cruzi* induced IL-12 production by mouse macrophages (26). *Toxoplasma gondii* tachyzoites have also been demonstrated to stimulate mouse macrophages for IL-12 production (27). The IL-12 inducing components of *T. cruzi* and *T. gondii* have been partially characterized and appear to be glycosylphosphatidylinositol-anchored glycoproteins (23, 26). To date, the only cloned and expressed parasite molecule shown to stimulate IL-12 production is a *L. braziliensis* protein (LeIF) (28). LeIF is homologous to the eukaryotic ribosomal protein eIF4A and induces IL-12 production from human peripheral blood mononuclear cells (PBMC) (28).

The fungus *Candida albicans* is an example of a microbe that has been shown to stimulate IL-12 production from both macrophages and neutrophils (29, 30). Herpes simplex virus and HIV-1 are examples of viruses that stimulate IL-12 mRNA expression in human PBMC (31).

The cell surface receptors involved in protozoal, fungal and virus-induced IL-12 production have not been identified. Binding of bacterial LPS or lipoteichoic acids to CD14 is the only receptor-coupling event associated with IL-12 stimulation, thus far (16, 17). In contrast, several cell surface receptors have been linked to inhibition of IL-12 production. LPS-stimulated IL-12 production from human monocytes was inhibited by binding of measles virus to its receptor CD46, a complement regulatory protein (32). Cross-linking of CD46 with antibody or C3b also inhibited IL-12 production. In addition, ligation of complement receptor 3 (CD11b/CD18), FcγRI or scavenger receptors inhibits IL-12 induction (33-35).

2.2.2 T cell-dependent stimulation

An alternate pathway for IL-12 induction involves ligation of CD40 on macrophages or dendritic cells to CD154 (CD40L) on activated Th1 cells (36). Naïve T cells and Th2 cells do not participate in this pathway of IL-12 induction (36, 37). CD40-

CD154-ligation is especially important for initiating IL-12 production in response to protein antigens that do not directly stimulate phagocytes to produce IL-12 (38). In CD40 knockout mice, this T cell-dependent pathway was shown to be crucial for IL-12 production in *L. major* but not *Mycobacterium tuberculosis* or *Histoplasma capsulatum* infections (39-41). Microbial products may co-operate with CD40 stimulation for enhanced IL-12 production. LPS has been shown to act as a co-factor with CD40 stimulation for IL-12 production (37). In addition, *L. braziliensis* LeIF synergized with soluble CD154 in inducing macrophage and dendritic cell IL-12 secretion (42).

2.3 Cytokine Regulation of IL-12 Production

Microbial and T cell-dependent IL-12 production is subject to positive and negative regulatory effects of a variety of cytokines. IFN-γ priming strongly augments both neutrophil and macrophage IL-12 production (7, 43). IFN-γ priming is actually a requirement for IL-12 production in response to *L. major* amastigotes and HIV glycoprotein 120 (25, 44). At the clonal level, mouse macrophage hybridomas stimulated with LPS demonstrated differential requirements for IFN-γ priming (45). In addition, IFN-γ priming appears to be required for T cell-dependent IL-12 production (37, 46). IL-12 and IFN-γ form a positive amplification loop *in vivo* and *in vitro* (15). This makes it difficult to determine which cytokine actually initiates the response. NK cells and neutrophils are potential early sources of IFN-γ production (15, 47). Similar to IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) primes macrophages for IL-12 production (48).

Macrophage-derived TNF-α may also contribute to IL-12 production. Anti-TNF-α serum blocked *M. bovis* and IFN-γ-induced IL-12 production from murine macrophages *in vitro* (49). In addition, mice lacking TNF receptors do not produce IL-12 in response to *M. bovis*, *L. monocytogenes* or *B. abortus* and experience exacerbated infections (49, 50).

When added concurrently with LPS or *Staphylococcus aureus,* the Th2 cytokines IL-4 and IL-13 inhibit IL-12 production from human PBMC (51). However, pretreatment of PBMC with IL-4 or IL-13 enhances IL-12 production in response to LPS or *S. aureus* (51). Further investigation of the priming effect of IL-13 revealed that it was additive with that of IFN-γ and, strikingly, was completely abolished by anti-TNF-α antibodies (52). In contrast to IL-4 and IL-13, the cytokines IL-6, IL-10, IL-11 and transforming growth factor-ß (TGF-ß) only have inhibitory effects on microbial and T cell-dependent IL-12 production (53-56).

2.4 Regulation of IL-12 Gene Expression

While IL-12 p40 gene expression has been more extensively studied, p35 gene expression is just as crucial for production of bioactive IL-12 p70 protein. As IL-12 p40 protein is produced in excess of p70, regulated expression of the p35 gene determines the level of p70 protein produced by stimulated cells (5, 48, 57). The relevance of IL-12 p35 expression was shown in a study of HIV-infected patients. Decreased expression of the IL-12 p35 but not the p40 gene correlated with defective IL-12 p70 production in the early stages of infection (58). Both IL-12 genes are primarily regulated at the transcriptional level (48, 56). Microbial stimuli such as LPS directly stimulate IL-12 p40 and p35 gene transcription (48). However, there is also evidence for some post-transcriptional regulation. TGF-ß inhibits LPS-stimulated IL-12 p40 gene transcription and mRNA stability in mouse macrophages (59). In a comparative study, LPS-stimulated IL-12 p40 transcription was found to be equivalent in adult PBMC and umbilical cord blood mononuclear cells (CBMC). However, CBMC IL-12 p40 gene expression was less stable and CBMC produced lower levels of IL-12 p70 protein (60).

Ets-2 and NF-κB binding motifs are present in the IL-12 p40 gene promoter and appear to be critical for responsiveness to both IFN-γ and LPS (61, 62). IFN-γ directly

stimulates transcription of the IL-12 p35 gene and primes the p40 gene for increased transcription in response to LPS (61). IFN-γ enhances the binding of nuclear complexes to the ets-2 and NF-κB sequences (62, 63). Indeed, LPS-stimulated IL-12 p40 transcription depends on activation of both ets-2 and NF-κB sequences (64). NF-κB activation was also shown to be involved in CD40-dependent IL-12 production (65). Interestingly, IL-11 inhibition of LPS-stimulated IL-12 production is associated with reduced NF-κB activation (66). The importance of IFN-γ in IL-12 induction was demonstrated in studies of interferon consensus sequence-binding protein (ICSBP) deficient mice. ICSBP is stimulated by IFN-γ and binds to the interferon-stimulated response element of gene promoters carrying this element. IL-12 production and Th1 responses are markedly impaired in ICSBP deficient mice (67, 68).

2.5 Effects of IL-12

The major effects of IL-12 are on NK and T cells (15). These cells demonstrate remarkably similar responses to IL-12. IL-12 also has limited stimulatory effects on neutrophils, B cells and macrophages (69-71). The most significant effect of IL-12 is its ability to stimulate high levels of IFN-γ production from NK and T cells (72). IFN-γ stimulation is central to most of the effects of IL-12 *in vivo* (15). IL-12 can synergize with other cytokines such as IL-2, IL-15, IL-1ß, and TNF-α for stimulation of maximal IFN-γ production (72-75). IL-12 can also synergize with B7/CD28 ligation during macrophage-T cell interaction for enhanced T cell IFN-γ production (76). One report has demonstrated that murine macrophages secrete IFN-γ in response to IL-12, suggesting an autocrine role for macrophage IL-12 (71). IL-12 induces lower-level production of several other cytokines including TNF-α and GM-CSF (76). Paradoxically, IL-12 stimulates production of the Th2 cytokine IL-10 which, in turn, inhibits IL-12 production (77). IL-12-induced IL-10 acts as a negative feedback mechanism, preventing the immunopathology associated

with excessive inflammatory responses (78). In contrast, IL-12 inhibits the production of TGF-ß (79).

IL-12 is a potent stimulator of NK and T cell cytotoxic activity and acts as a short term growth factor for these cells (80). IL-12 also stimulates T cell MHC-nonrestricted cytotoxic activity which occurs by a poorly understood mechanism (81). In addition, IL-12 treatment stimulates neutrophil reactive oxygen intermediate (ROI) production (69). ROI may contribute to the cytotoxic activity of neutrophils. Apart from stimulating cytotoxic activity, IL-12 protects human Th1 cells from activation-induced apoptosis (82). This may be of special importance in the maintenance of established Th1 responses.

The most distinctive of IL-12's functions is its ability to regulate the balance between Th1 and Th2 responses. The production of Th1 cytokines IFN-γ and TNF-ß promotes the activation of macrophages for nitric oxide (NO)-mediated microbicidal activity. Th2 cytokines predominantly induce humoral responses and promote mast cell and eosinophil activation (83). The type of antigen-presenting cell and antigen dose are among the factors that influence the differentiation path of antigen-stimulated T cells (84, 85). However, the dominant factor is the local cytokine milieu. IL-4 produced by basophils and mast cells promotes Th2 development while IL-12 directs the development of Th1 responses (86, 87). Both antigen-stimulated naïve T cells and resting T cells re-exposed to antigen develop into Th1 IFN-γ producing cells in the presence of IL-12 (80). IL-12-dependent Th1 development can be enhanced by IL-18 (88). IL-18 shares many common functions with IL-12; however, IL-18 can not stimulate Th1 development on its own (88).

2.6 Role of IL-12 in Infectious Diseases

Failure to control or resolve infectious diseases often results from the development of an inappropriate Th response rather than an insufficient immune response (89). In this

regard, IL-12 is essential for resistance to diseases controlled by Th1 responses. *L. major* infection in mice is a classic example of Th1-mediated resistance to infection. C57BL/6 mice mount a Th1 response and are resistant, while BALB/c mice mount a Th2 response and succumb to the infection (90). Several experimental approaches have demonstrated that IL-12 plays a critical role in developing Th1-mediated protection against *L. major* in mice. Antibody-neutralization of IL-12 at the time of infection was found to reverse resistance to infection (91). In addition, IL-12-deficient mice derived from a resistant background were susceptible to *L. major* infection (92). In agreement with these results, susceptible BALB/c mice developed Th1 responses and were cured following injection with IL-12 during the first week of infection (93). IL-12 may also be capable of reversing an established Th2 response to *L. major*. Combined treatment of BALB/c mice with Pentostam and IL-12 induced healing and switch from a Th2 response to a Th1 response (94). These data suggested that decreasing antigen levels were required for IL-12 to inhibit a Th2 response and generate or enhance a Th1 response. In humans, a Th2 response is believed to be responsible for a lack of protection against visceral leishmaniasis (95).

Evidence from murine models of toxoplasmosis and trypanosomiasis (Chagas' disease) indicates that IL-12 induces protective immunity in these diseases as well. IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *T. gondii* in mice. Treatment with anti-IL-12 antibodies increased the susceptibility of C57BL/6, BALB/c and severe combined immunodeficient (SCID) mice to acute infection, resulting in 100% mortality during the first two weeks of infection (27). *T. cruzi* -infected C57BL/6 mice also had significantly increased parasitaemia and mortality following treatment with an anti-IL-12 monoclonal antibody (96). Malaria is another protozoal disease in which IL-12 appears to have a protective role. Prophylactic treatment of *Plasmodium cynomolgi*-infected monkeys with IL-12 protected these animals from disease (97). Protection was associated with elevated plasma IFN-γ levels. A study of *P. chabaudi* AS-infected mice demonstrated that IL-12-induced protection required IFN-γ and TNF-α and was partially

dependent on NO (98). IL-12 may also act to correct the severe anaemia associated with blood stage malaria. Injection of *P. chabaudi* AS-susceptible mice with IL-12 during infection enhanced bone marrow and splenic erythropoiesis (99).

There is considerable evidence to support a role for IL-12 in resistance to *Schistosoma mansoni* infections in mice. Immunity induced by attenuated cercariae was shown to be dependent on IFN-γ production from Th1 cells (100). In addition, failure of mice to respond to vaccination against schistosomiasis correlated with impaired IL-12 production and enhanced Th2 responses (101). IL-12 inhibits the Th2-associated granulomatous response to *S. mansoni* eggs indicating that it acts to decrease the pathology of *Schistosoma* infections (102). Protection against other helminths (e.g.*Nippostrongylus brasiliensis*) is associated with Th2 responses and administration of exogenous IL-12 exacerbates these infections (103).

Resistance to *Mycobacterium* spp. in both humans and animal models is dependent on IL-12-induced Th1 responses (104, 105). *M. leprae*-infected patients with tuberculoid leprosy control the growth of the bacteria and their skin lesions are characterized by a predominance of Th1 cells. Patients with lepromatous leprosy are unable to control the infection and their skin lesions are characterized by predominance of Th2 cells (106). Recently, it was also shown that patients with IL-12R deficiency were impaired in their ability to control mycobacterial infections (104). IL-12 can stimulate cell-mediated immunity in viral infections as well. For example, IL-12 down-regulates HIV replication in human macrophages *in vitro* (107). Respiratory syncytial virus and pseudorabies virus infections in mice are examples of protective immunity mediated by Th1 humoral responses. In both cases, immunization with inactivated virus and exogenous IL-12 generated protective IgG2a antibody responses (108, 109). Fungal infections, in contrast, are controlled by cell-mediated Th1 responses that are dependent on IL-12 (110).

2.7 IL-12 as a Vaccine Adjuvant

Because of its ability to induce Th1 differentiation, IL-12 has been studied as a vaccine adjuvant to promote Th1 responses during immunization. The adjuvant effect of IL-12 was first demonstrated in vaccination of mice against *L. major*. Normally susceptible BALB/c mice were vaccinated simultaneously with leishmanial antigens and IL-12 and developed protective Th1 immune responses (111). IL-12 may be especially beneficial in the context of antigens that are weakly to nonimmunogenic. In one study, Th1-mediated resistance to *L. monocytogenes* was induced in mice following vaccination with a nonimmunogenic synthetic peptide and IL-12 (112). The adjuvant-like effect of IL-12 in mice has been demonstrated in vaccinations against several other pathogens including *Mycobacterium avium* (113). Vaccination of antigen with an IL-12-inducing compound also acts to stimulate murine Th1-mediated immunity. Co-administration of bacterial CpG oligodeoxynucleotides and hen egg lysozyme resulted in a Th1-dominated response characterized by IFN-γ production (114). Vaccination with hen egg lysozyme in incomplete Freund's adjuvant stimulated a Th2 response. In mice, prior sensitization with *S. mansoni* eggs and IL-12 partly inhibited granuloma formation and dramatically reduced tissue fibrosis induced by a natural infection with *S. mansoni* (115). The reduced liver pathology achieved in this study suggests that vaccination with IL-12 may have application in reducing Th2-associated pathological responses. Taken together, these data highlight the potential use of IL-12 in human vaccination as an adjuvant to induce Th1 responses.

2.8 References

1. Magram J., Connaughton S.E., Warrier R.R., Carvajal D.M., Wu C-Y., Ferrante J., Stewart C., Sarmiento U., Faherty D.A., and Gately M.K. (1996) IL-12 deficient mice are defective in IFN-γ production and type 1 cytokine reponses. *Immunity* 4: 471-481.

2. Kobayashi M, Fitz L., Ryan M., Hewick R.M., Clark S.C., Chan S., London R., Sherman F., Perussia B., and Trinchieri G. (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170: 827-846.

3. Stern A.S., Podlaski F.J., Hulmes J.D., Pan Y.E., Quinn P.M., Wolitzky A.G., Familletti P.C., Stremio D.L., Truitt T., Chizzonite R., and Gately M.K. (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA* 87: 6808-6812.

4. Guéry J-C., Ria F., Galbiati F., and Adorini L. (1997) Normal B cells fail to secrete interleukin-12. *Eur. J. Immunol.* 27: 1632-1639.

5. D'Andrea A., Rengaraju M., Valiante N.M., Chehimi J., Kubin M., Aste-Amezaga M., Chan S.H., Kobayashi M., Young D., Nickbarg E., Chizzonite R., Wolf S.F., and Trinchieri G. (1992) Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176: 1387-1398.

6. Cella M., Scheidegger D., Palmer-Lehmann K., Lane P., Lanzavecchia A., and Alber G. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* 184: 747-752.

7. Cassatella M.A., Meda L., Gasperini S., D'Andrea A., Ma X., and Trinchieri G. (1995) Interleukin-12 production by human polymorphonuclear leukocytes. *Eur. J. Immunol.* 25: 1-5.

8. Gubler U., Chua A.O., Schoenhaut D.S., Dwyer C.M., McComas W., Motyka R., Nabavi N., Wolitzky A.G., Quinn P.M., Familletti P.C., Gately M.K. (1991) Co-expression of two distinct genes is required to generate secreted, bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA* 88: 4143-4147.

9. Presky D.H., Gubler U., Chizzonite R.A., and Gately M.K. (1995) IL-12 receptors and receptor antagonists. *Res. Immunol.* 146: 439-445.

10. Desai B.B., Quinn P.M., Wolitzky A.G., Mongini P.K.A., Chizzonite R., and Gatley M.K. (1992) IL-12 receptor. II. Distribution and regulation of receptor expression. *J. Immunol.* 148: 3125-3132.

11. Presky D.H., Yang H., Minetti L.J., Chua A.O., Nabavi N., Wu C.Y., Gately M.K., and Gubler U. (1996) A functional interleukin-12 receptor complex is composed of two ß-type cytokine receptor subunits. *Proc. Natl. Acad. Sci. USA* 93: 14002-14007.

12. Gazzinelli R.T. (1996) Molecular and cellular basis of interleukin-12 activity in prophylaxis and therapy against infectious diseases. *Mol. Med. Today* June: 258-267.

13. Rogge L., Barberis-Maino L., Biffi M., Passini N., Presky D.H., Gubler U., and Sinigaglia F. (1997) Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J. Exp. Med.* 185: 825-831.

14. Szabo S.J., Dighe A.S., Gubler U., and Murphy K.M. (1997) Regulation of the interleukin-12R ß2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 185: 817-824.

15. Romani L., Puccetti P., and Bistoni F. (1997) Interleukin-12 in infectious diseases. *Clin. Microbiol. Rev.* 10: 611-636.

16. Zaitseva M., Golding H., Manischewitz J., Webb D., and Golding B (1996) *Brucella abortus* as a potential vaccine candidate: Induction of interleukin-12 secretion and enhanced B7.1 and B7.2 and intracellular adhesion molecule 1 surface expression in elutriated human moncytes stimulated by heat-inactivated *B. abortus*. *Infect. Immun.* 64: 3109-3117.

17. Cleveland M.G., Gorham J.D., Murphy T.L., Tuomanen E., and Murphy K.M. (1996) Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infect. Immun.* 64: 1906-1912.

18. Radolf J.D., Arndt L.L., Akins D.R., Curetty L.L., Levi M.E., Shen Y., Davis

L.S., and Norgard M.V. (1995) *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytes/macrophages. *J. Immunol.* 154: 2866-2877.

19. Chong C., Bost K.L., and Clements J.D. (1996) Differential production of interleukin-12 mRNA in response to viable or killed *Salmonella* spp. *Infect. Immun.* 64: 1154-1160.

20. Bohn E., and Autenrieth I.B. (1996) IL-12 is essential for resistance against *Yersinia enterocolitica* by triggering IFN-γ production in NK cells and CD4$^+$ T cells. *J. Immunol.* 156: 1458-1468.

21. Jakob T., Walker P.S., Krieg A.M., Udey M.C., and Vogel J.C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of TH1 responses by immunostimulatory DNA. *J. Immunol.* 161: 3042-3049.

22. Krieg A.M., Love-Homan L., Yi A-K., and Harty J.T. (1998) CpG DNA induces sustained IL-12 expression *in vivo* and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* 161: 2428-2434.

23. Gazzinelli R.T., Camargo M.M., Almeida I.C., Morita Y.S., Giraldo M., Acosta-Serrano A., Hieny S., Englund P.T., Ferguson A.J., Travassos L.R., and Sher A. (1997) Identification and characterization of protozoan products that trigger the synthesis of IL-12 by inflammmatory macrophages. *Chem. Immunol.* 68: 136-152.

24. Carrera L., Gazzinelli R.T., Badolato R., Hieny S., Müller W., Kühn R., and Sacks D. (1996) *Leishmania* promastigotes selectively inhibit interleukin-12 induction in bone marrow-derived macrophages from susceptible and resistant mice. *J. Exp. Med.* 183: 515-526.

25. von Stebut E., Belkaid Y., Jakob T., Sacks D.L., and Udey M.C. (1998) Uptake of *Leishmania major* amastigotes results in activation and interleukin-12 release from murine skin-derived dendritic cells: Implications for the initiation of anti-*Leishmania* immunity. *J. Exp. Med.* 188: 1547-1552.

26. Camargo M.M., Almeida I.C., Pereira M.E.S., Ferguson M.A.J., Travassos L.R., and Gazzinelli R.T. (1997) Glycophosphatidylinositol-anchored mucin-like glycoproteins isolated from *Trypanosoma cruzi* trypomastigotes initiate the synthesis of proinflammatory cytokines by macrophages. *J. Immunol.* 158: 5890-5901.

27. Gazzinelli R.T., Wysocka M., Hayashi S., Denkers E.Y., Hieny S., Caspar P., Trinchieri G., and Sher A. (1994) Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. *J. Immunol.* 153: 2533-2543.

28. Skeiky Y.A.W., Guderian J.A., Benson D.R., Bacelar O., Carvalho E.M., Kubin M., Badaro R., Trinchieri G., and Reed S.G. (1995) A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin-12. *J. Exp. Med.* 181:1527-1537.

29. Harrison T.S., and Levitz S.M. (1996) Role of IL-12 in peripheral blood mononuclear cell responses to fungi in persons with and without HIV infection. *J. Immunol.* 156: 4492-4497.

30. Romani L., Mencacci A., Cenci E., Puccetti P., and Bistoni F. (1997) Neutrophils and the adaptive immune response to *Candida albicans*. *Res. Immunol.* 147: 512-518.

31. Ghaekar S., Zheng L., Logar A., Navratil J., Borowski L., Gupta P., and Rinaldo C. (1996) Cytokine expression by human peripheral blood dendritic cells stimulated *in vitro* with HIV-1 and herpes simplex virus. *J. Immunol.* 157: 4028-4036.

32. Karp C.L., Wysocka M., Wahl L.M., Ahearn J.M., Cuomo P.J., Sherry B., Trinchieri G., and Griffin D.E. (1996) Mechanism of suppression of cell-mediated immunity by measles virus. *Science* 273: 228-231.

33. Marth T., and Kelsall B.L. (1997) Regulation of interleukin-12 by complement receptor 3 signaling. *J. Exp. Med.* 185: 1987-1995.

34. Sutterwala F.S., Noel G.J., Salgame P., and Mosser D.M. (1998) Reversal of proinflammatory responses by ligating the macrophage Fcγ receptor type 1. *J. Exp.*

Med. 188: 217-222.

35. Sutterwala F.S., Noel G.J., Clynes R., and Mosser D.M. (1997) Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J. Exp. Med.* 185: 1977-1985.

36. Ria F., Penna G., and Adorini L. (1998) Th1 cells induce and Th2 inhibit antigen-dependent IL-12 secretion by dendritic cells. *Eur. J. Immunol.* 28: 2003-2016.

37. Snijders A., Kalinski P., Hilkens C.M.U., and Kapsenberg M.L. (1998) High-level IL-12 production by human dendritic cells requires two signals. *Int. Immunol.* 10: 1593-1598.

38. DeKruyff R.H., Gieni R.S., and Umetsu D.T. (1997) Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40. *J. Immunol.* 158: 359-366.

39. Campbell K.A., Ovendalw P.J., Kennedy M.K., Fanslow W.C., Reed S.G., and Maliszewski C.R. (1996) CD40 ligand is required for protective cell-mediated immunity to *Leishmania major. Immunity* 4: 283-289.

40. Campos-Neto A., Ovendale P., Bement T., Koppi T.A., Fanslow W.C., Rossi M.A., and Alderson M.R. (1998) Cutting edge: CD40 ligand is not essential for the development of cell-mediated immunity and resistance to *Mycobacterium tuberculosis. J. Immunol.* 160: 2037-2041.

41. Zhou P., and Seder R.A. (1998) CD40 ligand is not essential for induction of type 1 cytokine responses or protective immunity after primary or secondary infection with *Histoplasma capsulatum. J. Exp. Med.* 187: 1315-1324.

42. Probst P., Skeiky Y.A., Steeves M., Gervassi A., Grabstein K.H., and Reed S.G. (1997) A *Leishmania* protein that modulates interleukin (IL)-12, IL-10 and tumor necrosis factor-α production and expression of B7-1 in human monocyte-derived antigen-presenting cells. *Eur. J. Immunol.* 27:2634-2642.

43. Kubin M., Chow J.M., and Trinchieri G. (1994) Differential regulation of

interleukin-12 (IL-12), tumor necrosis factor-α, and IL-1ß production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. *Blood* 83: 1847-1855.

44. Fantuzzi L., Gessani S., Borghi P., Varano B., Conti L., Puddu P., and Belardelli F. (1996) Induction of interleukin-12 (IL-12) by recombinant glycoprotein gp120 of human immunodeficiency virus type 1 in human monocytes/macrophages: Requirement of gamma interferon for IL-12 secretion. *J. Virol.* 70: 4121-4124.

45. Taneichi M., Horino A., Naito S., Sakurai S., Komuro K., and Uchida T. (1997) Differential stimulation requirements for IL-12 production among clones of macrophage hybridomas. *Journal of Interferon and Cytokine Research* 17: 603-607.

46. Hilkens C.M.U., Kalinski P., de Boer M., and Kapsenberg M.L. (1997) Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naïve T-helper cells toward the Th1 phenotype. *Blood* 90: 1920-1926.

47. Yeaman G.R., Collins J.E., Currie J.K., Guyre P.M., Wira C.R., and Fanger M.W. (1998) IFN-γ is produced by polymorphonuclear neutrophils in human uterine endometrium and by cultured peripheral blood polymorphonuclear neutrophils. *J. Immunol.* 160: 5145-5153.

48. Hayes M.P., Wang J., and Norcross M.A. (1995) Regulation of interleukin-12 expression in human monocytes: Selective priming by interferon-γ of lipopolysaccharide-inducible p35 and p40 genes. *Blood* 86: 646-650.

49. Flesch I.E.A., Hess J.H., Huang S., Aguet M., Rothe J., Bluethmann H., and Kaufmann S.H.E. (1995) Early interleukin-12 production by macrophages in response to mycobacterial infection depends on interferon-γ and tumor necrosis factor-α. *J. Exp. Med.* 181: 1615-1621.

50. Zhan Y., and Cheers C. (1998) Control of IL-12 and IFN-γ production in response to live or dead bacteria by TNF and other factors. *J. Immunol.* 161: 1447-1453.

51. D'Andrea A., Ma X., Aste-Amezaga M., Paganin C., and Trinchieri G. (1995)

Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: Priming for IL-12 and tumor necrosis factor α production. *J. Exp. Med.* 181: 537-546.

52. Minty A., Ferrara P., and Caput D. (1997) Interleukin-13 effects on activated monocytes lead to novel cytokine secretion profiles intermediate between those induced by interleukin-10 and by interferon-γ. *European Cytokine Network* 8: 189-201.

53. Takenaka H., Maruo S., Yamamoto N., Wysocka M., Ono S., Kobayashi M., Yagita H., Okumura K., Hamaoka T., Trinchieri G., Fujiwara H. (1997) Regulation of T cell-dependent and -independent IL-12 production by the three Th2-type cytokines IL-10, IL-6, and IL-4. *J. Leukoc. Biol.* 61: 80-87.

54. Aste-Amezaga M., Ma X., Sartori A., and Trinchieri G. (1998) Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J. Immunol.* 160: 5936-5944.

55. Leng S.X., and Elias J.A. (1997) Interleukin-11 inhibits macrophage interleukin-12 production. *J. Immunol.* 159: 2161-2168.

56. Trinchieri G., and Gerosa F. (1996) Immunoregulation of interleukin-12. *J. Leukoc. Biol.* 59: 505-511.

57. Snijders A., Hilkens C.M.U., van der Pouw Kraan T.C.T.M., Engel M., Aarden L.A., and Kapsenberg M.L. (1996) Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *J. Immunol.* 156: 1207-1212.

58. Chougnet C., Wynn T.A., Clerici M., Landay A.L., Kessler H.A., Rusnak J., Melcher G.P., Sher A., and Shearer G.M. (1996) Molecular analysis of decreased interleukin-12 production in persons infected with human immunodeficiency virus. *J. Infect. Dis.* 174: 46-53.

59. Du C., and Sriram S. (1998) Mechanism of inhibition of LPS-induced IL-12 p40 production by IL-10 and TGF-ß in ANA-1 cells. *J. Leukoc. Biol.* 64: 92-97.

60. Lee S.M., Suen Y., Chang L., Bruner V., Qian J., Indes J., Knoppel E., van de Ven C., and Cairo M.S. (1996) Decreased interleukin-12 (IL-12) from activated cord versus adult peripheral blood mononuclear cells and upregulation of Interferon-γ, natural killer, and lymphokine-activated killer activity by IL-12 in cord blood mononuclear cells. *Blood* 88: 945-954.

61. Ma X., Chow J.M., Gri G., Carra G., Gerosa F., Wolf S.F., Dzialo R., Trinchieri G. (1996) The interleukin-12 p40 gene promoter is primed by interferon-γ in monocytic cells. *J. Exp. Med.* 183: 147-157.

62. Murphy T.L., Cleveland M.G., Kulesza P., Magram J., and Murphy K.M. (1995) Regulation of interleukin-12 p40 expression through an NF-κB half-site. *Mol. Cell. Biol.* 15: 5258-5267.

63. Ma X., Neurath M., Gri G., and Trinchieri G. (1997) Identification and characterization of a novel ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. *J. Biol. Chem.* 272: 10389-10395.

64. Gri G., Savio D., Trinchieri G., and Ma X. (1998) Synergistic regulation of the human interleukin-12 p40 promoter by NF-κB and ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. *J. Biol. Chem.* 273: 6431-6438.

65. Yoshimoto T., Nagase H., Ishida T., Inoue J-I., and Nariuchi H. (1997) Induction of interleukin-12 p40 transcript by CD40 ligation via activation of nuclear factor-κB. *Eur. J. Immunol.* 27: 3461-3470.

66. Trepicchio W., Wang L., Bozza M., and Dorner A.J. (1997) IL-11 regulates macrophage effector function through the inhibition of nuclear factor-κB. *J. Immunol.* 159: 5661-5670.

67. Giese N.A., Gabriele L., Doherty T.M., Klinman D.M., Tadesse-Heath L., Contursi C., Epstein S.L., and Morse H.C. III (1997) Interferon (IFN) consensus sequence-binding protein, a transcription factor of the IFN regulatory factor family, regulates immune responses in vivo through control of interleukin-12 expression. *J. Exp.*

Med. 186: 1535-1546.

68. Scharton-Kersten T., Contursi C., Masumi A., Sher A., and Ozato K. (1997) Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin-12 p40 induction. *J. Exp. Med.* 186: 1523-1534.

69. Collison K., Saleh S., Parhar R., Meyer B., Kwaasi A., Al-Hussein K., Al-Sedairy S., and Al-Mohanna F. (1998) Evidence for IL-12-activated Ca^{2+} and tyrosine signaling pathways in human neutrophils. *J. Immunol.* 161: 3737-3745.

70. Metzger D.W., Vogel L.A., van Cleave V.H., Lester T.L., and Buchanan J.M. (1995) The effects of IL-12 on B-cell subset function. *Res. Immunol.* 146: 499-505.

71. Puddu P., Fantuzzi L., Borghi P., Varano B., Rainaldi G., Guillemard E., Malorni W., Nicaise P., Wolf S.F., Belardelli F., and Gessani S. (1997) IL-12 induces IFN-γ expression and secretion in mouse peritoneal macrophages. *J. Immunol.* 159: 3490-3497.

72. Chan S.H., Perussia B., Gupta J.W., Kobayashi M., Pospisil M., Young H.A., Wolf S.F., Young D., Clark S.C., Trinchieri G. (1991) Induction of interferon-γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173: 869-879.

73. Avice M-N., Demeure C.E., Delespesse G., Rubio M., Armant M., Sarfati M. (1998) IL-15 promotes IL-12 production by human monocytes via T cell-dependent contact and may contribute to IL-12-mediated IFN-γ secretion by $CD4^+$ T cells in the absence of TCR ligation. *J. Immunol.* 161: 3408-3415.

74. Hunter C.A., Chizzonite R., Remington J.S. (1995) IL-1ß is required for IL-12 to induce production of IFN-γ by NK cells. *J. Immunol.* 155: 4347-4354.

75. Tripp C.S., Wolf S.F., and Unanue E.R. (1993) Interleukin-12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency with listeriosis and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA* 90: 3725-3729.

76. Kubin M., Kamoun M., and Trinchieri G. (1994) Interleukin-12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J. Exp. Med.* 180: 211-222.

77. Meyaard L., Hovenkamp E., Otto S.A., and Miedema F. (1996) IL-12-induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. *J. Immunol.* 156: 2776-2782.

78. Gazzinelli R.T., Wysocka M., Hieny S., Scharton- Kersten T., Cheever A., Kühn R., Müller W., Trinchieri G., and Sher A. (1996) In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on $CD4^+$ T cells and accompanied by overproduction of IL-12, IFN-γ, and TNF-α. *J. Immunol.* 157: 798-805.

79. Marth T., Strober W., Seder R.A., Kelsall B.L. (1997) Regulation of transforming growth factor-ß production by interleukin-12. *Eur. J. Immunol.* 27: 1213-1220.

80. Gately M.K., Renzetti L.M., Magram J., Stern A.S., Adorini L., Gubler U., and Presky D. H. (1998) The interleukin-12/interleukin-12-receptor system: Role in normal and pathologic immune responses. *Annu. Rev. Immunol.* 16: 495-521.

81. Cesano A., Visonneau S., Clark S.C., and Santoli D. (1993) Cellular and molecular mechanisms of activation of MHC nonrestricted cytotoxic cells by IL-12. *J. Immunol.* 151: 2943-2957.

82. Radrizzani M., Accornero P., Amidei A., Aiello A., Delia D., Kurrle R., Colombo M.P. (1995) IL-12 inhibits apoptosis induced in a human Th1 clone by gp120/CD4 cross-linking and CD3/TCR activation or by IL-2 deprivation. *Cell. Immunol.* 161: 14-21.

83. Mosmann T.R., and Coffman R.L. (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7: 145-173.

84. Desmedt M., Rottiers P., Dooms H., Fiers W., and Grooten J. (1998) Macrophages

induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. *J. Immunol.* 160: 5300-5308.

85. Bretscher P., Wei G., and Menon J. (1992) Establishment of stable cell mediated immunity that makes 'susceptible' mice resistant to *Leishmania major. Science* 257: 539-542.

86. Seder R.A., Paul W.A., Davis M.M., and Fazekas de St. Groth B. (1992) The presence of interleukin-4 during in vitro priming determines the lymphocyte-producing potential of CD4+ T cells from T cell receptor transgenic mice. *J. Exp. Med.* 176: 1091-1098.

87. Scott P. (1993) IL-12: Initiation cytokine for cell-mediated immunity. *Science* 260: 496-497.

88. Stoll S., Jonuleit H., Schmitt E., Müller G., Yamauchi H., Kurimoto M., Knop J., and Enk A.H. (1998) Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development. *Eur. J .Immunol.* 28: 3231-3239.

89. Powrie F., and Coffman R.L. (1993) Cytokine regulation of T-cell function: potential for therapeutic intervention. *Immunol. Today* 14: 270-274.

90. Reiner S.L., and Locksley R.M. (1995) The regulation of immunity to *Leishmania major. Annu. Rev. Immunol.* 13: 151-177.

91. Heinzel F.P., Rerko R.M., Ahmed F., Pearlman E. (1995) Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. *J. Immunol.* 155: 730-739.

92. Mattner F., Di Padova K., Alber G. (1997) Interleukin-12 is indispensible for protective immunity against *Leishmania major. Infect. Immun.* 65: 4378-4383.

93. Heinzel F.P., Schoenhaut D.S., Rerko R.M., Rosser L.E., and Gately M.K. (1993) Recombinant interleukin-12 cures mice infected with *Leishmania major. J. Exp. Med.* 177: 1505-1509.

94. Nabors G.S., Afonso L.C.C., Farrell J. P., and Scott P. (1995) Switch from a type 2 to a type 1 T helper cell response and cure of established *Leishmania major* infection in mice is induced by combined therapy with interleukin-12 and Pentostam. *Proc. Natl. Acad. Sci. USA* 92: 3142-3146.

95. Badaro R., Falcoff E., Badaro F.S., Carvalho E.M., Pedral-Sampaio D., Barral A., Carvalho J.S., Barral-Netto M., Brandely M., Silva L., Bina J.C., Teixeira R., Falcoff R., Rocha H., Ho J.L., and Johnston W.D. (1990) Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. *N. Engl. J. Med.* 322: 16-21.

96. Aliberti J.C.S., Cardoso M.A.G., Martins G.A., Gazzinelli R.T., Viera L.Q., Silva J.S. (1996) Interleukin-12 mediates resistance to *Trypanosoma cruzi* in mice and is produced by murine macrophages in response to live trypomastigotes. *Infect. Immun.* 64: 1961-1967.

97. Hoffman S.L., Crutcher J.M., Puri S.K., Ansari A.A., Villinger F., Franke E.D., Singh P.P., Finkelman F., Gately M.L., Dutta G.P., and Sedegah M. (1997) Sterile protection of monkeys against malaria after administration of interleukin-12. *Nature Medicine* 3: 80-83.

98. Stevenson M.M., Tam M.F., Wolf S. F., Sher A. (1995) IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via a nitric oxide-dependent mechanism. *J. Immunol.* 155: 2545-2556.

99. Mohan K., and Stevenson M.M. (1998) Interleukin-12 corrects severe anemia during blood-stage *Plasmodium chabaudi* AS in susceptible A/J mice. *Exp. Hematol.* 26: 45-52.

100. Smythies I.E., Coulson P.S., and Wilson R.A. (1992) Monoclonal antibody to IFN-γ modifies pulmonary inflammatory responses and abrogates immunity to *Schistosoma mansoni* in mice vaccinated with attenuated cercariae. *J. Immunol.* 149: 3654-3658.

101. Oswald I.P., Casper P., Wynn T.A., Scharton-Kersten T., Williams M.E., Hieny

S., Sher A., and James S.L. (1998) Failure of P strain mice to respond to vaccination against schistosomiasis correlates with impaired production of IL-12 and up-regulation of Th2 cytokines that inhibit macrophage activation. *Eur. J. Immunol.* 28: 1762-1772.

102. Wynn T.A., Eltoum I., Oswald I.P., Cheever A.W., and Sher A. (1994) Endogenous interleukin-12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. *J. Exp. Med.* 179: 1551-1561.

103. Finkelman F.D., Madden K.B., Cheever A.W., Katona I.M., Morris S.C., Gately M.K., Hubbard B.R., Gause W.C., and Urban J.F. Jr. (1994) Effects of interleukin-12 on immune responses and host protection in mice infected with intestinal nematode parasites. *J. Exp. Med.* 179: 1563-1572.

104. Altare F., Durandy A., Lammas D., Emile J-F., Lamhamedi S., Le Deist F., Drysdale P., Jouanguy E., Döffinger R., Bernaudin F., Jeppsson O., Gollob J.A., Meinl E., Segal A.W., Fischer A., Kumararantne D., and Casanova J-L. (1998) Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 280: 1432-1435.

105. Cooper A.M., Magram J., Ferrante J., and Orme I.M. (1997) Interleukin-12 is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J. Exp. Med.* 186: 39-45.

106. Modlin R.L. (1994) Th1-Th2 paradigm: insights from leprosy. *J. Invest. Dermatol.* 102: 828-832.

107. Akridge R.E., and Reed S.G. (1996) Interleukin-12 decreases human immunodeficiency virus type 1 replication in human macrophage cultures reconstituted with autologous peripheral blood mononuclear cells. *J. Infect. Dis.* 173: 559-564.

108. Tang Y.W., and Graham B.S. (1995) Interleukin-12 treatment during immunization elicits a T helper cell type 1-like immune response in mice challenged with respiratory syncytial virus and improves vaccine immunogenicity. *J. Infect. Dis.* 172: 734-738.

109. Schijns V.E.C.J., Haagmans B.L., and Horzinek M.C. (1995) IL-12 stimulates an antiviral type 1 cytokine response but lacks adjuvant activity in IFN-γ-receptor-deficient mice. *J. Immunol.* 155: 2525-2532.

110. Mencacci A., Spaccapelo R., Del Sero G., Enssle K-H., Cassone A., Bistoni F., and Romani L. (1996) CD4+ T-helper cell responses in mice with low-level *Candida albicans* infection. *Infect. Immun.* 64: 4907-4914.

111. Afonso L.C.C., Scharton T.M., Vieira L.Q., Wysocka M., Trinchieri G., and Scott P. (1994) The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major. Science* 263: 235-237.

112. Miller M.A., Skeen M.J., and Ziegler H.K. (1997) A synthetic peptide administered with IL-12 elicits immunity to *Listeria monocytogenes. J. Immunol.* 159: 3675-3679.

113. Silva R.A., Pais T.F., Appelberg R. (1998) Evaluation of IL-12 in immunotherapy and vaccine design in experimental *Mycobacterium avium* infections. *J. Immunol.* 161: 5578-5585.

114. Chu R.S., Targoni O.S., Krieg A.M., Lehmann P.V., and Harding C.V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper (Th1) immunity. *J. Exp. Med.* 10: 1623-1631.

115. Wynn T.A., Cheever A.W., Jankovic D., Poindexter R.W., Casper P., Lewis F.A., and Sher A. (1995) An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. *Science* 376: 594-596.

SECTION II: MANUSCRIPTS I, II, AND III

MANUSCRIPT I

Interleukin (IL-) 2, IL-4 and tumor necrosis factor-α responses during *Entamoeba histolytica* liver abscess development in gerbils*

Darren Campbell and Kris Chadee

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ABSTRACT

To determine cytokine production patterns during hepatic amebiasis, we infected gerbils with *Entamoeba histolytica* in the liver and quantified spleen and hepatic lymph node cell proliferation, interleukin-2 (IL-2, a Th1 marker), IL-4 (a Th2 marker) and tumor necrosis factor-α (TNF-α) production in response to concanavalin A and amebic antigen *in vitro*. Early abscess development (day 5 after inoculation) coincided with IL-2, IL-4 and low TNF-α production and strong lymphoproliferative responses, whereas suppression of IL production and lymphoproliferation occurred during acute disease (day 20). Proliferative responses and IL-2 production increased at days 30 and 60 after inoculation, but IL-4 levels remained low. Animals drug-treated at day 20 after inoculation demonstrated high IL-2 and low IL-4 production and resistance to reinfection. While acute hepatic amebiasis in gerbils is accompanied by transient immunosuppression, late infection and resistance to reinfection are associated with IL-2 production but low IL-4 and TNF-α production (Th-1-like response).

INTRODUCTION

Entamoeba histolytica is an enteric protozoan parasite of humans and the causative agent of invasive amebiasis. *In vitro* and *in vivo* studies indicate that macrophages are potent effector cells for killing *E. histolytica* trophozoites. Macrophages activated with interferon-γ (IFN-γ), IFN-γ and tumor necrosis factor-α (TNF-α) or IFN-γ and lipopolysaccharide (LPS) demonstrated potent contact-dependent amebicidal activity [1, 2] attributed to nitric oxide (NO) production [3]. Recently, we demonstrated that TNF-α, produced by activated macrophages, acts in an autocrine fashion to elevate NO release and amebicidal activity by enhancing transcription of the inducible-nitric oxide synthase (iNOS) gene [4]. However, macrophages isolated from amebic liver abscess (ALA) are functionally deficient and the host is unable to control the infection. ALA-derived macrophages were unresponsive to IFN-γ and LPS-activating signals for production of TNF-α and NO and cytotoxicity against amebae [5] and were deficient in their ability to develop a respiratory burst and secrete interleukin (IL)-1 [6]. Pretreatment of naive macrophages *in vitro* with amebic proteins down-regulated TNF-α production and Ia molecule expression [7, 8]. ALA-derived macrophages produced elevated levels of prostaglandin E_2 (PGE_2) [9], which was responsible in part for inhibiting TNF-α production and Ia molecule expression [7, 8]. It is also possible that macrophage down-regulating cytokines such as IL-4, IL-10, and transforming growth factor-ß (TGF-ß) are released and play modulatory roles in amebic infections.

Amebic infections also modulate T cell responses. T cells from infected patients have reduced Concanavalin A (ConA) and antigen-induced proliferative responses *in vitro* [10, 11]. The potential contribution of T cell-derived cytokines to resistance to reinfection with amebae and to the suppression associated with uncontrolled invasive amebiasis remains to be elucidated. Cytokine responses to infectious agents tend to segregate into distinct patterns. Th1 responses are characterized by IL-2, IFN-γ and TNF-α and -ß

production, Th2 responses are marked by production of IL-4, -5, -6, -9 and -10 and Th0 responses are characterized by a mixed cytokine release profile [12]. Protozoan infections tend to be susceptible to Th1 responses, while helminth infections are usually controlled by Th2 responses. Activation of the inappropriate response can lead to exacerbation of disease [12]. IL-2, IFN-γ and TNF are involved in activating macrophages for microbicidal activity [13, 14]. As macrophages are potent cells for amebicidal activity, it is probable that a Th1 cytokine response is central in controlling invasive amebiasis, whereas production of macrophage-down-regulating cytokines, such as IL-4 and IL-10, may inhibit the cellular immune response to *E. histolytica.*

In this study, we used the gerbil model of ALA [15] to quantify lymphocyte proliferative responses during hepatic amebiasis, to determine the relative activation of T cell subsets (as measured by differential cytokine production) during initial ALA development and in challenged animals, and to determine how cytokine production relates to the immunomodulation observed during the disease and in resistance to re-infection.

MATERIALS AND METHODS

E. histolytica cultivation and preparation of soluble amebic antigen (SAA). Axenic *E. histolytica* HMI:IMSS was maintained in our laboratory in TYI-S-33 medium. Culture conditions and harvesting of trophozoites for preparation of soluble proteins were as previously described [16]. The amebae were subpassaged through gerbil livers prior to initiation of experiments to ensure high rates of infectivity (>90%).

To prepare SAA, the amebae were harvested and washed prior to resuspension in cold PBS. Amebae were lysed by 3 cycles of freeze-thaw (-120°C, 37°C). The lysed suspension was centrifuged at 15,000 *g* (4°C) for 10 min to remove cell debris, and the supernatant was used as SAA. Protein concentrations were determined by the method of Bradford [17] (Bio-Rad, Mississauga, Canada) by use of bovine albumin as a standard.

SAA was stored at -120°C until use.

Animals and infection procedures. Male gerbils (Charles River, St. Constant, Canada), 50-60 days old, were used in all experiments. After laparotomy, gerbils were infected intrahepatically (left lobe) with $5x10^5$ amebic trophozoites in mid-log growth phase (72 h) as described previously [16]. For intrahepatic challenge of previously infected gerbils, animals with 20-day-old ALA were treated with 200 mg/kg metronidazole (Rhone-Poulenc, Montreal) by oral gavage for 6 consecutive days to terminate the infection. Ten days after the last dose, gerbils were again laparotomized and reinfected intrahepatically with $5x10^5$ amebic trophozoites in the median liver lobe. One group of gerbils was not challenged after the drug cure. The nonchallenged drug-cured gerbils were examined at the same time as the drug-cured group, 10 days after challenge. All gerbils were confirmed to have ALA by gentle palpitation of the abdominal cavity prior to metronidazole treatment. ALA was further confirmed because remains of the original abscesses were evident at autopsy for all gerbils.

In vitro lymphocyte proliferation assay. Proliferative responses of spleen and hepatic lymph node (HLN) cells were determined by use of standard methods. Single-cell suspensions in PBS were prepared for both spleens and HLNs of 5 gerbils/time point (pooled within the same group). Cell suspensions were generated by teasing apart spleens or lymph nodes with needles, followed by passing disrupted organs through a wire mesh. Erythrocytes were lysed by osmotic shock (NH_4CL, 0.17M; Sigma, St Louis) and cells were resuspended at $5x10^6$/ml in complete RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 24 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate, and 50 μM 2-mercaptoethanol (Sigma). [^{3}H]thymidine (specific activity, 6.7 μCi/mmol; ICN, Montreal) incorporation was used to quantify cell proliferation as follows: Cells were plated at $5x10^5$/well in a total volume of 200 μl in flat-bottomed 96 well culture plates (Falcon; Becton Dickinson, Lincoln Park,

NJ) and incubated at 37°C and 5% CO_2. Cells (5 wells/condition) were incubated with no stimulus, 50 μg/ml SAA, or 2.5 μg/ml ConA (Pharmacia, Uppsala, Sweden) for 48 h. [^{3}H]thymidine (1 μCi/well) was added for an additional 18 h of incubation, after which the cells were harvested onto glass fiber filters using an automated cell harvester (Skatron, Lier, Norway). ^{3}H activity was measured by scintillation counting (LKB Wallac; Pharmacia). Results are expressed as mean counts per minute ± SE of 5 replicates after subtraction of background values.

Cytokine bioassays. Single cell suspensions were prepared as described above, and cells were plated at 1x10^7/well in a total volume of 2 ml in 24 well culture plates (Falcon; Becton Dickinson). Cells were incubated with no stimulus, 50 μg/ml SAA, or 5 μg/ml ConA, and supernatants were collected at 48 h for all cytokines measured. To eliminate residual ConA, 15 μg/ml α-methylmannoside (Sigma) was added to ConA-containing supernatants. Supernatants were stored at -120°C until cytokine quantification.

IL-2 levels were measured by quantification of the proliferation of murine CTLL cells in response to gerbil cell supernatants [18]. This assay has been reported previously for quantification of gerbil IL-2 [19]. Briefly, CTLL cells (2x10^4/well) in complete RPMI were incubated for 24 h in 96-well culture plates at 37°C and 5% CO_2 with different dilutions of gerbil cell supernatants containing rat anti-mouse IL-4 monoclonal antibody 11B11 (ATCC HB188; American Type Culture Collection, Rockville, MD) to ensure that the cells were proliferating in response to IL-2 only. Monoclonal antibody 11B11 neutralizes gerbil IL-4 activity (data not shown). Recombinant murine (rm) IL-2 from cultures of X63Ag8-653 myeloma cells transformed with murine IL-2 cDNA [20] was used to construct a standard curve. In this assay, 1 U of gerbil IL-2 is equal to 1 U of rmIL-2. CTLL cell proliferation in response to IL-2 was quantified using an MTT colorimetric assay [21]; 10 μl of a MTT-PBS solution (5 mg/ml) was added to each well after 24 h of incubation, and plates were incubated a further 4 h. The cells were then

solubilized with 0.04N HCL and absorbance measured by use of dual wavelengths of 570 and 630nm. All gerbil IL-2 activity in supernatants was completely neutralized with a polyclonal rabbit anti-gerbil rIL-2 antibody (provided by T.R. Klei, Louisiana State University, Baton Rouge), demonstrating specificity for IL-2.

IL-4 levels were determined by measuring proliferation of murine CT.4S cells in response to gerbil cell supernatants. The assay used was identical to that used for IL-2 quantification except that CT.4S cells were used as the responding cells [22] and rmIL-4 from cultures of X63Ag8-653 myeloma cells transformed with murine IL-4 cDNA [20] was used to construct a standard curve. In this assay, 1 U of gerbil IL-4 is equal to 1 U of rmIL-4. The polyclonal rabbit anti-gerbil rIL-2 antibody was added to the gerbil cell supernatants to ensure that the cells were proliferating in response to IL-4 only. This is the first report of gerbil IL-4 quantification. We were able to completely neutralize gerbil IL-4 activity using the rat anti-mouse IL-4 monoclonal antibody 11B11, demonstrating specificity for IL-4 in this assay.

TNF levels were determined by measuring the cytotoxic effects of cytokine in gerbil cell supernatants on TNF-sensitive murine L929 fibroblasts as previously described [7]. In brief, L929 cells ($4x10^4$ in 100 μl) were plated with serially diluted supernatants in 96-well culture plates at 37°C with 5% CO_2 in the presence of actinomycin D (8 μg/ml; Sigma). After 18 h of incubation, the cells were fixed with 2.5% gluteraldehyde (Sigma) and stained with 0.1% methylene blue (Sigma). The dye was extracted with 0.1 *N* HCL, and absorbance was measured at 600nm. Recombinant mouse TNF-α (Genzyme, Cambridge, MA) was used as the reference standard. In this assay, 1 U of gerbil TNF is equal to 1 U of rmTNF. Gerbil TNF activity was fully neutralized by polyclonal rabbit anti-mouse rTNF-α antibody (Genzyme).

Statistical analysis. Student's *t* test was used to identify differences between control and experimental conditions. $P < .05$ was considered significant. All values are expressed as mean ± SE.

RESULTS

Spleen and HLN proliferative responses during ALA development. Clinical studies have demonstrated suppression of T cell responses to mitogen during amebiasis [10, 11]. However, it is difficult to determine exactly when humans were infected and, hence, the stage of the disease. For this reason, we examined the kinetics of gerbil spleen and HLN T cell proliferative responses during early (days 5-10 after inoculation), acute (day 20 after inoculation) and late disease (days 30-60 after inoculation). Acute disease was denoted by maximal liver-abscess size. As shown in figure 1, proliferative responses of HLN (draining lymph nodes for the liver) cells were stronger than that for spleen cells. SAA-induced (i.e., antigen specific) proliferation (Fig. 1) was demonstrated by spleen and HLN cells beginning at the earliest time point examined (5 days). Interestingly, infected gerbils' spleen cell responses to ConA were lower than those of controls throughout the infection. While HLN cell responses to SAA were relatively constant from day 5-60, spleen cell proliferative responses to SAA were noticeably reduced at day 20, recovering during days 30-60 to levels seen at days 5-10. Both spleen and HLN cells showed reduced mitogenesis in response to ConA at day 20. Suppressed proliferative responses at the acute phase of infection were not restricted to T cells, as B cells were also suppressed in their proliferative responses to LPS at day 20 and recovered thereafter (data not shown).

Kinetics of IL-2 and IL-4 production during ALA development. Cytokine production from patients with ALA or from animal models of the disease has not been previously reported. We examined the kinetics of IL-2 (a Th1 marker) and IL-4 (a Th2 marker) production from spleen and HLN cells of gerbils with ALA as an indication of the T cell response phenotype (Th1, Th2, or Th0) during amebiasis.

Early in the infection (days 5 and 10), spleen and HLN cells from infected gerbils secreted a mixture of IL-2 and IL-4 (Th0 response) (Fig. 2). Significant levels of both cytokines were produced at this stage, although IL-2 levels were low. A comparable

cytokine release pattern was seen in response to ConA and SAA in both spleen and HLN cell preparations. Interestingly, ConA-stimulated spleen cells from control gerbils produced both IL-2 (3.3 U/ml) and IL-4 (1.6 U/ml), but at the onset of infection (day 5), IL-2 production dramatically declined (0.4 U/ml) while IL-4 levels remain unchanged (1.5 U/ml) (Fig. 2, left).

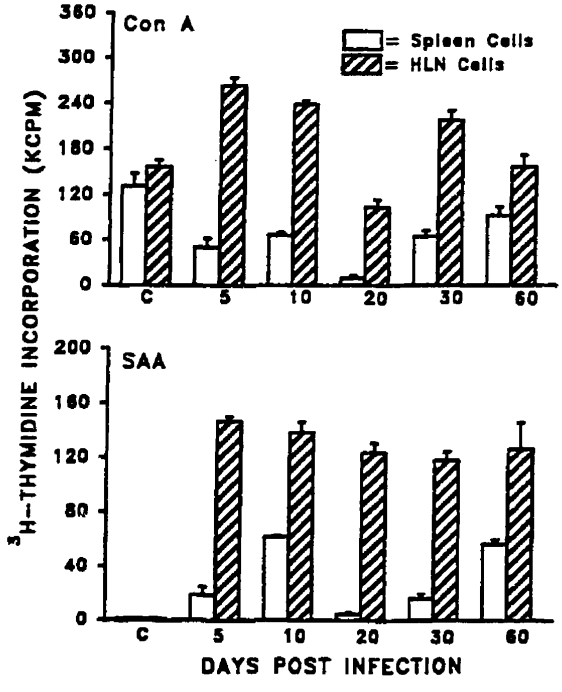

Figure 1. *In vitro* proliferative responses of spleen and hepatic lymph node (HLN) cells from gerbils with amebic liver abscess and uninfected (C) gerbils stimulated with concanavalin A (ConA) or soluble amebic antigen (SAA). Data are representative of duplicated experiments and are expressed as mean kcpm (cpm x 1000) ± SE (5 wells/condition). kcpm values for nonstimulated cells (mean background: spleen cells=5.35 kcpm, HLN cells=2.16 kcpm) were subtracted from kcpm generated in response to ConA or SAA.

Marked suppression of both IL-2 and IL-4 production was seen during the acute stage of infection (day 20). Thus, suppression of T cell responses accompanies suppression of macrophage functions and maximal abscess size and splenomegaly [23] at acute disease. At 30 and 60 days after inoculation, the proliferative responses of spleen

and HLN cells equaled or surpassed the levels achieved at days 5 and 10 (Fig. 1). However, a qualitative change in the cytokines produced by these cells occurred along with restored proliferative responses. IL-2 production rose at days 30 and 60, while IL-4 levels remained low in response to ConA and SAA (Th1 response) (Fig. 2). Peak IL-2 levels occurred at day 60 with HLN cells stimulated with ConA (9.8 U/ml) (Fig. 2, top right).

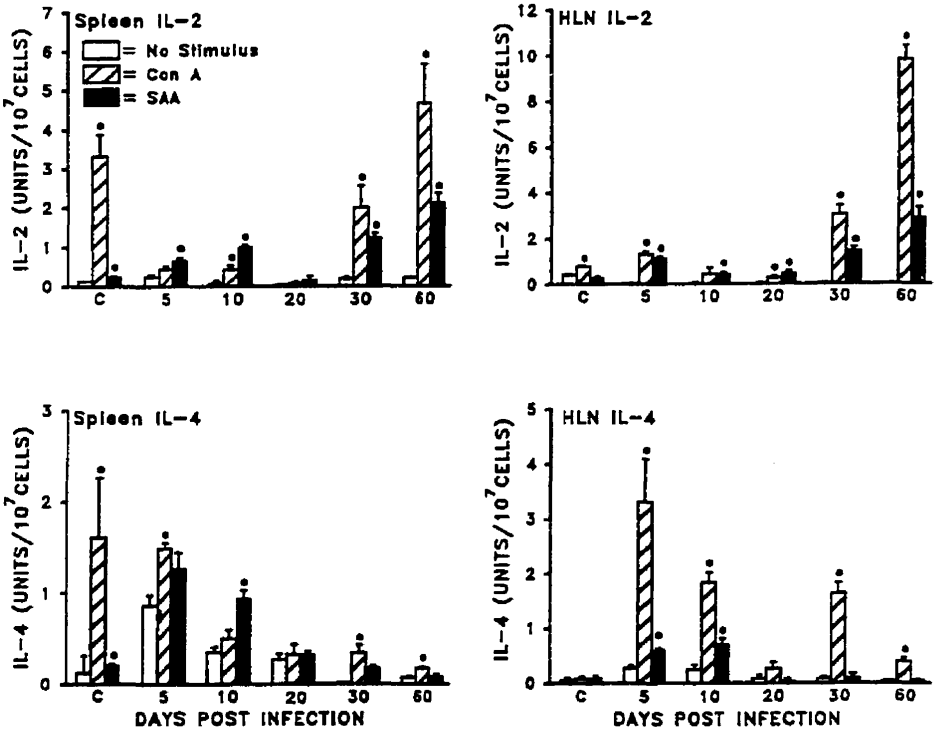

Figure 2. Kinetics of IL-2 and IL-4 production by spleen and hepatic lymph node (HLN) cells from gerbils with amebic liver abscess and uninfected gerbils (C) in response to concanavalin A (ConA) or soluble amebic antigen (SAA). Data are representative of duplicated experiments and are expressed as mean ± SE (4 wells/condition). * $P < .05$ vs. unstimulated homologous controls.

Kinetics of TNF-α production during ALA development. Macrophages are major producers of TNF-α during *E. histolytica* infections, and macrophage TNF-α production is modulated by the parasite *in vitro* and *in vivo* [7, 24, 25]. However, T cell production of TNF-α and -ß during ALA development has not been investigated. For this reason, we measured TNF activity in the supernatants of lymphoid cells from infected gerbils. In this

assay, all gerbil TNF activity was neutralized by a polyclonal anti-mouse rTNF-α antibody that does not react with mouse TNF-ß, suggesting that the gerbil TNF activity may be largely ascribed to TNF-α rather than TNF-ß.

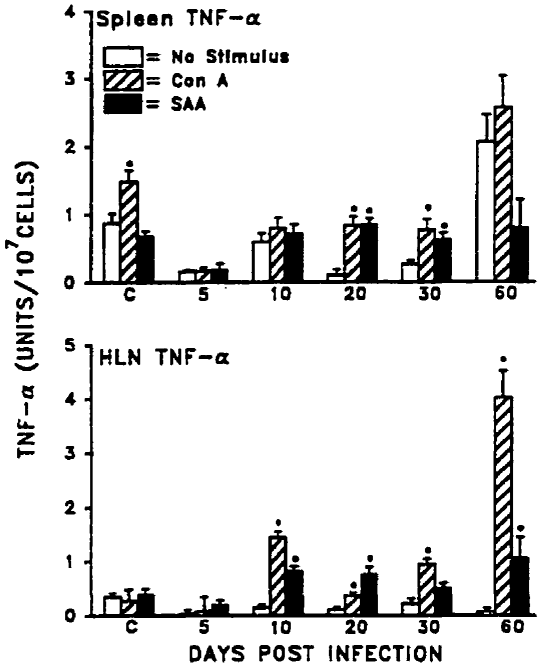

Figure 3. Kinetics of TNF-α production by spleen and hepatic lymph node (HLN) cells from gerbils with amebic liver abscess and uninfected gerbils (C) in response to concanavalin A (ConA) or soluble amebic antigen (SAA). Data are representative of duplicated experiments and are expressed as mean ± SE (4 wells/condition). * $P < .05$ vs.unstimulated homologous controls.

TNF-α production was lowest at day 5 of infection, whereas significant but low levels of TNF-α were produced by spleen (Fig. 3, top) and HLN cells (Fig. 3, bottom) in response to the T cell mitogen ConA and SAA from days 10-30 after inoculation. Similar to IL-2 production by spleen cells in response to ConA, TNF-α production decreased at the onset of infection. The highest TNF-α levels were produced by HLN cells in response to ConA (4.0 U/ml) at day 60 (Fig. 3, bottom).

Drug cure and challenge infections. Studies using amebic antigen-stimulated peripheral blood lymphocytes of cured patients indicated that supernatants of these cells

contain IL-2 and IFN-γ and activate macrophages for amebicidal activity [26]. However, it is not known whether these cells also produce Th2 cytokines in response to antigen stimulation. In this study, we drug-cured gerbils with ALA at day 20 after inoculation, then challenged the animals by reinoculation of trophozoites in the median liver lobe. This was done to determine whether the animals were resistant to reinfection and to investigate their lymphoproliferative responses and cytokine production patterns. All challenged gerbils were resistant to reinfection.

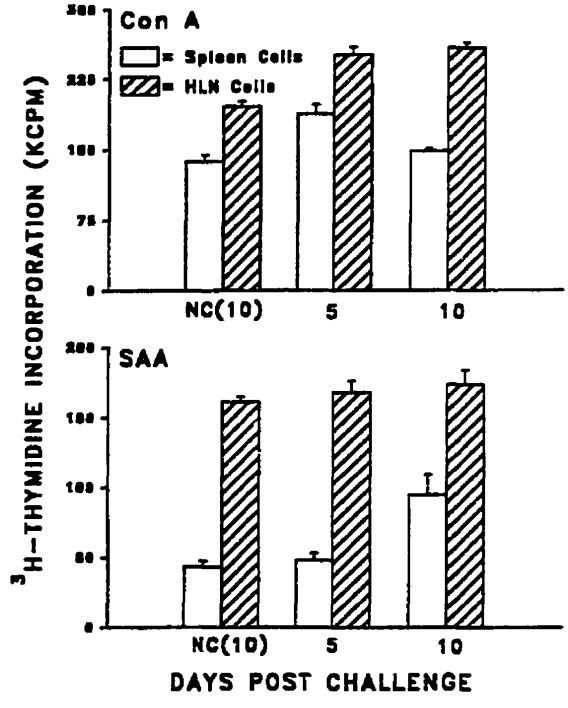

Figure 4. *In vitro* proliferative responses of spleen and hepatic lymph node (HLN) cells from drug-cured and challenged gerbils in response to concanavalin A (ConA) or soluble amebic antigen (SAA). NC(10)= nonchallenged gerbils sacrificed concurrently with group sacrificed 10 days after challenge. Data are representative of duplicated experiments and are expressed as mean kcpm (cpm x 1000) ± SE (5 wells/condition). kcpm values for nonstimulated cells (mean background: spleen=8.02 kcpm, HLN=8.16 kcpm) were subtracted from kcpm generated in response to ConA or SAA.

Spleen and HLN cell proliferative responses in drug-cured and challenged gerbils.

The mitogenic responses of spleen and HLN cells from drug-cured and challenged gerbils

are summarized in figure 4. Proliferative responses to both ConA and SAA were equivalent in the nonchallenged and challenged groups. A challenge infection with *E. histolytica* trophozoites did not suppress proliferative responses. Levels of [^{3}H]thymidine incorporation were comparable to or higher than those during a primary infection (Fig. 1).

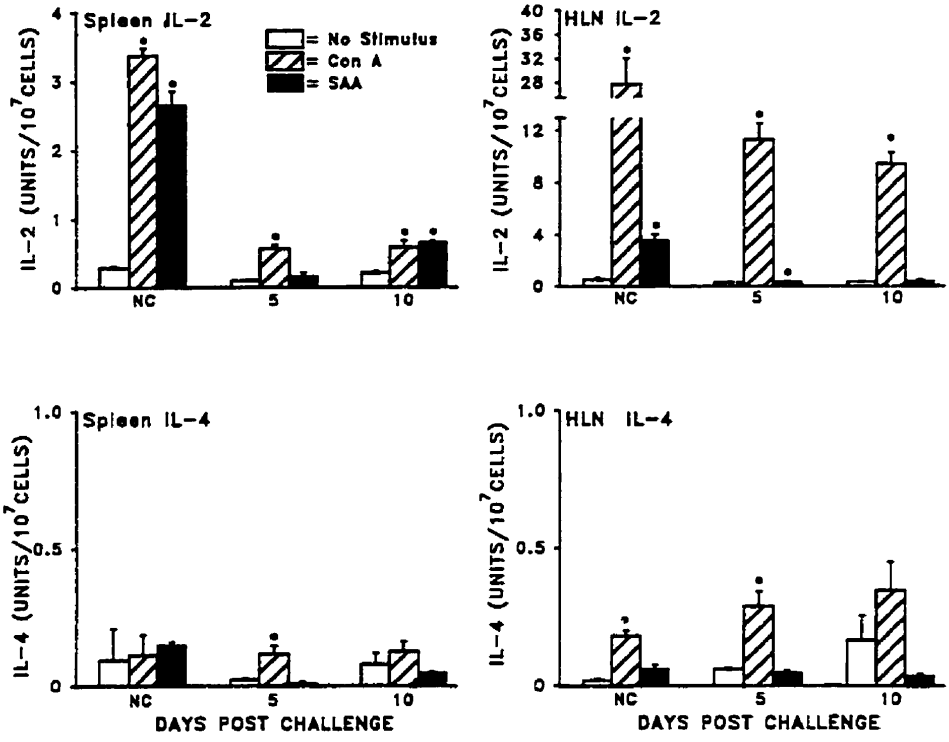

Figure 5. IL-2 and IL-4 production by spleen and hepatic lymph node (HLN) cells from drug-cured and challenged gerbils in response to concanavalin A (ConA) or soluble amebic antigen (SAA). NC(10), nonchallenged gerbils sacrificed concurrently with group sacrificed 10 days after challenge. Data are representative of duplicated experiments and are expressed as mean ± SE (4 wells/condition). $*P < .05$ vs. unstimulated homologous controls.

IL-2 and IL-4 production in drug-cured and challenged gerbils. Subsequent to drug-cure, spleen and HLN cells from gerbils produced high levels of IL-2 in response to ConA or SAA (Fig. 5). The highest level of IL-2 production was by HLN cells of nonchallenged gerbils in response to ConA (27.7 U/ml) (Fig. 5, top right). In contrast, negligible amounts of IL-4 (<0.4 U/ml) were produced by cells in response to either

stimulus (Th1 response) (Fig. 5, bottom). Lower levels of IL-2 were produced when animals were sacrificed 5 and 10 days after the intrahepatic challenge; however, these animals showed no evidence of established infection in the liver. IL-2 production was also, nonetheless, higher than IL-4 production, suggesting that a Th1-like response was maintained (although weakened) after the challenge inoculation.

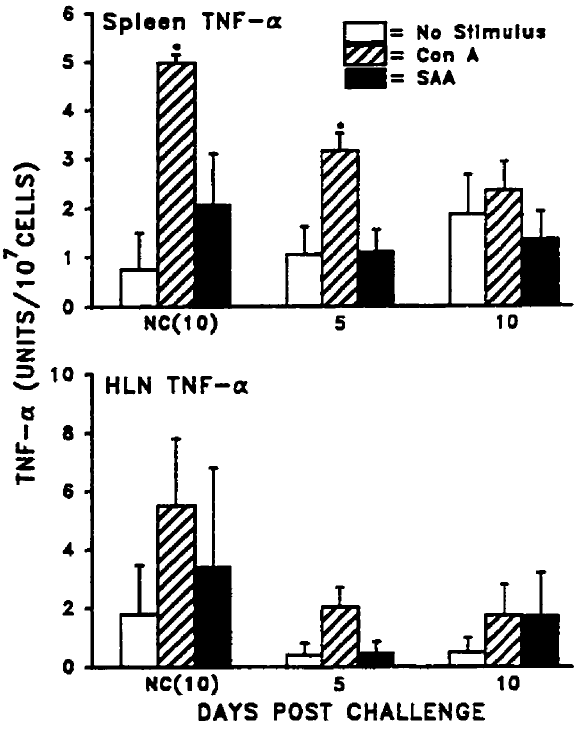

Figure 6. TNF-α production by spleen and hepatic lymph node (HLN) cells from drug-cured and challenged gerbils in response to concanavalin A (ConA) or soluble amebic antigen (SAA). NC(10)= nonchallenged gerbils sacrificed concurrently with group sacrificed 10 days after challenge. Data are representative of duplicated experiments and are expressed as mean ± SE (4 wells/condition). $^*P < .05$ vs. unstimulated homologous controls.

TNF-α production in drug-cured and challenged gerbils. Similar to levels of TNF-α produced during a primary hepatic infection (Fig. 3), low levels of this cytokine were produced by spleen and HLN cells from nonchallenged and challenged gerbils (Fig. 6). Significant levels of TNF-α (Fig. 6, top) were produced only in response to the pan-T cell stimulant ConA by spleen cells of nonchallenged gerbils (5.0 U/ml) and those from gerbils

examined 5 days after challenge (3.1 U/ml). However, no significant production of TNF-α was evident from cells isolated from the draining HLN of nonchallenged gerbils or from gerbils 5 and 10 days after challenge (Fig. 6, bottom). Similar to the phenomenon with IL-2 (Fig. 5), challenge infection lowered TNF-α production compared to that from cells taken from nonchallenged gerbils.

DISCUSSION

The role of T cell-derived cytokines in amebiasis has been largely unexplored because of lack of reagents for immunologic studies in the gerbil, the principal animal model for hepatic amebiasis. While the precise mechanisms contributing to resistance to the disease have not been identified, Th1 cytokines IL-2, IFN-γ and TNF may be involved in activation of macrophages for amebicidal activity and resistance to infection. Supernatants of splenocytes from amebic galactose-specific adherence lectin (Gal-lectin)-immunized gerbils demonstrated IL-2-like and IFN-γ-like activity when the cells were stimulated with the Gal-lectin *in vitro* [27]. These immunized animals were resistant to a challenge infection [28]. Similarly, drug-cured patients appear to have a low level of recurrence [29], and supernatants of their Gal-lectin-stimulated lymphocytes were found to contain IL-2 and IFN-γ [26] and to activate macrophages for amebicidal activity [10]. In contrast, Th2 cytokines IL-4 and IL-10 may be involved in the down-regulation of macrophage amebicidal activity that is characteristic of amebic infections [3].

In this report, we were able to utilize murine cytokine bioassays to determine the kinetics of IL-2, IL-4 and TNF production during hepatic amebiasis and in animals resistant to reinfection. By use of lymphocyte proliferation assays as a parallel indicator of lymphocyte functionality, we found that amebic infections stimulated mixed IL-2, IL-4, and TNF-α production (Th0-like response), followed by suppression of cytokine production and proliferation at acute disease. Lymphoid cells obtained during days 30-60

after inoculation and from resistant animals were characterized by IL-2 production (Th1-like response) and restored proliferative responses.

Antigen-specific proliferative responses were evident in lymphoid cells at the onset of infection (day 5); however, ConA-induced splenocyte proliferation was reduced compared with that for uninfected gerbils, which is consistent with previous reports [30]. Notably, establishment of infection lead to a reduction in IL-2 and TNF-α but not IL-4 production by ConA-stimulated splenocytes, suggesting that *E. histolytica* promotes a mixed Th1- and Th2- or Th0-like response over a Th1-like response at the time of infection. The nature of the antigens recognized may be important in selecting the Th phenotype that develops [12]. A 220-kDa surface protein of *E. histolytica*, for example, has been shown to induce IL-4- and IL-10-secreting cells when injected in mice, whereas peptides of the molecule induce Th1 responses [31]. PGE_2 down-regulates IL-2 transcription and up-regulates IL-4 transcription [32] and is stimulated by amebae early in the infection [9]. Hence, PGE_2 production may also be involved in promoting IL-4 over IL-2 production at the onset of infection. IL-4 was the only Th2 cytokine we were able to quantify in our gerbil model. IL-4 production at days 5-10 after inoculation may assist amebic survival in the liver through inhibition of macrophage NO release [33].

While we found that early infection is linked to a Th0-like response, proliferative responses and lymphokine production were markedly suppressed during acute disease (day 20). This was less evident with TNF-α. Production of this cytokine was generally low throughout early and acute infection. This may suggest that T cells are not a significant source of TNF-α in amebic infections. Also, T cell-derived TNF-α production may be inhibited by macrophage PGE_2 production early in the infection [7]. Either the parasite itself or the host response to the infection (or both) may be responsible for the T cell suppression during acute disease in these animals. This suppression coincides with near-maximal suppression of several macrophage functions during acute disease. Down-regulation of both T cell and macrophage functions in amebiasis possibly has additive or

even synergistic effects on the cellular response to the parasite, as these cells must cooperate in the orchestration of immune defense.

The T cell proliferative responses were quantitatively similar before and after acute disease; however, there was a significant qualitative change in the cytokines produced. IL-4 production did not recover, while IL-2 production reached higher levels than were achieved early in the infection and TNF-α was higher at day 60. That is, a shift from a Th0- to a Th1-like response occurred after day 20. Liver abscesses at day 30-60 were also smaller than at day 20 (data not shown), perhaps in response to increased cell-mediated immunity associated with the Th0-to-Th1 shift. The mechanism(s) involved in altering established cytokine responses is not understood. In our study, perhaps the suppression of IL-4 at day 20 allowed the subsequent outgrowth of Th1 cells from a previously mixed Th1 and Th2 or Th0 population. Whether a Th0 response occurs in human amebiasis followed by a switch to a Th1 response is unknown. Continuous growth of the ALA in humans may be representative of a failure to switch to a Th1 response.

To clarify the link between a Th1 response and resistance to infection, we challenged drug-cured gerbils by intrahepatic inoculation with *E. histolytica*. Even though T cells from these animals were markedly suppressed at the time of drug-treatment (day 20), the gerbils were completely resistant to a challenge infection. Clearance of the amebae appeared to reverse the suppression occurring at the acute stage of the disease. This argues that the parasite, or components thereof, may be directly mediating the reduction in cytokine secretion and proliferative responses. Previous studies on macrophage functions during ALA development in gerbils support the concept of *E. histolytica* directly inducing suppression in a localized manner. Macrophages in the ALA demonstrate reduced activation as measured by IL-1-like activity, TNF-α and NO production, and amebicidal activity [5-7]. In contrast, macrophages distal from the abscess and not directly exposed to amebae, such as those in the peritoneum or the spleen, are up-regulated in these functions [5-7]. *In vitro*, amebic components inhibit macrophage TNF-α and IL-1-like activity,

production of NO, amebicidal activity, and Ia molecule expression [6, 5, 8], highlighting their role in directly modulating cellular immune responses.

In this study, prior exposure of gerbils to amebae was sufficient to enable them to overcome the parasite's immunomodulatory effects upon challenge. Lymphoid cells from drug-cured nonchallenged animals show high production of IL-2 but low IL-4 production, suggesting that at the time of challenge, amebae encounter a Th1-like response that may have helped prevent infection. A challenge infection with *E. histolytica* may, however, have had some suppressive effects. Compared with cytokine production in cells from the nonchallenged group, cells from gerbils examined 5 and 10 days after challenge produced lower levels of IL-2 and TNF-α. Nevertheless, IL-2 production was higher than IL-4 production, and the gerbils were resistant to reinfection. IFN-γ may be produced in conjunction with IL-2 in resistant gerbils and function to activate macrophages for amebicidal activity.

In summary, we have shown that early hepatic amebiasis is accompanied by a Th0 or mixed Th1- and Th2-like response in gerbils, which may facilitate amebic survival in the liver. Down-regulation of lymphoid cell proliferation and production of IL-2 and IL-4 occurred during acute disease. This may combine with suppression of macrophage functions [5, 7] to severely cripple the immune response against the parasite. Suppression was transient, however, as proliferative responses and IL-2 (but not IL-4) production recovered at the late stage of the disease. This switch from a Th0-like response to a Th1-like response may help control the infection in gerbils. A Th1-like response was evident in the drug-cured gerbils that were resistant to challenge infections.

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REFERENCES

1. Salata RA, Murray HN, Rubin BY, Ravdin JI. The role of gamma-interferon in the generation of human macrophages cytotoxic for *Entamoeba histolytica* trophozoites. Am J Trop Med Hyg **1987**;37:72-8.

2. Denis M, Chadee K. Cytokine activation of murine macrophages for *in vitro* killing of *Entamoeba histolytica* trophozoites. Infect Immun **1989**;57:1750-6.

3. Lin JY, Chadee K. Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. J Immunol **1992**;148:3999-4005.

4. Lin JY, Seguin R, Keller K, Chadee K. Tumor necrosis factor alpha augments nitric oxide-dependent macrophage cytotoxicity against *Entamoeba histolytica* by enhanced expression of the nitric oxide synthase gene. Infect Immun **1994**;62:1534-41.

5. Wang W, Keller K, Chadee K. *Entamoeba histolytica* modulates the nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxicity against amoebae and tumour cells. Immunology **1994**;83:601-10.

6. Denis M, Chadee K. *In vitro* and *in vivo* studies of macrophage functions in amebiasis. Infect Immun **1988**;56:3126-31.

7. Wang W, Keller K, Chadee K. Modulation of tumor necrosis factor production by macrophages in *Entamoeba histolytica* infection. Infect Immun **1992**;60:3169-74.

8. Wang W, Chadee K. *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatability complex Ia molecule and I-Aß mRNA expression by a prostaglandin E_2-dependent mechanism. Infect Immun **1995**;63:1089-94.

9. Wang W, Chadee K. *Entamoeba histolytica* alters arachidonic acid metabolism in

macrophages *in vitro* and *in vivo*. Immunology **1992**;76:242-50.

10. Salata RA, Martinez-Palomo A, Murray HN et al. Patients treated for amebic liver abscess develop cell-mediated immune responses effective *in vitro* against *Entamoeba histolytica*. J Immunol **1986**;136:2633-9.
11. Ganguly NK, Mahajan RC, Gill NJ, Koshy A. Kinetics of lymphocyte subpopulations and their functions in cases of amoebic liver abscess. Trans R Soc Trop Med Hyg **1981**;75:807-10.
12. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today **1996**;17:138-46.
13. Nacy CA, Meierovics AI, Belosevic M, Green SJ. Tumor necrosis factor-alpha: central regulatory cytokine in the induction of macrophage antimicrobial activities. Pathobiol **1990**;59:182-4.
14. Espinoza-Delgado I, Bosco MC, Musso T, Gusella, Longo DL, Varesio L. Interleukin-2 and human monocyte activation. J Leuk Biol **1995**;57:13-9.
15. Chadee K, Meerovitch E. The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica*. Am J Trop Med Hyg **1984**;33:46-54.
16. Chadee K, Meerovitch E. The pathogenesis of experimentally induced amebic liver abscess in the gerbil (*Meriones unguiculatus*). Am J Pathol **1984**;117:71-80.
17. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **1976**;72:248-54.
18. Gillis S, Ferm MM, Ou W, Smith KA. T cell growth factor: parameters of production and a quantitative microassay for activity. J Immunol **1978**;120:2027-32.
19. Mai Z, Kousoulas KG, Horohov DW, Klei TR. Cross-species PCR cloning of gerbil (*Meriones unguiculatus*) interleukin-2 cDNA and its expression in COS-7 cells. Vet Immunol Immunopath **1994**;40:63-71.
20. Karasuyama H, Melchers F. Establishment of mouse cells which constitutively secrete

large quantities of interleukin 2,3,4 or 5 using modified cDNA expression vectors. Eur J Immunol **1988**;18:97-104.

21. Mosmann TR. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **1983**;65:55-63.
22. Hu-Li J, Ohara J, Watyson C, Tsang W, Paul WE. Derivation of a T cell line that is highly reponsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). J Immunol **1989**;142:800-7.
23. Chadee K, Meerovitch E. *Entamoeba histolytica*: antibody responses and lymphoreticular changes in gerbils (*Meriones unguiculatus*) in response to experimental liver abscess and amebic abstract injection. Z Parasitenkd **1984**;70:781-95.
24. Seguin R, Keller K, Chadee K. *Entamoeba histolytica* stimulates the unstable transcription of c-fos and tumour necrosis factor-α by protein kinase C signal transduction in macrophages. Immunology **1995**;86:49-57.
25. Seguin R, Mann BJ, Keller K, Chadee K. Identification of the galactose-adherence lectin epitopes of *Entamoeba histolytica* that stimulate tumor necrosis factor-α production by macrophages. Proc Natl Acad Sci (USA) **1995**;92:12175-9.
26. Schain DC, Salata RA, Ravdin JI. Human T lymphocyte proliferation, lymphokine production and amebicidal activity elicited by the galactose-inhibitable adherence protein of *Entamoeba histolytica*. Infect Immun **1992**;60:2143-6.
27. Schain DC, Salata RA, Ravdin JI. Development of amebicidal cell-mediated immunity in gerbils (*Meriones unguiculatus*) immunized with the galactose-inhibitable adherence lectin of *Entamoeba histolytica*. J Parasitol **1995**;81:563-8.
28. Petri WA, Ravdin JI. Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of *Entamoeba histolytica*. Infect Immun **1991**;59:97-101.
29. Deleon A. Pronostico tardio en el absceso hepatico amibiano. Arch Invest Med

1970;1:205-6.

30. Vinayak VK, Sharma P. Kinetics of the immune responses during the course of hepatic amoebic infection: an experimental study. Trans R Soc Trop Med Hyg **1989**;83:349-53.

31. Talamas-Rohana P, Schlie-Guzman MA, Hernandez-Ramirez VI, Rosales-Encina JL. T-cell suppression and selective *in vivo* activation of Th2 subpopulation by the *Entamoeba histolytica* 220-Kilodalton lectin. Infect Immun **1995**;63:3953-8.

32. Lacour M, Arrighi JF, Muller KM, Carlberg C, Sauret JH, and Hauser C. cAMP up-regulates IL-4 and IL-5 production from activated CD4+ T cells while decreasing IL-2 release and NF-AT induction. Int Immunol **1994**;6:1333-43.

33. Taub DD, Cox GW. Murine Th1 and Th2 clones differentially regulate macrophage nitric oxide production. J Leuk Biol **1995**;58:80-9.

CONNECTING STATEMENT I

In Manuscript I, antigen and mitogen-induced T cell proliferative responses were shown to be transiently, but markedly, suppressed at day 20 of amoebic liver abscess development in gerbils. The mechanisms responsible for this suppression are not known. In contrast, mechanisms involved in suppression of macrophage functions during amoebiasis have been extensively characterized. While macrophage suppression is localized to the vicinity of the amoebic abscess, both hepatic lymph node and spleen T cell proliferative responses were suppressed in our study. This is indicative of systemic suppression which may be mediated by a serum-borne factor(s). Accordingly, we next investigated the suppressive effect of day 20 serum on mitogen-stimulated proliferative responses of naïve gerbil spleen cells and the potential mechanisms operating to mediate this suppression.

MANUSCRIPT II

Serum from *Entamoeba histolytica*-Infected Gerbils Selectively Suppresses T Cell Proliferation by Inhibiting Interleukin-2 Production*

Darren Campbell, Denis Gaucher and Kris Chadee

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ABSTRACT

Suppression of T and B cell responses during invasive amebiasis may be serum-mediated. In this report, the mechanism of serum-mediated suppression of spleen cell lymphoproliferation from gerbils with amebic liver abscess was examined. Compared with uninfected gerbil serum (10%), serum samples collected at days 10, 30 and 60, but not day 20 after infection augmented both concanavalin A (ConA; T cell mitogen)- and lipopolysaccharide (LPS; B cell mitogen)-induced proliferation of homologous spleen cells. Only day 20 serum (>5%) inhibited ConA- but not LPS-induced proliferation of spleen cells from uninfected gerbils. The suppressive mechanism was independent of nitric oxide and prostaglandin, but involved reduced IL-2 production. Addition of exogenous IL-2 reversed the suppressive effect of day 20 serum on ConA-stimulated proliferation. These results identify a mechanism whereby serum may contribute to transient suppression of T cell responses during *E. histolytica* infections.

INTRODUCTION

Entamoeba histolytica infection leads to intestinal and extra-intestinal amebiasis, most commonly manifested as amebic liver abscess. In the absence of clinical intervention, amebic infections are progressive, and cause considerable tissue pathology [1]. The disease has been estimated to cause 40,000 deaths annually [2]. Upon diagnosis of amebiasis, metronidazole provides effective treatment and there is some evidence to suggest that drug-treated humans and animals may be resistant to reinfection [3, 4]. In addition, immunization of gerbils with portions of recombinant amebic molecules, such as the galactose adherence lectin (Gal-lectin), protects against challenge infections [5].

E. histolytica is susceptible to cell-mediated immunity. Activation of murine macrophages with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) resulted in potent *in vitro* amebicidal activity mediated by nitric oxide (NO) [6, 7]. Human macrophages kill amebae *in vitro* following stimulation with supernatants from Concanavalin A (ConA)-activated human T cells [8]. *In vivo*, activated Th1 cells, which secrete IFN-γ, TNF-ß and interleukin-2 (IL-2), represent the principal source for macrophage-stimulating cytokines. Accordingly, a Th1-like response in gerbils was associated with resistance to reinfection with *E. histolytica* following drug-abbreviated infections [4]. In addition, when stimulated with the Gal-lectin *in vitro*, T cells from cured patients produced IFN-γ and IL-2, and splenocytes from immune gerbils demonstrated IL-2- and IFN-γ-like activity [9, 10]. Recent passive immunization studies with anti-*E. histolytica* antibodies suggest that humoral responses may contribute to resistance to infection by an undefined mechanism [11]. However, *E. histolytica* has been shown to cap antibodies bound to its surface [12], to produce proteases which degrade bound antibodies [13] and to resist complement-mediated lysis [14].

During an initial episode of invasive amebiasis, down-regulation of immune

defenses occurs. This may contribute to prolonged and exacerbated infections because of an inability to respond effectively to the parasite. Macrophages derived from the amebic liver abscess were unresponsive to IFN-γ and lipopolysaccharide (LPS)-activation for TNF-α and NO production, and cytotoxicity against amebae [15, 16]. However, spleen and peritoneal macrophages from infected gerbils were upregulated in these functions, arguing that macrophage suppression may be localized to the site of infection. Different mechanisms may be responsible for suppressing macrophage and lymphocyte functions. Suppression of lymphocyte proliferative responses and cytokine production appears to be systemic. Amebiasis patient peripheral blood T cells demonstrated depressed proliferative responses to ConA [17]. In addition, ConA-induced gerbil spleen cell proliferative responses were profoundly suppressed at day 20 of amebic liver abscess development [4]. ConA-induced spleen cell IL-2 and IL-4 production was also suppressed at day 20 of amebic liver abscess development [4]. Such systemic suppression of lymphocyte responses can be mediated by suppressive serum factors [18, 19] and there is evidence suggesting a role for serum-mediated suppression in amebiasis. *E. histolytica* antigen-stimulated T cells from drug-cured amebiasis patients demonstrated a 63% reduction in proliferation and a 93% reduction in IFN-γ production in the presence of infected patient serum, as compared with control serum [20]. However, the suppressive mechanism(s) involved and whether serum-mediated suppression affects both T and B cell responses is not known.

In this study, we used the gerbil model of amebiasis [21] to examine the mechanism of serum-mediated suppression of lymphocyte responses during the course of amebic liver abscess development. Because myriad suppressive events may occur during amebic infections [22], we also examined the effect of serum on lymphoproliferative responses of spleen cells from uninfected animals. Our results indicate that serum-mediated suppression was a transient phenomenon only present during the acute phase of infection (day 20) and was specific for ConA-induced T cell proliferation. The suppressive effect was not

mediated by nitric oxide or prostaglandin, but involved reduced IL-2 production.

MATERIALS AND METHODS

E. histolytica cultivation. Axenic *E. histolytica* trophozoites (HMI:IMSS strain) were cultured in TYI-S-33 medium as previously described [21]. Amebae were subpassaged through gerbil livers prior to initiation of experiments to ensure high levels of infectivity (>90%).

Animals and infection procedures. Male gerbils (*Meriones unguiculatus*; Charles River, St. Constant, Canada), 50-60 days old, were used in all experiments. Amebic liver abscesses were initiated, following laparotomy, by direct inoculation of 5 x 10^5 *E. histolytica* trophozoites in the left liver lobe of anesthetized gerbils as previously described [21]. Gerbils were confirmed to have amebic liver abscesses at autopsy.

Serum collection and processing. Serum samples were collected from gerbils at time of sacrifice. For each time point, the blood of 5 gerbils was pooled, allowed to clot and centrifuged at 2000 *g* for 10 min. The pooled sera were then heated at 56°C for 0.5 h to inactivate complement and filter sterilized prior to use on the day of collection. For some experiments, serum samples were frozen at -80°C until required. Experiments were repeated with serum samples collected from different groups of infected gerbils .

In vitro lymphocyte proliferation assay. Single-cell suspensions in PBS were prepared from spleens of uninfected (control) gerbils and animals infected for 10, 20, 30 and 60 days (3 gerbils/time point). Cell suspensions were generated by teasing apart spleens with needles and passing the disrupted organs through a wire mesh. Erythrocytes were lysed by Gay's salts [23]. Remaining cells were resuspended at 5 x 10^6/ml in RPMI-1640 (Gibco, Burlington, Canada) supplemented with 10% serum from infected or uninfected gerbils, 24 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate and 50 µM ß2-mercaptoethanol (Sigma, St. Louis, MO). [^{3}H]thymidine (^{3}H-TdR; specific

activity = 6.7 μCi/mmol; ICN, Montreal, Canada) incorporation was used to quantify cell proliferation. The cells were plated at 5 x 10^5/well in a total volume of 200 μl in flat-bottomed 96-well culture plates (Falcon; Becton Dickinson, Lincoln Park, NJ) and incubated at 37°C with 5% CO_2. Cells (3 wells/condition) were incubated with no stimulus, 2.5 μg/ml ConA (Pharmacia, Uppsala, Sweden) or 50 μg/ml LPS (*Escherichia coli* serotype 0111:B4; Sigma) for 48 h. ^{3}H-TdR (1 μCi/well) was added for an additional 18 h of incubation, after which the cells were harvested onto glass fiber filters by means of an automated cell harvester (Skatron, Lier, Norway). ^{3}H-TdR incorporation was measured by scintillation counting (LKB Wallac; Pharmacia). Results are expressed as mean kilo counts per minute (kcpm=cpm x 1000) ± SE after subtraction of background (unstimulated) values.

IL-2 bioassay. For IL-2 quantification, single cell suspensions were prepared as described above except that cells were plated at 5 x 10^6/well in a volume of 1 ml in 24-well culture plates (Falcon; Becton Dickinson). Cells were incubated with no stimulus or 5 μg/ml ConA for 24 h prior to supernatant collection. To eliminate residual ConA, 15 μg/ml α-methylmannoside (Sigma) was added to the supernatant aliquots to be quantified for IL-2. Supernatants were frozen at -80°C until required.

IL-2 levels were measured by quantification of murine CTLL cell proliferation in response to gerbil cell supernatants [24]. This assay has been used previously to measure gerbil IL-2 activity [25]. CTLL cells were cultured in RPMI-1640, supplemented as above but with 10% fetal calf serum (Hyclone Laboratories; Logan, UT) instead of gerbil serum. CTLL cells (2 x 10^4/well) were incubated for 24 h in 96-well culture plates at 37 °C/5% CO_2 with different dilutions of gerbil spleen cell supernatants. Rat anti-mouse IL-4 monoclonal antibody 11B11 (ATCC HB188; American Type Culture Collection, Rockville, MD), which neutralizes gerbil IL-4 activity [4], was added to the supernatants to ensure that CTLL cells were proliferating in response to IL-2 only. IL-2 from cultures

of X63Ag8-653 myeloma cells transfected with murine IL-2 cDNA [26] was used to construct a standard curve. In this assay, 1 U of gerbil IL-2 is equal to 1 U of murine IL-2. At the dilutions used in this assay, gerbil serum did not inhibit the ability of CTLL cells to proliferate in response to murine IL-2 (data not shown). CTLL cell proliferation was quantified using an MTT colorimetric assay [27]; 10 μL of an MTT-PBS solution (5 mg/ml) was added to each well after 24 h of incubation, and plates were incubated for a further 4 h. The cells were then solubilized with 0.04 N HCL in isopropanol and absorbance was measured by use of dual wave lengths of 570 and 630 nm. All gerbil IL-2 activity in supernatants was completely neutralized with a polyclonal rabbit anti-gerbil rIL-2 antibody (a kind gift of T. R. Klei, Louisiana State University, Baton Rouge), demonstrating specificity for IL-2.

Statistical analysis. Student's *t* test was used to identify differences between control and experimental conditions. $P < 0.05$ was considered significant.

RESULTS

The effect of serum from gerbils with amebic liver abscess on lymphoproliferation. Serum-mediated suppression of lymphoproliferative responses in amebiasis was originally identified using serum from *E. histolytica* infected human patients [20]. Compared to control serum, serum taken from infected patients suppressed proliferation and IFN-γ production of antigen-stimulated peripheral blood mononuclear cells (PBMC) from treated patients. This effect diminished as time between drug therapy and serum collection increased. However, at what stage of infection this suppression occurs or the mechanism(s) involved was not identified.

To determine when serum-mediated suppression of lymphoproliferation occurs, we examined spleen cell proliferative responses to the T cell mitogen ConA and the B cell mitogen LPS. Splenocytes isolated from gerbils with amebic liver abscess at 10, 20, 30

and 60 days post infection were stimulated *in vitro* in the presence of control serum (from uninfected animals) or homologous serum day post infection from infected gerbils.

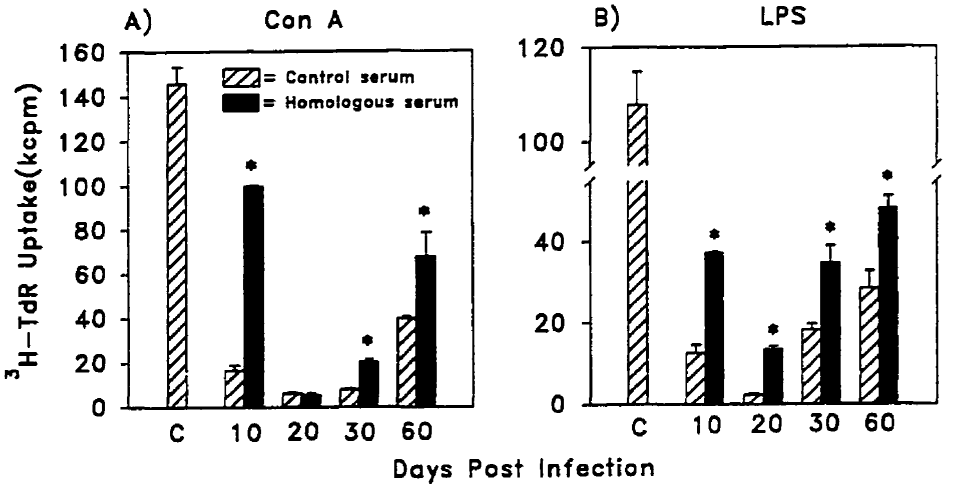

Figure 1. Effect of homologous serum on proliferative responses of spleen cells harvested from *E. histolytica*-infected gerbils at different times post infection. Spleen cells from infected or uninfected animals (C) were incubated *in vitro* with serum (10%) from uninfected (control serum) or infected gerbils (homologous serum). Cells were stimulated with **A)** concanavalin A (ConA), or **B)** lipopolysaccharide (LPS) and proliferation measured by uptake of [^{3}H]thymidine (^{3}H-TdR). The results are expressed as mean kcpm (cpm x 1000) of triplicate cultures (±SE) after the subtraction of ^{3}H-TdR uptake in the absence of stimuli (<1 kcpm). *Denotes significant difference compared to response with control serum at that time point ($P< 0.05$). Similar results were obtained from two independent experiments using different batches of serum.

As shown in figure 1A and B, lymphoproliferative responses to ConA and LPS, in the presence of control serum, were suppressed as early as day 10. Mitogenic responses were lowest at day 20 and recovered slightly thereafter, but never reached control levels. Interestingly, homologous serum from infected animals significantly augmented lymphoproliferative responses to both ConA and LPS except for ConA-stimulated day 20 spleen cells (Fig. 1A and B). Day 20 serum also failed to augment day 20 spleen cell proliferative responses to soluble amebic proteins (data not shown). Similar proliferative responses were obtained with hepatic lymph node cells (draining lymph nodes) stimulated with ConA, LPS, or amebic proteins (data not shown). Therefore, the results with spleen

cells were indicative of both local and systemic responses to the amebic liver abscess. These results suggest that the stimulatory factor(s) present at other times during infection was absent in day 20 serum or that a suppressive effect was competing with the stimulatory effect.

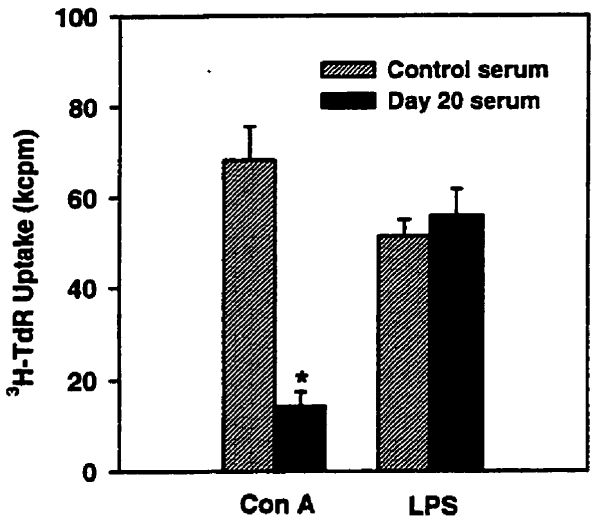

Figure 2. Effect of day 20 serum on ConA- and LPS-induced proliferative responses of normal gerbil spleen cells. Spleen cells from uninfected gerbils were stimulated with concanavalin A (ConA) or lipopolysaccharide (LPS) in the presence of serum (10%) from uninfected gerbils (control serum) or serum from gerbils infected with *E. histolytica* for 20 days (day 20 serum). Proliferative responses were measured by uptake of [^{3}H]thymidine (^{3}H-TdR). The results are expressed as mean kcpm (cpm x 1000) of triplicate cultures (±SE) after the subtraction of ^{3}H-TdR uptake in the absence of stimuli (<1 kCPM). *Denotes significant difference compared to response with control serum ($P < 0.05$). Similar results were obtained from three independent experiments using different batches of serum.

Day 20 serum inhibits Con A- but not LPS-induced lymphoproliferative responses of spleen cells from normal gerbils. Amebic liver abscess development is accompanied by a range of suppressive effects on cell-mediated immunity [22] which may affect lymphocyte proliferation. Indeed, infected gerbil spleen cell proliferative responses in the presence of control serum were suppressed compared to responses of spleen cells from normal animals (Fig. 1A and 1B). Therefore, to specifically examine the potential for serum-mediated suppression, we investigated the effect of day 20 serum on proliferative

responses of spleen cells from uninfected gerbils. As shown in figure 2, ConA-induced spleen cell proliferation was significantly inhibited in the presence of day 20 serum as compared to controls. In contrast, day 20 serum did not affect LPS-induced proliferation. These data suggest that day 20 serum specifically inhibited T cell, but not B cell, lymphoproliferative responses.

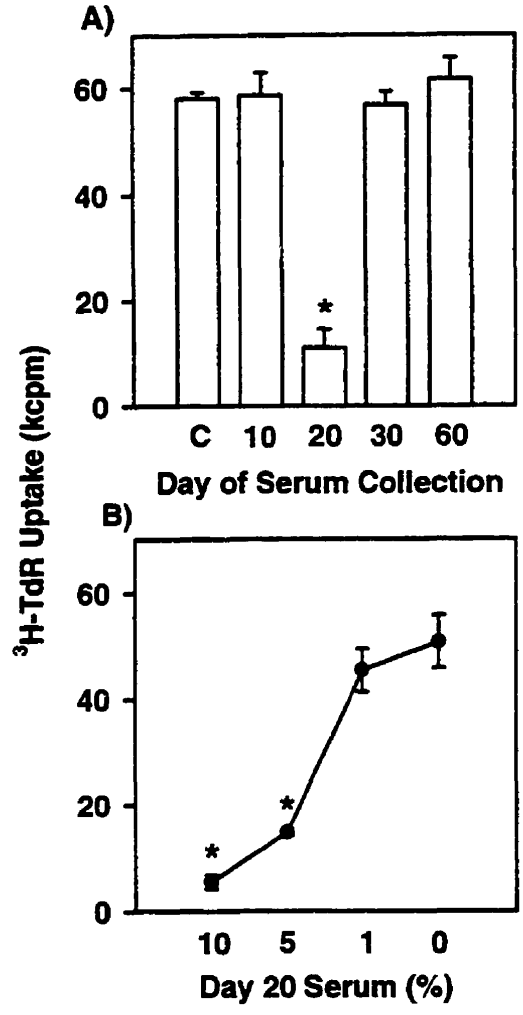

Figure 3. Kinetics and dose-dependence of suppression mediated by serum from *E. histolytica*-infected gerbils. **A)** Spleen cells from uninfected gerbils were stimulated with concanavalin A (ConA) in the presence of serum (10%) from uninfected gerbils (C) or serum from gerbils infected with *E. histolytica* for 10, 20, 30 or 60 days. **B)** Spleen cells from uninfected gerbils were stimulated with ConA in the presence of 0-10% day 20 serum (final serum concentration was made to 10% with control serum). Proliferative responses were measured by uptake of [^{3}H]thymidine (^{3}H-TdR). The results are expressed as mean kcpm (cpm x 1000) of triplicate cultures (±SE) after the subtraction of ^{3}H-TdR uptake in the absence of stimulus (<1 kcpm). *Denotes significant difference compared to response with 10% control serum ($P < 0.05$). Similar results were obtained from two independent experiments using different batches of serum.

Suppression of ConA-induced spleen cell proliferative responses is restricted to serum collected at day 20 of infection and is dose-dependent. Day 20 of amebic liver abscess development in gerbils appears to correlate with maximal suppression of T cell responsiveness. In the presence of control serum, lymphoproliferative responses to ConA were lowest at this time of infection (Fig. 1A) and ConA-induced spleen cell cytokine production was found to be markedly suppressed at day 20 [4]. As day 20 serum suppressed ConA-induced proliferative responses (Fig. 2), we investigated if serum collected before or after this time point also mediated suppression of ConA-induced proliferation of uninfected gerbil spleen cells. Incubation of spleen cells with day 10, 30 or 60 post infection serum did not affect ConA-stimulated proliferation as compared to controls (Fig. 3A). In contrast, day 20 serum markedly inhibited ConA-induced lymphoproliferation in a dose-dependent manner (Fig. 3B). As little as 5% serum was required to significantly inhibit spleen cell lymphoproliferative responses. These data clearly suggest that serum-mediated inhibition of T cell responses is a potent suppressive mechanism transiently present at day 20 of infection.

Day 20 serum suppresses ConA-stimulated IL-2 production from gerbil spleen cells. A variety of mechanisms may operate to inhibit T cell proliferation. These include macrophage production of prostaglandin E_2 (PGE_2) and reactive nitrogen intermediates such as NO [28, 29] or, alternatively, inhibition of T cell IL-2 production [30]. We investigated the potential ability of the PGE_2 inhibitor indomethacin (Sigma) and the NO inhibitor aminoguanidine (Sigma) to reverse the suppressive effect of day 20 serum. As indicated in Table 1, these inhibitors had no effect on the suppression of lymphoproliferation in response to day 20 serum. However, day 20 serum markedly inhibited IL-2 production from uninfected gerbil spleen cells in response to ConA. In two independent experiments using different batches of day 20 serum, IL-2 production was inhibited by 80% and 71% respectively (Table 2). The same batches of day 20 serum also strongly inhibited lymphoproliferative responses to ConA (Fig. 2 and 3). These data

clearly suggest that day 20 serum inhibited T cell lymphoproliferative responses, independent of PGE_2 and NO, by suppressing production of the T cell growth factor IL-2.

Table 1. Effect of aminoguanidine and indomethacin on day 20 serum-mediated suppression

		ConA-stimulated ^{3}H-TdR Uptake (kcpm ± SE)*	
Inhibitor	Concentration	Control Serum	Day 20 Serum
—	—	48.77 ± 0.71	7.69 ± 0.84§
AG	0.5 mM	51.19 ± 2.62	7.35 ± 0.62§
	1.0 mM	47.91 ± 1.92	7.88 ± 0.96§
INDO	5 μg/ml	50.43 ± 1.58	7.40 ± 0.15§
	10 μg/ml	49.94 ± 1.83	6.02 ± 0.99§
AG (0.5 mM) + INDO (5μg/ml)		50.44 ± 2.62	7.85 ± 0.78§

*Spleen cells from uninfected gerbils were stimulated with Concanavalin A (ConA) in the presence of control or day 20 serum ± inhibitors aminoguanidine (AG) and indomethacin (INDO) for 48 h followed by 18 h incubation with [^{3}H]thymidine (^{3}H-TdR). Results are expressed as mean kcpm (cpm x 1000) of triplicate determinations (± SE) after subtraction of ^{3}H-TdR uptake in the absence of stimuli (< 1 kcpm). Similar results were obtained in two independent experiments using different batches of serum.
§Denotes significant difference compared to ConA-stimulated response in the presence of control serum ($P<0.05$).

Table 2. Effect of day 20 serum on ConA-stimulated spleen cell IL-2 production

		IL-2 (U/ml) in spleen cell supernatants*	
		ConA	
Expt. No.	Serum	+	—
1	Control	3.40 ± 0.85	0.25 ± 0.01
	Day 20	0.66 ± 0.10§	0.14 ± 0.09
2	Control	1.48 ± 0.26	0.24 ± 0.04
	Day 20	0.46 ± 0.20§	0.26 ± 0.10

*Spleen cells from uninfected gerbils were incubated with control or day 20 serum ± Concanavalin A (ConA) for 24 h prior to supernatant collection. Interleukin-2 (IL-2) levels were determined in a CTLL/MTT colorimetric assay.
§Denotes significant difference compared to ConA-induced response in the presence of control serum ($P < 0.05$).

Exogenous IL-2 reverses day 20 serum-mediated suppression of Con A-stimulated lymphoproliferation. To confirm that reduced availability of IL-2 was linked to the suppression of T cell proliferation observed in the presence of day 20 serum, we determined whether exogenously added IL-2 could overcome the suppressive effect. As shown in figure 4, supernatants from ConA-stimulated uninfected gerbil spleen cells (containing IL-2 at 2.2-2.3 U/ml), significantly induced proliferation of spleen cells incubated with control or day 20 serum. This stimulatory effect was neutralized by polyclonal anti-gerbil IL-2 antibody, indicating that the cells were proliferating specifically in response to IL-2 rather than any residual ConA in the supernatant. The addition of exogenous IL-2 partially reversed the suppressive effect of day 20 serum on Con A-stimulated spleen cell proliferation, and this effect was ablated by anti-IL-2 (Fig. 4). This

indicates that the cells retained responsiveness to IL-2 in the presence of day 20 serum. Taken together with the data presented in Table 2, the results suggest that the suppressive mechanism associated with day 20 serum involved inhibited production of IL-2 which decreased the levels of IL-2 available to support T cell proliferation.

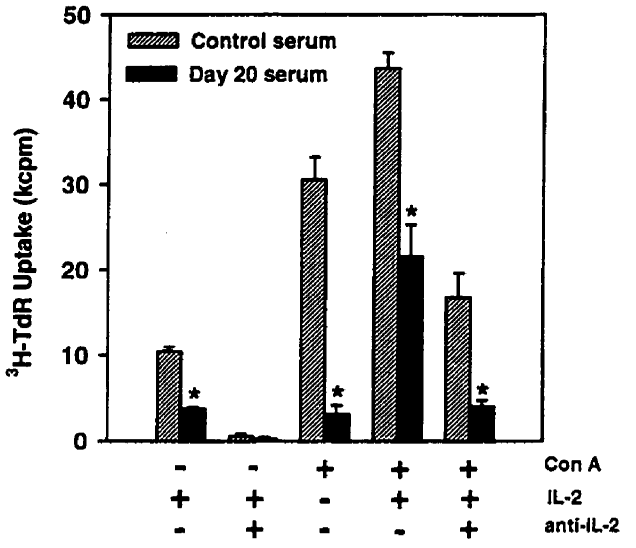

Figure 4. Effect of exogenous IL-2 on day 20 serum-mediated suppression. Spleen cells from uninfected gerbils were stimulated with combinations of concanavalin A (ConA), 10% supernatant of ConA-stimulated uninfected gerbil spleen cells (containing 2.2-2.3 U/ml IL-2) and anti-gerbil IL-2, as indicated. Cells were stimulated in the presence of serum (10%) from uninfected gerbils (control serum) or serum from gerbils infected with *E. histolytica* for 20 days (day 20 serum). Proliferative responses were measured by uptake of [^{3}H]thymidine (^{3}H-TdR). The results are expressed as mean kcpm (cpm x 1000) of triplicate cultures (±SE) after the subtraction of ^{3}H-TdR uptake in the absence of stimuli (<1 kcpm). *Denotes significant difference compared to response with control serum ($P < 0.05$). Similar results were obtained from two independent experiments using different batches of serum.

DISCUSSION

Suppression of T cell responsiveness commonly occurs during infection with a variety of parasitic protozoans; including *Trypanosoma cruzi*, *Toxoplasma gondii* and *Leishmania donovani* [30, 31, 18]. There is evidence that T cell responses are also systemically suppressed during *E. histolytica* infection in both humans and animal models of the disease [17, 4]. In addition, macrophages in the vicinity of the amebic liver abscess,

or directly exposed to amebic components *in vitro*, are profoundly suppressed in their accessory and effector cell functions [22]. While the mechanisms involved in the suppression of macrophages during amebic infections have been partially characterized, how T and B cell responses are down-regulated is not known. Suppressive factors in the circulation are a potential means of systemic lymphocyte inhibition. Previous studies [20] have found that serum from patients with amebic liver abscesses suppressed *in vitro* amebic-antigen specific proliferation and IFN-γ production from PBMCs of drug-treated patients. In the present study, we clearly demonstrate that the suppressive activity of serum is transient and is confined to day 20 of infection, a time corresponding to maximal growth of the amebic liver abscess in gerbils [4, 21]. In addition, the serum suppressive effect was specific for T cell mitogenesis, and involved marked inhibition of IL-2 production by day 20 serum.

Certain potential inhibitory mechanisms appear unlikely to be involved in the serum-mediated suppression observed in amebiasis. Absorption of serum against viable *E. histolytica* trophozoites did not ablate the suppressive activity of amebiasis patient serum, suggesting that immune complexes were not causing the suppressed T cell proliferation [20]. The production of NO or PGE_2 by macrophages upon exposure to serum is also unlikely to be involved as we found no decrease in serum suppressive activity in the presence of specific inhibitors for these molecules (Table 1). Reduction in antigen-specific IFN-γ production by human amebiasis serum also points to a NO-independent mechanism of suppression [20]. The results of our study suggest that the mechanism of serum-mediated suppression in *E. histolytica* infection involves impaired IL-2 production. Spleen cell IL-2 release in response to ConA was notably inhibited in the presence of day 20 serum as compared to control serum (Table 2). Because IL-2 is the principal autocrine growth factor for T cells, inhibition of its production or activity would markedly suppress T cell responses. The ability of IL-2 to support T cell activation and proliferation may be negatively regulated by direct suppression of IL-2 production [32] or by decreased surface

expression of IL-2 receptors [33]. The fact that exogenous IL-2 could partially rescue day 20 serum-suppressed lymphoproliferative responses suggests that reduced IL-2 receptor expression was not a factor in our study. Instead, day 20 serum may have been directly suppressing IL-2 production. This could be affected by parasite components in serum or host factors released during infection. The cytokine IL-10, for example, suppresses T cell proliferation and IL-2 production [34] and was induced *in vivo* in response to a 220kD lectin molecule of *E. histolytica* [35]. At this time, we cannot rule out the additional possibility that soluble IL-2 receptors were present in day 20 serum and sequestered IL-2 produced by T cells [18]. The gerbil IL-2 receptor has not been cloned and sequenced, so we were unable to examine soluble IL-2 receptor levels in serum or surface expression of IL-2 receptors. The inability of day 20 serum to suppress LPS-induced B cell proliferation (Fig. 2) may be related to the fact that a variety of other cytokines also have B cell growth-promoting activity. Deficiency in one particular cytokine has minimal effects on B cell proliferation [36]. At present, how serum from amebiasis patients suppresses T cell responses is not known. Perhaps a mechanism analogous to that operating in the gerbil is responsible.

Compared with spleen cells from uninfected gerbils, those harvested from infected animals and incubated with control serum had suppressed ConA- and LPS-induced proliferative responses *ex vivo*, with maximal suppression occurring at day 20 (Fig. 1A and B). Day 20 serum-mediated inhibition of T cell proliferation and IL-2 production in response to ConA was a potent suppressive mechanism (Fig. 3). There may be a lingering effect of day 20 serum on *ex vivo* ConA-induced proliferation of day 20 spleen cells incubated with control serum. This does not, however, explain the suppressed LPS-stimulated responses throughout infection and suppressed Con A-stimulated responses at days 10, 30 and 60 post infection. Other mechanisms may have been involved in suppressing T and B cell proliferative responses during infection. A role for macrophage-produced PGE_2 in this suppression is an attractive possibility. PGE_2 production is

elevated during *E. histolytica* infection in gerbils [37] and this arachidonic acid metabolite has been implicated in suppression of both T and B cell proliferative responses [28, 29]. *E. histolytica* has been shown to suppress IFN-γ-induced expression of class II major histocompatibility complex antigens on the surface of macrophages by a PGE_2-dependent mechanism [38]. Reduced antigen-presenting capacity may well contribute to suppressed T cell activation *in vivo*.

Transient suppression of lymphocyte responses at acute disease has been reported previously in animal models of *E. histolytica* infection [39, 40]. In the gerbil model of amebiasis, day 20 may be considered acute infection. We have found that this time point corresponds to peak liver abscess size and maximal suppression of antigen-specific and mitogen-induced T cell proliferation and cytokine production [4]. Both T and B cell proliferative responses recovered after day 20 (Fig. 1A and B), as does IL-2 production from stimulated hepatic lymph node and spleen cells [4]. Strikingly, the suppressive effect of serum on ConA-stimulated mitogenesis was restricted to day 20 of infection (Fig. 3). Serum collected at all other time points enhanced *ex vivo* proliferative responses (Fig. 1A and B), perhaps by stimulatory cytokines. These results suggest that serum-mediated suppression in amebiasis is associated with acute infection. The suppressive effect of serum from human amebiasis patients decreased with time between drug treatment and serum collection, suggesting that serum-mediated suppression in humans may also be a transient phenomenon related to the extent of the disease [20]. However, humans may experience a more prolonged acute phase and relentless progression of the disease in the absence of clinical intervention [41]. In this scenario, serum-mediated suppression of T cell responses may continue indefinitely.

In summary, our data indicate that serum-mediated suppression occurs during acute hepatic amebiasis in gerbils. Day 20 serum specifically inhibited ConA-induced spleen T cell proliferation, independent of NO or PGE_2. The mechanism of suppression was associated with decreased IL-2 production in the presence of day 20 serum. Reduced T cell

responses during acute amebiasis would result in inhibited production of cytokines for activation of macrophage amebicidal activity, and reduced T cell help for B cell antibody responses. Inhibited proliferative responses and IFN-γ production by human PBMC in the presence of serum from amebiasis patients [20] suggests that an analogous IL-2-dependent suppressive mechanism may occur during human infections. Serum-mediated suppression of T cell responsiveness in *E. histolytica* -infected humans or animals could exacerbate and prolong infections by adding to the multiple suppressive phenomena occurring during infection with *E. histolytica*.

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REFERENCES

1. Ravdin JI. Pathogenesis of disease caused by *Entamoeba histolytica*: studies of adherence, secreted toxins, and contact-dependent cytolysis. Rev Infect Dis **1986**;8:247-60.
2. Walsh JA. Prevalence of *Entamoeba histolytica* infection. In: Ravdin JI. Amebiasis-human infection by *Entamoeba histolytica*.. New York: John Wiley and Sons, **1988**:93-105.
3. Deleon A. Pronostico tardio en el absceso hepatico amibiano. Arch Invest Med **1970**;1:205-6.

4. Campbell D, Chadee K. Interleukin (IL)-2, IL-4 and tumor necrosis factor-α responses during *Entamoeba histolytica* liver abscess development in gerbils. J Infect Dis **1997**;175:1176-83.

5. Soong CJ G, Kain KC, Abd-Alla M, Jackson TFHG, Ravdin JI. A recombinant cysteine rich section of the *Entamoeba histolytica* galactose-inhibitable lectin is efficacious as a subunit vaccine in the gerbil model of amebic liver abscess. J Infect Dis **1995**;171:645-51.

6. Denis M, Chadee K. Cytokine activation of murine macrophages for in vitro killing of *Entamoeba histolytica* trophozoites. Infect Immun **1989**;57:1750-6.

7. Lin JY, Chadee K. Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. J Immunol **1992**;148:3999-4005.

8. Salata RA, Pearson RD, Ravdin JI. Interaction of human leukocytes and *Entamoeba histolytica*: killing of virulent amebae by the activated macrophages. J Clin Invest **1985**;76:491-9.

9. Schain DC, Salata RA, Ravdin JI. Human T-lymphocyte proliferation, lymphokine production, and amebicidal activity elicited by the galactose-inhibitable adherence protein of *Entamoeba histolytica*. Infect Immun **1992**;60:2143-6.

10. Schain DC, Salata RA, Ravdin JI. Development of amebicidal cell-mediated immunity in gerbils (*Meriones unguiculatus*) immunized with the galactose-inhibitable adherence lectin of *Entamoeba histolytica*. J Parasitol **1995**;81:563-8.

11. Lotter H, Zhang T, Seydal KB, Stanley SL, Tannich E. Identification of an epitope on the *Entamoeba histolytica* 170-kD lectin conferring antibody-mediated protection against invasive amebiasis. J Exp Med **1997**;185:1793-1801.

12. Espinosa-Cantellano M, Martinez-Palomo A. *Entamoeba histolytica*: mechanism of surface receptor capping. Exp Parasitol **1994**;79:424-35.

13. Tran VQ, Herdman DS, Torian BE, Reed SL. The neutral cysteine proteinase of *Entamoeba histolytica* degrades IgG and prevents its binding. J Infect Dis

1998;177:508-11.

14. Braga LL, Ninomiya H, McCoy JJ et al. Inhibition of the complement membrane attack complex by the galactose-specific adhesin of *Entamoeba histolytica*. J Clin Invest **1992**;90:1131-7.
15. Wang W, Keller K, Chadee K. Modulation of tumor necrosis factor production by macrophages in *Entamoeba histolytica* infection. Infect Immun **1992**; 60:3169-74.
16. Wang W, Keller K, Chadee K. *Entamoeba histolytica* modulates the nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxicity against amoebae and tumour cells. Immunology **1994**;83:601-10.
17. Salata RA, Martinez-Palomo A, Murray HW et al. Patients treated for amebic liver abscess develop cell-mediated immune responses effective in vitro against *Entamoeba histolytica*. J Immunol **1986**;136:2633-9.
18. Barral-Netto M, Barral A, Santos SB et al. Soluble IL-2 receptor as an agent of serum-mediated suppression in human visceral leishmaniasis. J Immunol **1991**;147:281-4.
19. Tomar RH, Hinds P, Katz D, Schultz K. Sera from simian immunodeficiency virus-infected rhesus macaques inhibit lymphocyte proliferation. J Clin Lab Analysis **1992**;6:297-301.
20. Salata RA, Martinez-Palomo A, Canales L, Murray HW, Trevino N, Ravdin JI. Suppression of T-lymphocyte responses to *Entamoeba histolytica* antigen by immune sera. Infect Immun **1990**;58:3941-6.
21. Chadee K, Meerovitch E. The pathogenesis of experimentally induced amebic liver abscess in the gerbil (*Meriones unguiculatus*). Am J Pathol **1984**;117:71-80.
22. Campbell D, Chadee K. Survival strategies of *Entamoeba histolytica*: modulation of cell-mediated immune responses. Parasitol Today **1997**;13:184-190.
23. Mishell BB, Shiigi SM. Selected methods in cellular immunology. San Francisco: W. H. Freeman and Co., **1980**.
24. Gillis S, Ferm MM, Ou W, Smith KA. T cell growth factor: parameters of production

and a quantitative microassay for activity. J Immunol **1978**;120:2027-32.

25. Mai Z, Kousoulas KG, Horohov DW, Klei TR. Cross-species PCR cloning of gerbil (*Meriones unguiculatus*) interleukin-2 cDNA and its expression in COS-7 cells. Vet Immunol Immunopathol **1994**;40:63-71.
26. Karasuyama H, Melchers F. Establishment of mouse cells which constitutively secrete large quantities of interleukin-2, 3, 4 or 5 using modified cDNA expression vectors. Eur J Immunol **1988**;18:97-104.
27. Mosmann TR. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **1983**;65:55-63.
28. Santoli D, Philips PD, Colt TL, Zurier RB. Suppression of interleukin-2-dependent human T cell growth *in vitro* by prostaglandin E (PGE) and their precursor fatty acids. J Clin Invest **1990**;85:424-32.
29. Albina JE, Abate JA, Henry WL. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-γ in the induction of the nitric oxide-synthesizing pathway. J Immunol **1991**;147:144-8.
30. Harel-Bellan A, Joskowicz M, Fradelizi D, Eisen H. Modification of T cell proliferation and interleukin-2 production in mice infected with *Trypanosoma cruzi*. Proc Natl Acad Sci (USA) **1983**;80:3466-9.
31. Haque S, Haque A, Kasper LH. A *Toxoplasma gondii* derived factor(s) stimulates immune downregulation: an *in vitro* model. Infect Immun **1995**;63:3442-7.
32. Majumder S, Kierszenbaum F. *Trypanosoma cruzi* immunosuppressive factor decreases the interleukin-2 mRNA level in cultured normal activated human lymphocytes. Infect Immun **1995**;63:4546-9.
33. Sileghem M, Darji A, Remels L, Hamers R, De Baetselier P. Different mechanisms account for the suppression of interleukin-2 production and interleukin-2 receptor expression in *Trypanosoma brucei* infected mice. Eur J Immunol **1989**;19:119-24.

34. Taga K, Tosato G. IL-10 inhibits human T cell proliferation and IL-2 production. J Immunol **1992**;148:1143-8.

35. Talamas-Rohana P, Schlie-Guzman MA, Hernandez-Ramirez VI, Rosales-Encina JL. T cell suppression and selective *in vivo* activation of TH2 subpopulation by the *Entamoeba histolytica* 220-kilodalton lectin. Infect Immun **1995**; 63:3953-8.

36. Banchereau J, Roussell F. Human B lymphocytes: phenotype, proliferation, and differentiation. Adv Immunol **1992**;52:125-262.

37. Wang W, Chadee K. *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages *in vitro* and *in vivo*. Immunology **1992**;76:242-50.

38. Wang W, Chadee K. *Entamoeba histolytica* suppresses gamma interferon-induced macrophage class II major histocompatibility complex Ia molecule and I-Aß mRNA expression by a prostaglandin E_2-dependent mechanism. Infect Immun **1995**;63:1089-94.

39. Gill NJ, Ganguly NK, Dilawari JB, Mahajan RC. Lymphocyte subpopulations transformation studies in an experimental model of intestinal and hepatic amoebiasis. Trans R Soc Trop Med Hyg **1985**;79:618-23.

40. Vinayak VK, Sharma P. Kinetics of the immune responses during the course of hepatic amoebic infection-an experimental study. Trans R Soc Trop Med Hyg **1989**;83:349-53.

41. Salata RA, Ravdin JI. Review of the human immune mechanisms directed against *Entamoeba histolytica*. Rev Infect Dis **1986**;8:261-72.

CONNECTING STATEMENT II

In Manuscript I, it was demonstrated that a Th1 response is associated with resistance to amoebic infections. Likewise, immunosuppression associated with primary amoebic infections (Manuscript II) can be overcome by prior sensitization of the host. Vaccination with the native Gal-lectin or recombinant cysteine rich heavy subunit regions protect gerbils against a challenged infection. However, it is not known what regions of the heavy subunit are involved in stimulating Th1 and cell-mediated immunity for host defence against amoebiasis. Interleukin-12 (IL-12) is the key cytokine involved in stimulating Th1 responses *in vivo*. As the Gal-lectin has been previously shown to activate macrophages for TNF-α production, we investigated whether it could also stimulate macrophages to produce IL-12. In the following study, we characterize Gal-lectin stimulation of IL-12 and identify the region of the molecule responsible for mediating IL-12 induction in human macrophages.

MANUSCRIPT III

A subunit vaccine candidate region of the *Entamoeba histolytica* galactose-adherence lectin promotes Interleukin-12 gene transcription and protein production in human macrophages*

Darren Campbell, Barbara J. Mann, and Kris Chadee

*an abbreviated version of this manuscript has been submitted for publication in the *Proceedings of the National Academy of Sciences of the United States of America*

ABSTRACT

The cysteine-rich region of the 170-kDa subunit Gal-lectin of *Entamoeba histolytica* is a subunit vaccine candidate and a protective antigen in the gerbil model of amebiasis. However, it is not known what regions are important and the mechanisms by which it induces protective immunity. The Gal-lectin activates macrophage tumor necrosis factor-α production and nitric oxide-dependent amebicidal activity. As macrophage-mediated immunity is activated by Th1 cytokines and Th1 differentiation is promoted by interleukin-12 (IL-12), we investigated what portion of the Gal-lectin could stimulate IL-12 in human THP-1 macrophages. Native Gal-lectin stimulated IL-12 p40/p35 mRNA expression in a dose- and time-dependent manner as measured by reverse-transcription PCR. Human immune serum and Gal-lectin monoclonal antibody inhibition studies identified aa 596-998 of the cysteine-rich region as immunogenic and containing the IL-12 inducing domain. Interferon-γ (IFN-γ) priming augmented Gal-lectin-induced IL-12 mRNA expression independent of TNF-α and IL-1β, and was required for IL-12 p70 protein production from macrophages and human peripheral blood mononuclear cells. Gal-lectin plus IFN-γ stimulated IL-12 p40/p35 gene transcription with stable mRNA transcripts. Cycloheximide inhibited IL-12 p40 and caused superinduction of p35 mRNA expression, demonstrating a differential requirement for newly synthesized proteins. These results suggest that aa 596-998 of the Gal-lectin can confer protection against amebiasis through induction of IL-12 to initiate protective Th1 immunity.

INTRODUCTION

The protozoan parasite *Entamoeba histolytica* is the etiologic agent of amebiasis. Invasive amebiasis causes up to 110,000 deaths each year (1). Evidence from clinical and experimental amebiasis (2, 3) clearly indicates that resistance to reinfection occurs. The galactose-adherence lectin (Gal-lectin) of *E. histolytica* is the most promising vaccine candidate molecule. The Gal-lectin is a 260-kDa heterodimeric surface glycoprotein consisting of 170-kDa heavy and 31- to 35-kDa light subunits linked by disulfide bonds (4). *E. histolytica* adheres via the cysteine-rich region of the 170-kDa subunit to human colonic mucins for colonization and epithelial cells as a prerequisite for tissue invasion (5, 6, 7). Native Gal-lectin and recombinant fusion proteins containing the cysteine-rich region of the Gal-lectin protected gerbils from amebic infections with vaccine efficacy of 67% for the native molecule and 71-81% for the recombinant cysteine-rich region (8, 9, 10).

Cell-mediated immunity is crucial for host defense and resistance against amebiasis (11-15). Naive macrophages activated with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) express potent nitric oxide (NO)-dependent amebicidal activity (11). Many studies demonstrate that the Gal-lectin promotes cell-mediated immunity. The cysteine-rich region directly stimulates TNF-α production and activates IFN-γ-primed macrophages for NO production and amebicidal activity (12, 16). T lymphocytes from drug-cured amebiasis patients and Gal-lectin-immunized gerbils respond to the Gal-lectin with IFN-γ and interleukin-2 (IL-2) production and supernatants from antigen-activated T cells stimulate human macrophages to kill amebae (13-14). T lymphocyte production of macrophage-activating cytokines IFN-γ, IL-2 and TNF-β is a hallmark of Th1-type responses (17). We recently demonstrated that a Th1 response in amebiasis is associated with resistance to reinfection in gerbils following drug-abbreviated infections (3). Macrophage-derived IL-12 is the principal cytokine involved in the differentiation of

antigen-specific Th1 lymphocytes. IL-12 p70 is a heterodimeric cytokine composed of disulfide-linked products of the IL-12 p40/p35 genes. IL-12 acts on T and NK cells to stimulate IFN-γ production which promotes Th1 differentiation (17). Stimulation of IL-12 and consequent Th1 immunity by the Gal-lectin may represent the mechanism by which immunization with this molecule, or parts thereof, protects against amebiasis.

The purpose of this study was to determine what regions of the Gal-lectin could stimulate IL-12 induction in human macrophages, which could promote a protective Th1 response during vaccination. Herein, we demonstrate that native Gal-lectin, similar to lipopolysaccharide (LPS), stimulated IFN-γ-primed macrophages for IL-12 p40/p35 gene transcription with stable mRNA transcripts. Using a panel of monoclonal antibodies (mAbs) that recognize nonoverlapping epitopes on the cysteine rich region, we have identified a sequence containing aa 596-998 that mediates IL-12 mRNA induction. Bioactive IL-12 p70 protein was produced in IFN-γ primed macrophages and human peripheral blood mononuclear cells (PBMC) in response to the Gal-lectin. Induction of IL-12 by the immunoprotective cysteine-rich region of the Gal-lectin suggests that this subunit vaccine candidate may initiate Th1-mediated cellular immunity for host defense against amebiasis.

MATERIALS AND METHODS

Cultivation of *E.histolytica* and Preparation of Gal-lectin. *E.histolytica* (strain HMI-IMSS) was cultivated in our laboratory as previously described (18). Soluble amebic proteins (AP) were prepared from trophozoites in mid log growth phase (3 days) by freeze-thaw lysis. Lysed trophozoites were centrifuged at 15,000 *g* (4°C) for 10 min and protein concentration in the supernatants quantified by the method of Bradford (19). The Gal-lectin was purified by mAb affinity chromatography as described (4). Endotoxin contamination was undetectable by the E-Toxate assay (sensitive to 0.05-0.5 endotoxin

unit/ml; Sigma) in AP at 50 μg/ml and Gal-lectin at 1 μg/ml.

THP-1 Macrophage Cultivation and Preparation of Human PBMC. Human THP-1 macrophages were maintained at 37°C and 5% CO_2 in complete RPMI-1640 [RPMI-1640 (GIBCO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 20 mM HEPES (Sigma) and 10% heat-inactivated fetal calf serum (Hyclone Laboratories)]. To obtain adherent macrophages for preparation of RNA, 1 x 10^6 cells/well in 24-well culture plates were differentiated in the presence of 10 nM PMA (Sigma) for 3 days. Macrophages were washed and quiesced in complete RPMI-1640 for 24 hr prior to stimulation as described in the figure legends. For IL-12 protein quantification, macrophages were pretreated with 1.2% DMSO (Sigma) for 24 hr prior to stimulation, instead of PMA (20).

For PBMC isolation, blood was collected from amebiasis-free donors in EDTA-treated syringes and leucocytes purified by centrifugation over Ficoll (Pharmacia). Leucocytes were plated at 4 x 10^6 cells/ml and enriched for monocytes by adherence for 2 hr at 37°C. Non-adherent cells were washed off and monocyte-derived macrophages generated by culturing monocytes in complete RPMI-1640 for 3 days prior to stimulation.

Preparation of Polyclonal and mAbs Against the Gal-lectin. Normal human serum and pooled immune serum from amebic liver abscess patients was provided courtesy of J. Keystone (University of Toronto). Pre-immune serum and rabbit polyclonal anti-Gal-lectin serum was prepared as described (5). The production of murine anti-Gal-lectin mAbs has been previously described (21). The epitopes recognized by mAbs 1G7 (IgG2b), 8C12 (IgG1) and 7F4 (IgG2b) were mapped to the cysteine-rich region of the Gal-lectin using a series of successive deletion peptides of the 170-kDa Gal-lectin subunit (5). mAb R35 (IgG1) was used as an isotype-matched control for mAb 8C12 and recognizes human colonic mucins (a kind gift of D. Podolsky, Harvard University).

Construction of Competitors, RNA Preparation, and Semiquantitative Reverse-Transcription PCR (RT-PCR). Specific primers were designed to amplify

human IL-12 p40 (5'-GGGACAACAAGGAGTATGAG-3' sense, 5'-GATGCCCATTCGCTCCAAG-3' antisense) and previously published primer sequences were used to amplify human IL-12 p35 (22). The IL-12 p40/p35 PCR primer products were the predicted sizes (427 bp and 532 bp, respectively) and confirmed by restriction enzyme digestion. To construct competitors, the IL-12 p40/p35 products were individually subcloned into pGEM-T vector (Promega). After digestion of IL-12 p40/pGEM-T with *Eco*RI, a 360 bp fragment of irrelevant DNA was inserted to generate a 787 bp competitor. Conversely, the subcloned IL-12 p35 product was digested with *Hin*dIII and *Eco*RI to delete 178 bp and generate a 354 bp competitor.

Total RNA was isolated from cells with Trizol (GIBCO) and 1 μg RNA of each sample was used for RT to make cDNA for PCR. Semiquantitative RT-PCR was performed as previously described (23) with modifications. IL-12 p40/p35 primer pairs were used at 25 pmol and 50 pmol/reaction, respectively. Competitors in pGEM-T were used at 0.3 pg/pcr reaction. The PCR consisted of 94°C for 30s, 55°C for 1 min, and 72°C for 2 min for a total of 35 cycles followed by a final cycle of 94°C for 30s, 55°C for 1 min, and 72°C for 8 min. IL-12 p40/p35 products were accumulating exponentially in the presence or absence of competitors. PCR products (30 μl) were electrophoresed in 1.5% agarose gels containing ethidium bromide to visualize amplification and were photographed with Polaroid film (Polaroid). The results were analyzed using NIH image program 1.59 (FTP from zippy.nim.nih.gov). The IL-12 target/competitor ratio was determined for each sample as relative density. Relative differences in this ratio represent changes in mRNA levels. An intermediate size product (heteroduplex) was formed between the IL-12 target and competitor products. However, the heteroduplex does not interfere with densitometric scanning analysis of the homodimeric products (24). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The GAPDH primers amplify a 358 bp fragment (23).

Measurement of IL-12 p40 and p70, IL-1ß, and TNF-α Protein. IL-

12 p40 and p70 protein levels were measured by using specific ELISAs (R&D Systems) according to the manufacturer's protocols. IL-1ß and TNF-α protein levels were measured by ELISA (R&D Systems) as described in detail elsewhere (25). Samples were assayed in triplicate and cytokine levels calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

Nuclear Run-on Analysis. IL-12 p40/p35 gene transcription was detected in a PCR-based nuclear run-on assay (26). Following 4 hr stimulation, macrophages were washed and resuspended in PBS. Cells (8 x 10^6/sample) were resuspended in lysis buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCL, 1% Triton X-100, pH 7.5), incubated on ice for 10 min, and centrifuged at 1300 *g* for 15 min at 4°C. The nuclear pellets were washed once in lysis buffer and the nuclei divided into 2 aliquots, each resuspended in 100 µl of storage buffer (50 mM Tris-HCL, pH 8.0, 40% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT) and frozen in liquid nitrogen until use. Elongation of the nascent RNA *in vitro* was done by adding 100 µl of transcription buffer (20 mM Tris-HCL, pH 8.0, 300 mM KCL, 10 mM $MgCl_2$, 200 mM sucrose, 48 µM EDTA and 1 mM DTT) with or without ribonucleotides (1 mM each of rATP, rCTP, rGTP, and rUTP) to 100 µl of nuclei and incubating at 30°C for 30 min. The reactions were stopped by adding 1 ml Trizol and RNA extracted for RT-PCR analysis. A difference between samples incubated with and without rNTPs is indicative of active transcription.

RESULTS

Gal-lectin Stimulates IL-12 p40 and p35 mRNA Expression in Human Macrophages. Production of bioactive IL-12 p70 is dependent on the expression of both IL-12 p40 and p35 genes. To determine if macrophages express IL-12 p40 and p35 mRNA in response to the Gal-lectin, cells were stimulated for 6 hr with graded doses of native Gal-lectin. Relative IL-12 mRNA levels were detected by

semiquantitative competitive RT-PCR. As shown in Fig. 1, IL-12 p35 mRNA was constitutively expressed at low levels, whereas p40 mRNA was undetectable in unstimulated cells. However, IL-12 p40/p35 mRNA expression was stimulated by Gal-lectin in a dose-dependent manner. Soluble amebic proteins (AP), which contain Gal-lectin and other proteins (16), stimulated a moderate level of IL-12 p40/p35 mRNA expression, highlighting the relative potency of the Gal-lectin. In contrast, the positive control LPS stimulated strong IL-12 p40/p35 mRNA expression. Gal-lectin also stimulated a time-dependent increase in IL-12 mRNA expression with peak p40/p35 mRNA levels occurring after 6 hr (Fig. 2).

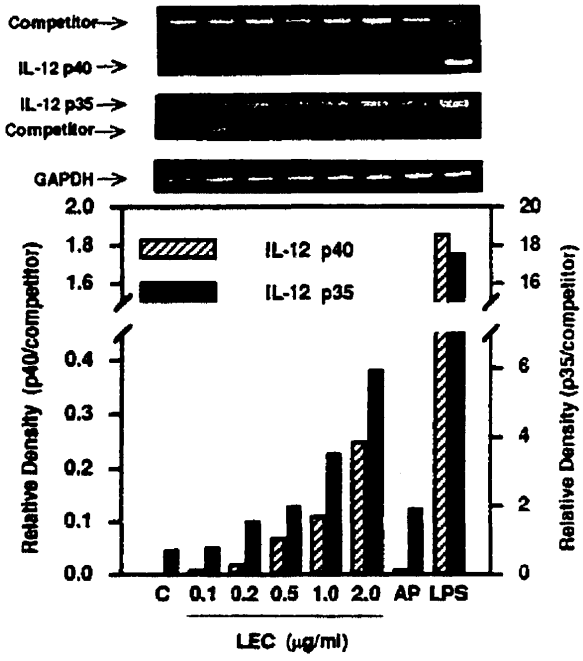

Figure 1. Expression of human IL-12 p40 and p35 mRNA in response to Gal-lectin is dose dependent. PMA-differentiated THP-1 macrophages (1 x10^6/ml) were stimulated with medium alone (C), various concentrations of purified native Gal-lectin (LEC), soluble amebic proteins (AP; 50 μg/ml), or LPS (1 μg/ml; *Escherichia coli* serotype 0111:B4; Sigma) for 6 hr prior to extraction of total RNA. Semi-quantitative competitive RT-PCR was performed on total RNA (1 μg/ml) as described in the materials and methods. PCR products were size-fractionated on 1.5% agarose gels containing ethidium bromide to visualize results. PCR lane designations are identical to those for histograms generated following NIH image analysis. The relative density (IL-12 p40 or p35/ competitor) is a representation of relative IL-12 mRNA levels. GAPDH was used as an internal control. Similar results were obtained in three independent experiments.

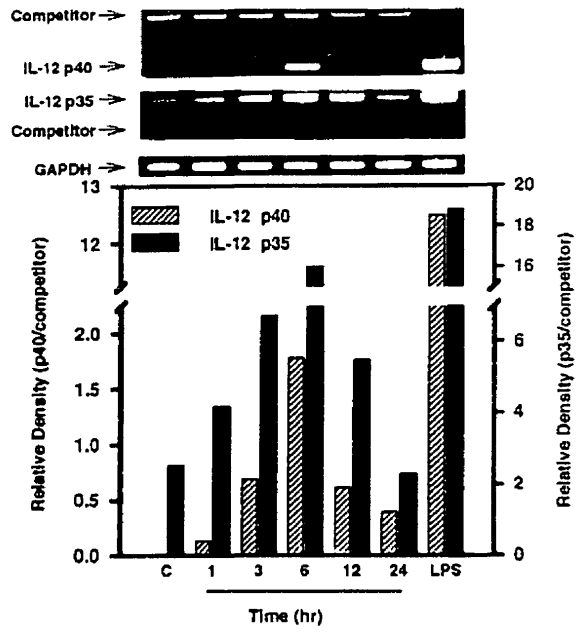

Figure 2. Kinetics of IL-12 p40 and p35 mRNA expression in macrophages stimulated with Gal-lectin. Cells were stimulated in medium alone (C), with LPS (1 μg/ml) for 6 hr, or with 1 μg/ml Gal-lectin for the time periods indicated. Total RNA was extracted and semi-quantitative RT-PCR performed. GAPDH was used as an internal control. Similar results were obtained in three independent experiments.

Polyclonal and mAbs Against the Cysteine-Rich Region of the Gal-lectin Inhibit IL-12 p40 mRNA Expression. As IL-12 p40 mRNA expression was highly inducible but not constitutively expressed, we examined levels of p40 mRNA in antibody inhibition studies. Pooled serum (1:500) isolated from patients with amebic liver abscess and rabbit serum raised against the native Gal-lectin (1:500) inhibited IL-12 p40 mRNA expression in response to the Gal-lectin (>50% inhibition compared to homologous controls) but not in response to LPS (Fig. 3). These results demonstrate that the portion(s) of the Gal-lectin responsible for IL-12 induction is immunogenic in a natural infection and following vaccination with the Gal-lectin. To identify the specific portion of the Gal-lectin that stimulates IL-12 mRNA expression, we examined the inhibitory effects of a panel of mAbs which map to non-overlapping epitopes on the cysteine-rich region of the Gal-lectin 170-kDa subunit. Both mAbs 1G7 (epitope aa 596-818) and 8C12 (epitope aa 895-998)

markedly inhibited IL-12 p40 mRNA expression in response to Gal-lectin. mAbs 7F4 (epitope aa 1082-1138) and R35 (an irrelevant isotype-matched control for 8C12) did not (Fig. 4). mAbs 1G7 and 8C12 also inhibited Gal-lectin-induced IL-12 p35 mRNA expression (data not shown). None of the mAbs, used at a range of concentrations, inhibited LPS-induced IL-12 p40 mRNA expression. Thus, aa 596-998 of the cysteine-rich region is responsible for stimulating IL-12 mRNA expression in macrophages.

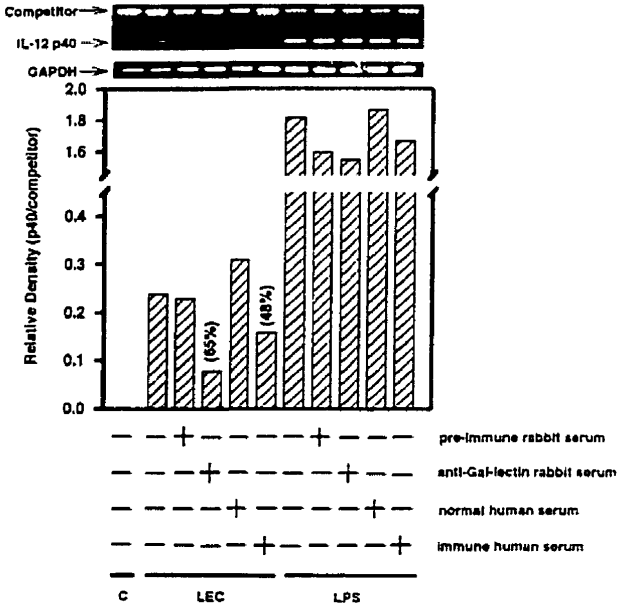

Figure 3. Serum from amebiasis patients and rabbit anti-Gal-lectin serum specifically inhibit IL-12 p40 mRNA expression in response to Gal-lectin. Gal-lectin (LEC; 1 μg/ml) and LPS (1 μg/ml) were pre-treated with medium or 1:500 dilutions of pre-immune rabbit serum, anti-Gal-lectin rabbit serum, normal human serum, or pooled immune serum from amebic liver abscess patients for 1 hr at 4°C. Macrophages were then incubated with medium alone (C) or the pre-treated stimulants for 6 hr prior to extraction of total RNA and semiquantitative RT-PCR analysis. GAPDH was used as an internal control. Values in parentheses indicate percent inhibition of relative IL-12 p40 levels as compared to controls. Similar results were obtained in two independent experiments.

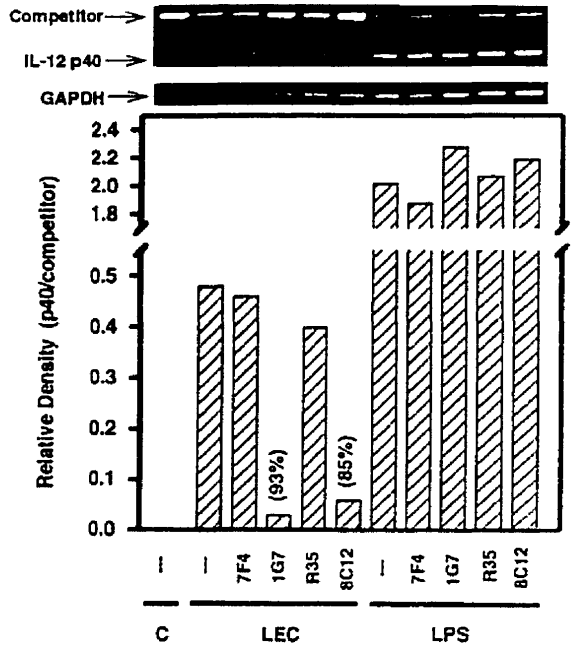

Figure 4. Anti-Gal-lectin mAbs specifically inhibit IL-12 p40 mRNA expression in response to the Gal-lectin. Gal-lectin (LEC; 1 μg/ml) and LPS (1 μg/ml) were pre-treated with medium or mAbs (1 μg/ml) for 1 hr at 4°C. Macrophages were incubated with medium alone (C) or the pre-treated stimulants for 6 hr prior to extraction of total RNA and semiquantitative RT-PCR analysis. GAPDH was used as an internal control. Values in parentheses indicate percent inhibition of relative IL-12 p40 levels as compared to controls. 7F4 and 1G7 are both IgG2b anti-Gal-lectin mAbs. R35 is an irrelevant control mAb against human colonic mucins and is isotype-matched to anti-Gal-lectin mAb 8C12 (both are IgG1). Similar results were obtained in three independent experiments.

IFN-γ Enhances IL-12 p40 and p35 mRNA Expression and IL-12 Protein Production in Response to the Gal-lectin. IFN-γ directly induces IL-12 p35 mRNA expression and primes macrophages for enhanced IL-12 p40/p35 mRNA induction in response to LPS (27). To determine the effect of IFN-γ on IL-12 p40/p35 mRNA expression in response to the Gal-lectin, macrophages were stimulated with Gal-lectin+IFN-γ for 6 hr and mRNA expression analyzed by semiquantitative RT-PCR. Preliminary studies demonstrated that simultaneous stimulation with IFN-γ produced optimal results. IFN-γ synergized with Gal-lectin to induce high levels of IL-12 p40/p35 mRNA expression, comparable to levels achieved with LPS alone (Fig.5). Similarly, IFN-γ synergized with LPS for enhanced IL-12 mRNA expression. As expected, IFN-γ alone

stimulated IL-12 p35 but not p40 mRNA expression. IL-12 p40 and p70 protein production were measured to determine if IFN-γ had a similar enhancing effect at the protein level. As shown in Table 1, Gal-lectin alone stimulated moderate IL-12 p40 but not p70 protein production from macrophages and human PBMC. However, IFN-γ synergized with the Gal-lectin for high levels of IL-12 p40 and p70 protein production. LPS±IFN-γ stimulated high levels of IL-12 p40 and p70 protein secretion from macrophages and PBMC.

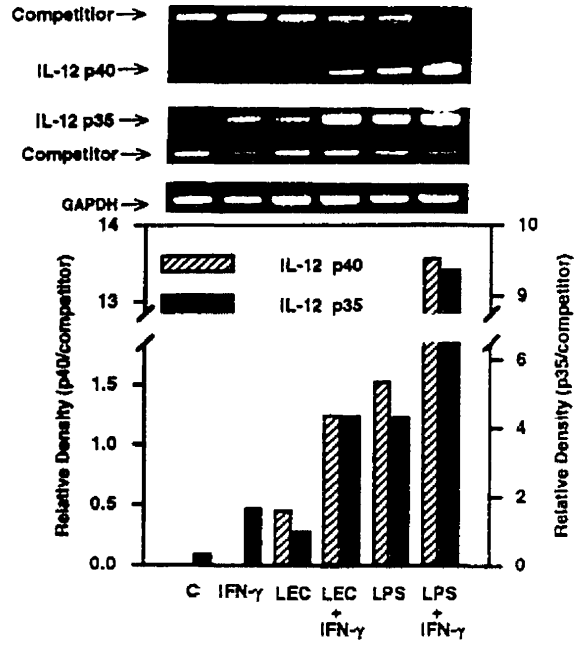

Figure 5. IFN-γ enhances IL-12 p40 and p35 mRNA expression in response to the Gal-lectin. Macrophages were stimulated with medium alone (C), IFN-γ (100 U/ml), Gal-lectin (LEC; 1 μg/ml)±IFN-γ, or LPS (1 μg/ml)±IFN-γ for 6 hr prior to isolation of total RNA and semiquantitative RT-PCR analysis. GAPDH was used as an internal control. Similar results were obtained in three independent experiments.

Table 1. IL-12 protein production from human THP-1 macrophages and PBMC.

	IL-12 production (pg/ml)					
	THP-1[a]		PBMC[b]			
			Donor 1		Donor 2	
Stimulus	p40	p70	p40	p70	p40	p70
Medium	<7[c]	<2[c]	<7	<2	<7	<2
IFN-γ	<7	<2	<7	<2	<7	<2
Gal-lectin	347±16	<2	146±15	<2	228±9	<2
Gal-lectin+IFN-γ	10,501±245	97±5	6,366±279	21±3	7,434±392	53±5
LPS	13,917±392	128±6	9,848±215	71±5	11,088±317	66±3
LPS+IFN-γ	48,988±2256	274±5	35,577±1331	132±8	41,751±1297	209±5

Macrophages were stimulated with medium alone, IFN-γ (100 U/ml), Gal-lectin (1 μg/ml)±IFN-γ or LPS (1 μg/ml)±IFN-γ and 24 hr supernatants measured for IL-12 p40 and p70 protein production by specific ELISAs.

[a] data represent mean of triplicate determinations+SEM from one representative experiment.

[b] data represent mean of triplicate determinations±SEM from each of the individual donors.

[c] lower limits of detection: IL-12 p40 =7 pg/ml, IL-12 p70=2 pg/ml.

Gal-lectin, in the presence of IFN-γ, stimulated macrophages to produce TNF-α (256±22 pg/ml) and IL-1β (160±6 pg/ml) protein as early as 6 hr post-stimulation. As TNF-α may contribute to IL-12 production in response to microbial stimuli (28), the effect of neutralizing antibodies (10 μg/ml) to TNF-α and IL-1β on Gal-lectin+IFN-γ-stimulated IL-12 p40 and p35 gene expression was examined. Neutralization of TNF-α, IL-1β, or both had no effect on IL-12 p40 or p35 mRNA expression in response to either Gal-lectin+IFN-γ or LPS+IFN-γ (Fig. 6), indicating that IL-12 mRNA expression occurred

independent of TNF-α and IL-1β.

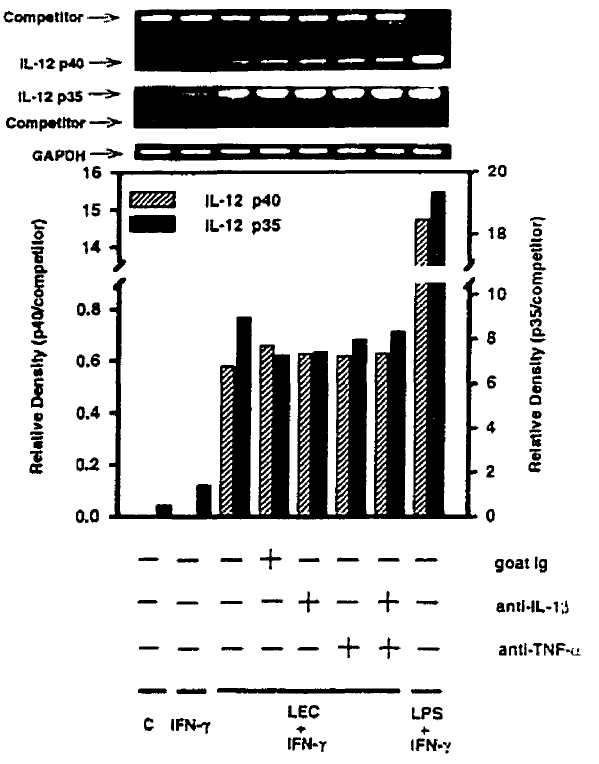

Figure 6. IL-1β and TNF-α do not contribute to IL-12 p40 or p35 mRNA expression in response to Gal-lectin+IFN-γ. Macrophages were stimulated for 6 hr with medium alone (C), IFN-γ (100 U/ml), Gal-lectin (LEC; 1 μg/ml)+IFN-γ, or LPS (1 μg/ml)+IFN-γ in the presence or absence of goat anti-human IL-1β or TNF-α antibodies or control goat Ig (all at 10 μg/ml). Total RNA was extracted and semiquantitative RT-PCR performed. GAPDH was used as an internal control. Similar results were obtained in two independent experiments.

Regulation of IL-12 p40 and p35 mRNA expression in response to Gal-lectin+IFN-γ. Because IFN-γ was required for IL-12 p70 protein production, the molecular regulation of IL-12 p40/p35 gene expression was determined in IFN-γ-primed macrophages. Stimulation with either Gal-lectin or LPS+IFN-γ, induced transcription of the IL-12 p40 gene and augmented the basal transcriptional levels of the IL-12 p35 gene (Fig. 7). As expected, IL-12 p40 was not transcribed in unstimulated cells or in response to IFN-γ alone. Basal IL-12 p35 transcription was enhanced by IFN-γ stimulation. GAPDH levels were equivalent among the +rNTP conditions, demonstrating equal

constitutive transcription in the unstimulated and stimulated nuclei.

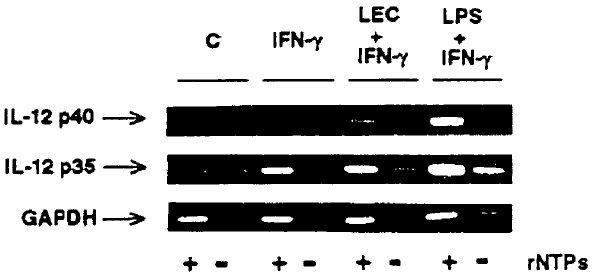

Figure 7. Gal-lectin+IFN-γ stimulates IL-12 p40 and p35 gene transcription in macrophages. Cells were stimulated for 4 hr with medium alone (C), IFN-γ (100 U/ml), Gal-lectin (LEC; 1 μg/ml)+IFN-γ, or LPS (1 μg/ml)+IFN-γ. Nuclei were isolated and transcription continued *in vitro* with (+) or without (-) rNTPs in transcription buffer. Total RNA was extracted and subjected to RT-PCR analysis. A difference between + and - rNTP conditions is an indication of active transcription. GAPDH was used as an internal control to ensure integrity of the nuclei and transcription machinery. Similar results were obtained in three independent experiments.

To determine whether *de novo* protein synthesis was required for IL-12 p40/p35 mRNA expression, the effect of the protein synthesis inhibitor cycloheximide was examined. As shown in Fig. 8, cycloheximide markedly inhibited IL-12 p40 mRNA expression, whereas IL-12 p35 was superinduced in response to Gal-lectin or LPS+IFN-γ. These data suggest that newly synthesized proteins are required for expression of IL-12 p40 mRNA, but not IL-12 p35 mRNA. IL-12 transcript stability was examined to determine if posttranscriptional regulation contributed to the relative differences in IL-12 p40/p35 mRNA expression seen in response to Gal-lectin+IFN-γ and LPS+IFN-γ (Fig. 5). However, results in Fig. 9 clearly demonstrate that Gal-lectin+IFN-γ induced IL-12 p40/p35 transcripts that were of comparable stability to those stimulated by LPS+IFN-γ.

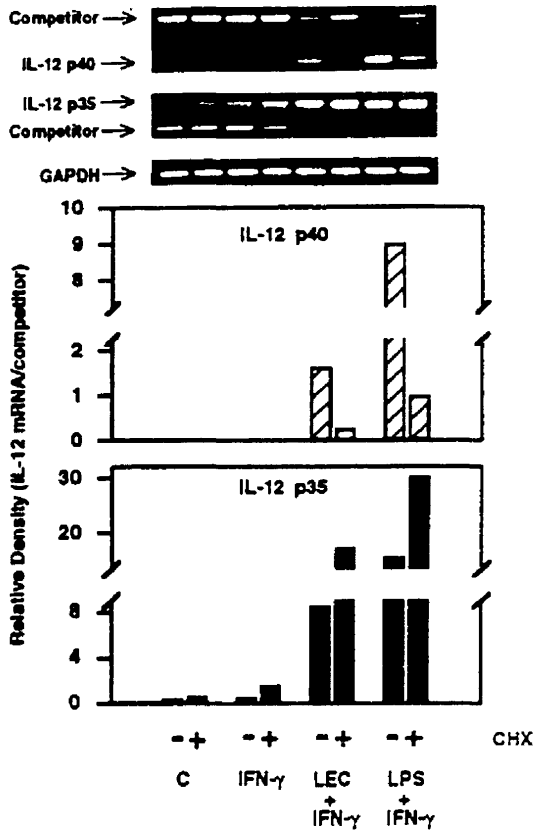

Figure 8. Cycloheximide differentially affects IL-12 p40 and p35 mRNA expression in response to Gal-lectin+IFN-γ. Macrophages were incubated for 2 hr in the presence or absence of cycloheximide (CHX; 10 μg/ml) prior to 6 hr stimulation with medium alone (C), IFN-γ (100 U/ml), Gal-lectin (LEC; 1 μg/ml)+IFN-γ, or LPS (1 μg/ml)+IFN-γ. Total RNA was extracted and subjected to semiquantitative RT-PCR analysis. GAPDH was used as an internal control. Similar results were obtained in two independent experiments.

DISCUSSION

Induction of IL-12 is crucial for developing protective immunity to pathogens susceptible to Th1-mediated immune responses (17). In addition, exogenous IL-12 can act as a natural adjuvant during vaccination with parasite components to induce protective Th1 responses (29). Evidence suggests that resistance to invasive amebiasis is associated with a Th1 response. Th1 cytokine production and mRNA expression predominate in gerbils resistant to reinfection with *E. histolytica* (3, unpublished results) and activation of

macrophages with Th1 cytokines results in potent amebicidal activity (11). In contrast, high titers of anti-amebic antibodies develop, but do not control the infection and are ineffective in preventing intestinal colonization with *E. histolytica* (30, 31).

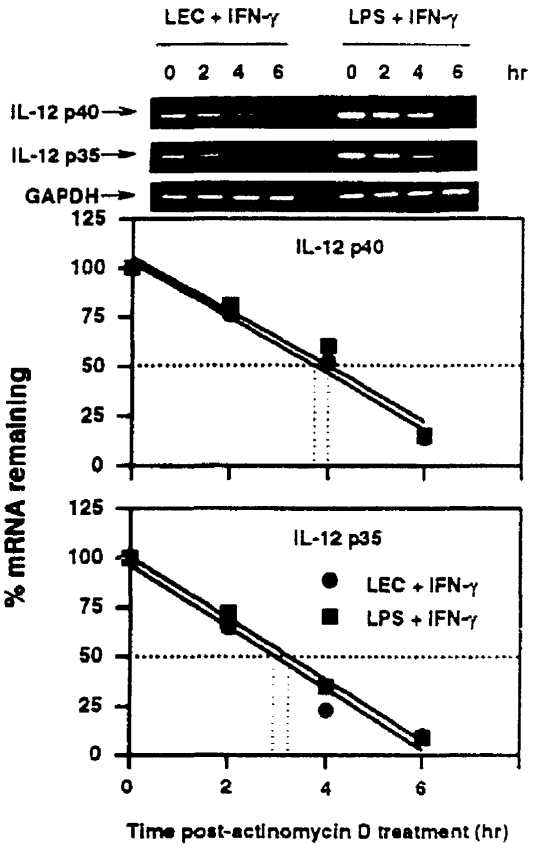

Figure 9. Comparative stability of IL-12 p40 and p35 mRNA in response to Gal-lectin+IFN-γ and LPS+IFN-γ. Macrophages were stimulated for 6 hr with Gal-lectin (LEC; 1 μg/ml)+IFN-γ (100 U/ml) or LPS (1 μg/ml)+IFN-γ. Actinomycin D (10μg/ml; GIBCO) was added and total RNA harvested at various times following actinomycin D treatment for RT-PCR analysis. Densitometry was measured by NIH image and IL-12 p40 and p35 levels normalized to GAPDH. IL-12 mRNA levels are expressed as percentage of mRNA remaining. 100% mRNA represents normalized IL-12 p40 or p35 mRNA expression in response to Gal-lectin+IFN-γ or LPS+IFN-γ at the time of actinomycin D treatment (0 hr). Similar results were obtained in two independent experiments.

A major goal in the development of a Gal-lectin-based vaccine against amebiasis is to identify the protective portions of the cysteine rich region and the immunological basis of the protective response. We hypothesized that distinct regions of the Gal-lectin induce

protective Th1 cell-mediated immunity *in vivo* through stimulation of macrophage IL-12 production. In this report, we demonstrate that the native Gal-lectin, similar to LPS, stimulates IL-12 p40/p35 mRNA expression in human macrophages. Previous mAb inhibition studies have shown that the cysteine-rich region (aa 379-1209) contains the Gal-binding epitopes for adherence to target cells (5). This region is also important antigenically. It contains B and T cell epitopes and stimulates macrophage TNF-α and NO production (5, 32, 16). Immunization with recombinant proteins containing aa 758-1134 and aa 649-1202 of the cysteine-rich region protected gerbils against intrahepatic challenge with *E. histolytica* (71% and 81% efficacy, respectively), demonstrating that the Gal-lectin shows potential for use as a subunit vaccine (9, 10). With a panel of mAbs that map to non-overlapping epitopes on the cysteine-rich region, we identified a portion of the Gal-lectin that stimulates IL-12 induction. Specific inhibition of Gal-lectin-stimulated IL-12 p40 mRNA expression by mAbs 1G7 and 8C12, but not 7F4, suggests that aa 596-998 contain the IL-12-inducing activity. Pooled human immune serum from amebiasis patients also inhibited Gal-lectin-stimulated IL-12 p40 mRNA expression, confirming that the IL-12-inducing region is immunogenic in natural infections. The fact that mAbs 1G7 and 8C12, but not 7F4, inhibit amebic adherence to target cells (5) suggests that binding of the Gal-lectin to macrophages is required to stimulate IL-12 mRNA expression. The lack of an effect of these mAbs on LPS-induced IL-12 mRNA expression suggests that the Gal-lectin and LPS stimulate IL-12 by binding to different macrophage surface molecules. The identification of the cysteine-rich region of the Gal-lectin as having both antigen-specific immunogenicity and IL-12-inducing activity further promotes its candidacy as a subunit vaccine against amebiasis. This is important as a minority of gerbils immunized with the native Gal-lectin had exacerbated infection compared to controls (8). The NH_2 terminal end of the molecule (aa 1-436) appears to be responsible for this effect (33).

We also demonstrated that cytokine regulation played a critical role in Gal-lectin-induced IL-12 mRNA expression and protein production. IFN-γ synergized with Gal-

lectin in stimulating IL-12 p40/p35 mRNA expression and IL-12 p70 protein production from macrophages and human PBMC. A requirement for IFN-γ has been demonstrated for IL-12 induction in response to other infectious organisms (34). Recent reports demonstrate that IFN-γ stimulates a transcription factor (interferon consensus sequence binding protein) that may be necessary for induction of IL-12 p40 mRNA and consequent control of infection in diseases such as toxoplasmosis (35). Although Gal-lectin plus IFN-γ also stimulated TNF-α and IL-1β production from macrophages, neutralization of these cytokines revealed that they did not act in an autocrine fashion to stimulate or augment IL-12 mRNA expression. While IL-12 itself is a potent inducer of IFN-γ, TNF-α and IL-1β may synergize with IL-12 to induce IFN-γ production from natural killer cells, a crucial early source of IFN-γ during infection (36, 37). During vaccination with the cysteine-rich region, cytokines from NK and other cells may play critical roles in the development of protective immunity.

Nuclear run-on analysis revealed that Gal-lectin+IFN-γ induced IL-12 p40 and p35 mRNA expression by stimulating transcription of the IL-12 p40 gene and augmenting IL-12 p35 gene transcription. As IL-12 p40 production generally exceeds IL-12 p70 production (by about 100 fold in this study), it has been suggested that the amount of bioactive IL-12 production is determined by the level of IL-12 p35 mRNA (38). In this respect, Gal-lectin+IFN-γ stimulation of IL-12 p35 gene expression is important in induction of bioactive IL-12 protein. Even though IL-12 p40 and p35 mRNA were co-ordinately expressed in response to stimuli in this study, the effects of the protein synthesis inhibitor cycloheximide suggest the involvement of different regulatory pathways for IL-12 p40 and p35 mRNA expression. Inhibition of protein synthesis prior to stimulation demonstrated that newly synthesized proteins are involved in IL-12 p40 mRNA expression, but not in IL-12 p35 mRNA expression. Cycloheximide pre-treatment caused superinduction of IL-12 p35 mRNA. This suggests that proteins involved in the degradation of the IL-12 p35 transcripts were inhibited. The stability of the IL-12 p40 and

p35 transcripts induced by Gal-lectin+IFN-γ was comparable to those induced by LPS+IFN-γ, even though LPS+IFN-γ induced relatively stronger expression of IL-12 mRNA. Taken together, these data suggest that IL-12 p40 and p35 genes were regulated primarily at the transcriptional level in response to Gal-lectin or LPS+IFN-γ.

In summary, we have shown that the Gal-lectin, in combination with IFN-γ, stimulates IL-12 p40/p35 gene transcription and IL-12 p70 protein production in human macrophages. mAb inhibition studies identified aa 596-998 of the cysteine-rich region as the IL-12-inducing domain. This portion of the Gal-lectin has proven subunit vaccine potential (9, 10). Our results suggest that the cysteine-rich region may have an adjuvant effect *in vivo* by inducing Th1-mediated immunity through IL-12 stimulation. These findings promote the use of this immunogenic portion of the Gal-lectin as a subunit vaccine candidate to prevent amebiasis.

ACKNOWLEDGEMENTS

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REFERENCES

1. World Health Organization (1995) *The World Health Report* (World Health Organization, Geneva).
2. Deleon, A. (1970) Pronostico tardio en el absceso hepatico amibiano. *Arch. Invest. Med.* 1, 205-206.

3. Campbell, D., & Chadee, K. (1997) Interleukin (IL)-2, IL-4 and tumor necrosis factor-α responses during *Entamoeba histolytica* liver abscess development in gerbils. *J. Infect. Dis.* **175**, 1176-1183.

4. Petri, W. A., Jr., Smith, R. D., Schlesinger, P. H., Murphy, C. F., & Ravdin, J. I. (1987) Isolation of the galactose-binding lectin which mediates the *in vitro* adherence of *Entamoeba histolytica. J. Clin. Invest.* **80**, 1238-1244.

5. Mann, B. J., Chung, C. Y., Dodson, J. M., Ashley, L. S., Braga, L. L., & Snodgrass, T. L. (1993) Neutralizing monoclonal antibody epitopes of the *Entamoeba histolytica* galactose adhesin map to the cysteine-rich extracellular domain of the 170-kilodalton subunit. *Infect. Immun.* **61**, 1772-1778.

6. Chadee, K., Petri, W. A., Jr., Innes, D. J., & Ravdin, J. I. (1987) Rat and human colonic mucins bind to and inhibit the adherence lectin of *Entamoeba histolytica*. *J. Clin. Invest.* **80**, 1245-1254.

7. Ravdin, J. I., & Guerrant, R. A. (1981) The role of adherence in cytopathogenic mechanism of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes. *J. Clin. Invest.* **68**, 1305-1313.

8. Petri, W. A., Jr., & Ravdin, J. I. (1991) Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of *Entamoeba histolytica. Infect. Immun.* **59**, 97-101.

9. Soong, C. J. G., Kain, K. C., Abd-Alla, M., Jackson, T. F. G. H., & Ravdin, J. I. (1995) A recombinant cysteine-rich section of the *Entamoeba histolytica* galactose-inhibitable lectin is efficacious as a subunit vaccine in the gerbil model of amebic liver abscess. *J. Infect. Dis.* **171**, 645-651.

10. Zhang, T., & Stanley, S. L., Jr. (1994) Protection of gerbils from amebic liver abscess by immunization with a recombinant protein derived from the 170-kilodalton surface adhesin of *Entamoeba histolytica. Infect. Immun.* **62**, 2605-2608.

11. Lin, J. -Y., Seguin, R., Keller, K., & Chadee, K. (1994) Tumor necrosis factor alpha augments nitric oxide-dependent macrophage cytotoxicity against *Entamoeba histolytica* by enhanced expression of the nitric oxide synthase gene. *Infect. Immun.* **62**, 1534-1541.

12. Séguin, R., Mann, B. J., Keller, K., & Chadee K. (1997) The tumor necrosis factor alpha-stimulating region of galactose-inhibitable lectin of *Entamoeba histolytica* activates gamma interferon-primed macrophages for amebicidal activity mediated by nitric oxide. *Infect. Immun.* **65**, 2522-2527.

13. Schain, D. C., Salata, R. A., & Ravdin, J. I. (1992) Human T lymphocyte proliferation, lymphokine production, and amebicidal activity elicited by the galactose-inhibitable adherence protein of *Entamoeba histolytica. Infect. Immun.* **60**, 2143-2146.

14. Salata, R. A., Martinez-Palomo, A., Murray, H. W., Conales, L., Trevino, N., Segovia, E., Murphy C. F., & Ravdin, J. I. (1986) Patients treated for amebic liver abscess develop cell-mediated immune responses effective *in vitro* against *Entamoeba histolytica. J. Immunol.* **136**, 2633-2639.

15. Schain, D. C., Salata, R. A., & Ravdin, J. I. (1995) Development of amebicidal cell-mediated immunity in gerbils (*Meriones unguiculatus*) immunized with the galactose-inhibitable adherence lectin of *Entamoeba histolytica. J. Parasitol.* **81**, 563-568.

16. Séguin, R., Mann, B. J., Keller, K., & Chadee K. (1995) Identification of the galactose-adherence lectin epitopes of *Entamoeba histolytica* that stimulate tumor necrosis factor-α production by macrophages. *Proc. Natl. Acad. Sci. USA* **92**, 12175-12179.

17. Romani, L., Puccetti, P., & Bistoni, F. (1997) Interleukin-12 in infectious diseases. *Clin. Microbiol. Rev.* **10**, 611-636.

18. Chadee, K., & Meerovitch, E. (1984) The pathogenesis of experimentally induced

amebic liver abscess in the gerbil (*Meriones unguiculatus*). *Am. J. Pathol.* **117**, 71-80.

19. Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
20. Kubin, M., Chow, J. M., & Trinchieri, G. (1993) Differential regulation of interleukin-12 (IL-12), tumor necrosis factor-α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. *Blood* 83, 1847-1855.
21. Petri, W. A., Jr., Snodgrass, T. L., Jackson, T. F. H. G., Gathiram, V., Simjee, A. E., Chadee, K., & Chapman, M. D. (1990) Monoclonal antibodies directed against the galactose-binding lectin of *Entamoeba histolytica* enhance adherence. *J. Immunol.* **144**, 4803-4809.
22. de Waal Malefyt, R., Figdor, C. G., Huijbens, R., Mohan-Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G., & de Vries, J. E. (1993) Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. *J. Immunol.* 151, 6370-6381.
23. Yu, Y., & Chadee, K. (1997) *Entamoeba histolytica* stimulates interleukin 8 from human colonic epithelial cells without parasite-enterocyte contact. *Gastroenterology* **112**, 1536-1547.
24. Gebhardt, A., Peter, A., Gerding, D., & Niendirf, A. (1994) Rapid quantitation of mRNA species in ethidium bromide-stained gels of competitive RT-PCR products. *J. Lipid Res.* **35**, 976-981.
25. Roberts, F. A., Richardson, G. J., & Michalek S. M. (1997) Effects of *Porphyromonas gingivalis* and *Escherichia coli* lipopolysaccharides on mononuclear phagocytes. *Infect. Immun.* **65**, 3248-3254.
26. Rolfe, F. G., & Sewell, W. A. (1997) Analysis of human interleukin-5 gene

transcription by a novel nuclear run-on method based on the polymerase chain reaction. *J. Immunol. Methods* **202**, 143-151.

27. Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. L., Dzialo, R. & Trinchieri, G. (1996) The interleukin-12 p40 gene promoter is primed by interferon-γ in monocytic cells. *J. Exp. Med.* **183**, 147-157.
28. Zhan, Y., & Cheers, C. (1998) Control of IL-12 and IFN-γ production in response to live or dead bacteria by TNF and other factors. *J. Immunol.* **161**, 1447-1453.
29. Afonso, L. C. C., Scharton, T. M., Vieira, L. Q., Wysocka, M., Trinchieri, G., & Scott, P. (1994) IL-12 functions as an effective adjuvant in a vaccine against *Leishmania major* by directing the development of leishmanial specific $CD4^+$ Th1 cells. *Science* **263**, 235-237.
30. Katzenstein, D., Rickerson, V., & Braude, A. (1982) New concepts of amebic liver abscess derived from hepatic imaging, serodiagnosis, and hepatic enzymes in 67 consecutive cases in San Diego. *Medicine* **61**, 237-246.
31. Braga, L. L., Mendonca, Y., Paiva, C. A., Sales, A., Cavalcante, A. L. M., & Mann, B. J. (1998) Seropositivity for and intestinal colonization with *Entamoeba histolytica* and *Entamoeba dispar* in individuals in northeastern Brazil. *J. Clin. Microbiol.* **36**, 3044-3045.
32. Velazquez, C., Valette, I., Cruz, M., Labra, M.- L., Montes, J., Stanley, S. L., Jr., & Calderon, J. (1995) Identification of Immunogenic epitopes of the 170 k-Da subunit adhesin of *Entamoeba histolytica* in patients with invasive amebiasis. *J. Euk. Microbiol.* **42**, 636-641.
33. Lotter, H., Zhang, T., Seydel, K. B., Stanley, S. L., Jr., & Tannich, E. (1997) Identification of an epitope on the *Entamoeba histolytica* 170-kD lectin conferring antibody-mediated protection against invasive amebiasis. *J. Exp. Med.* **185**, 1793-1801.
34. Flesch, I. E. A., Hess, J. H., Huang, S., Aguet, M., Rothe, J., Bluethmann, H., &

Kaufmann, S. H. E. (1995) Early interleukin-12 production by macrophages in response to mycobacterial infection depends on interferon-γ and tumor necrosis factor-α. *J. Exp. Med.* **181**, 1615-1621.

35. Scharton-Kersten, T., Contursi, C., Masumi, A., Sher, A., & Ozato, K. (1997) Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin-12 p40 induction. *J. Exp. Med.* **186**, 1523-1524.
36. Hunter, C. A., Chizzonite, R., & Remington, J. S. (1995) IL-1β is required for IL-12 to induce production of IFN-γ by NK cells. A role for IL-1β in the T cell-independent mechanism of resistance against intracellular pathogens. *J. Immunol.* **155**, 4347-4354.
37. Tripp, C. S., Wolf, S. F., & Unanue, E. R. (1993) Interleukin-12 and tumor necrosis factor-α are costimulators of interferon-γ production by natural killer cells in severe combined immunodeficiency with listeriosis and interleukin-10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA* **90**, 3725-3729.
38. Snijders, A., Hilkens, C. M. U., van der Pouw Kraan, T. C. T. M., Engel, M., Aarden, L. A., & Kapsenberg, M. L. (1996) Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *J. Immunol.* **156**, 1207-1212.

SECTION III: GENERAL DISCUSSION

The interaction between *Entamoeba histolytica* and the immune system is complex. While immune responses clearly develop, amoebae are able to modulate a variety of macrophage and T cell functions (1-4). The effect of this is to down-regulate cellular defences and favour the survival of *E. histolytica* in host tissues. Amoebic infections are sometimes difficult to diagnose or are mis-diagnosed because of the generalized symptoms associated with infection (e.g. diarrhoea at the gut level). Therefore, disease and associated tissue pathology may be well established before chemotherapy begins (5). The development of a suitable vaccine to prevent infection and its deleterious consequences should be a priority. Experimental vaccine studies in animals demonstrate the feasibility of this approach. Immunization with crude amoebic proteins or defined antigens results in high levels of protection against *E. histolytica* challenge (6-9).

To develop an effective preventative vaccine for use in humans, we must first identify the mechanisms involved in resistance to *E. histolytica*. Unfortunately, characterization of antigen-specific responses in amoebiasis has lagged behind that for other protozoal infections. This is principally because of the lack of an immunologically intact murine model for *E. histolytica*. The Mongolian gerbil serves as a reliable model of amoebic liver abscess but suffers from the lack of commercially available immunological reagents for study (10). SCID mice develop transient amoebic liver infections but the animals are deficient in T and B lymphocytes (11).

We hypothesized that activation of the appropriate T cell cytokine response (Th1 vs. Th2) would be key in orchestrating host defence against *E. histolytica*. The data we have provide indirect evidence that Th1/cellular immune responses are linked to control of amoebic infections. T cells from recovered amoebiasis patients produce Th1 cytokines IFN-γ and IL-2 upon *in vitro* stimulation with the Gal-lectin of*E. histolytica* (12).

Similarly, spleen cells from Gal-lectin-immunized gerbils demonstrate IFN-γ-like and IL-2-like activity *in vitro* (13). Pregnant women experience more severe *E. histolytica* infection compared to other women (14, 15). A successful pregnancy is characterized by a Th2 environment in the mother and this may exacerbate amoebiasis in infected women (16). Studies of *Leishmania major* infection, which is controlled by a Th1 response, indicate that pregnancy impairs resistance of C57BL/6 mice (17). Conversely, an established Th1 response to *L. major* jeopardizes concurrent pregnancy (18). A hallmark of Th1 responses is activation of macrophage anti-microbial immunity in response to Th1 cytokines (19). Activation of human and murine macrophages with IFN-γ and TNF-α results in amoebicidal activity, demonstrating a potent Th1 effector mechanism (20, 21).

To determine the balance of Th1 and Th2 responses occurring during amoebic liver abscess development, we quantified IL-2 (Th1 marker) and IL-4 (Th2 marker) cytokine production from gerbil hepatic lymph node and spleen cells in a kinetic study (Manuscript I). Onset of infection and growth of the amoebic abscess (days 5-10) was accompanied by a Th0 response. However, both cytokine production and T cell proliferative responses were profoundly suppressed at day 20 post-infection. At days 30-60, proliferative responses and IL-2 production recovered. A previous study of amoebiasis patients demonstrated that serum taken from these patients suppressed both *in vitro* proliferative responses and IFN-γ production of T cells from recovered patients (22). As the mechanism(s) of suppression was not identified in that study, we examined the effect of serum collected from infected gerbils on proliferative responses of spleen cells from naïve gerbils (Manuscript II). The suppressive effect was restricted to serum collected at day 20 post infection and was specific for Concanavalin A-stimulated T cell proliferation. IL-2, or a lack thereof, was central to the serum-mediated suppression. Reduced proliferation was associated with lower IL-2 levels in cell supernatants and was reversed by exogenous IL-2. T cell proliferation may be suppressed by inhibited IL-2 production, decreased cell surface expression of IL-2 receptors (IL-2R), production of soluble IL-2R (sIL-2R) or a

combination of these effects (23-25). In our study, the ability of spleen cells to respond to exogenously added IL-2 suggests that IL-2R expression was intact and that sIL-2R production was not a factor. Therefore, the reduced IL-2 levels indicate a defect associated with IL-2 production. As the suppressive effect of human patient serum decreased with time after chemotherapy (22), an analogous phenomenon may be occurring in human amoebiasis.

A number of different mechanisms operate to inhibit IL-2 production and T cell proliferation. Production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) was demonstrated to suppress T cell proliferation in experimental *Plasmodium chabaudi* infection (26). In murine toxoplasmosis, both NO-dependent and IL-10-dependent mechanisms inhibit T cell proliferative responses and IL-2 production (27). In our study, NO and PGE_2 inhibitors had no effect on the suppression mediated by day 20 serum *in vitro*. In addition, NO production is suppressed during amoebic liver abscess development (4). PGE_2 levels are, however, elevated during amoebic infection and may contribute to suppressed T cell responses *in vivo* by inhibiting the antigen-presentation function of macrophages (28, 29). A role for IL-10 in the serum-mediated suppression in our study is a possibility. A 220-kDa amoebic lectin molecule induces IL-10 production *in vivo* (30). However, IL-10 may be suppressed along with IL-4 at day 20 of amoebic liver abscess development in gerbils (Manuscript I). It is remarkable that the suppression of T cell responses is so transient in these animals. Day 30 serum does not inhibit T cell proliferation and *ex vivo* IL-2 production rises dramatically at days 30 and 60 of infection (Manuscripts I & II). Further investigation is required to determine the factors involved in this recovery.

At days 30-60 of amoebic liver abscess development in gerbils, lymphoid cell IL-2 production increased while IL-4 levels remained low (Manuscript I). In other words, a switch from a Th0 response (days 5-10) to a Th1 response occurred. Significantly, this coincided with control of the liver abscess in gerbils. In addition, drug-cure of gerbils at

day 20 resulted in resistance to reinfection and demonstrated Th1 responses. These data corroborate our hypothesis that a Th1 response is protective against *E. histolytica*. How the switch to a Th1 response occurs is not clear at this stage. Combined IL-12 and drug-treatment of *Leishmania major*-infected mice stimulated switch to a Th1 response in these animals (31). This prompted the authors of the study to suggest that decreasing antigen levels may have facilitated the switch. Also in *L. major*-infected mice, injection of CpG-containing synthetic oligodeoxynucleotides reverted an established Th2 response, demonstrating the elasticity of Th responses (32). In our infected gerbils, perhaps the suppression of IL-4 production at day 20 allowed the outgrowth of a Th1 response. There is also evidence to suggest that a Th2 response may, under certain circumstances, stimulate the subsequent development of a Th1 response. Eosinophils may play a role in this scenario. Human eosinophils produce biologically active IL-12 in response to the Th2 cytokines IL-4 and granulocyte-macrophage colony-stimulating factor (33). Alternatively, production of PGE_2 during *E. histolytica* infection may contribute to the switch to a Th1 response (28). PGE_2 generally inhibits LPS-stimulated macrophage IL-12 production (34). However, PGE_2 alone has been shown to stimulate IL-12 release from human dendritic cells and can synergize with TNF-α in this activity (35). The Th cytokine responses in *E. histolytica*-infected humans remain to be analysed. However, as stated above, there is indirect evidence that drug-cured and resistant human patients develop Th1-mediated immunity (12).

The most important factor promoting differentiation of antigen-stimulated T cells into the Th1 phenotype is IL-12 (36). The potency of this effect has been demonstrated in studies where vaccination along with exogenous IL-12 has an adjuvant-like effect in inducing a Th1 response to normally Th2-stimulating or non-immunogenic antigens (37, 38). Thus, the ability of an antigen to directly stimulate IL-12 production would be of benefit in vaccination against diseases controlled by Th1 responses. In this context, we examined the ability of the amoebic Gal-lectin to stimulate IL-12 production from human

macrophages (Manuscript III). Gerbils vaccinated with native Gal-lectin or recombinant portions of the cysteine-rich region of its 170-kDa subunit are protected against *E. histolytica* infection (8, 9, 39, 40). However, the nature of the protective immune response in these studies and the means by which it is generated remained to be clarified. In Manuscript I we showed that a Th1 response was associated with resistance to *E. histolytica*. In Manuscript III we demonstrate that Gal-lectin stimulated IL-12 p40 and p35 mRNA expression in human macrophages. In combination with IFN-γ, Gal-lectin stimulated transcription of the IL-12 genes and production of bioactive IL-12 p70 protein. In essence, this study identifies a mechanism by which vaccination with the Gal-lectin may act to induce protective Th1 responses *in vivo*. Induction of IL-12 production during immunization with the Gal-lectin would promote the development of protective Th1 immunity against *E. histolytica*. Preliminary results indicate that gerbils immunized with the native Gal-lectin plus Freund's adjuvant produce a prompt Th1 response and a 10-fold increase in lymphocyte proliferation in response to Gal-lectin stimulation (Gaucher and Chadee, personal communication).

Previous work in our laboratory has shown that mAb affinity-purified Gal-lectin stimulates TNF-α mRNA expression and protein production from murine macrophages (41). We were interested in determining whether proinflammatory cytokines contributed to IL-12 induction in human macrophages. In addition to IL-12, Gal-lectin and IFN-γ stimulated both TNF-α and IL-1ß production from human macrophages (Manuscript III). However, addition of neutralizing antibodies to TNF-α and IL-1ß revealed that neither of these proinflammatory cytokines played a role in inducing IL-12 mRNA expression in our system. The TNF-α and IL-1ß produced may be more important in augmenting the effects of IL-12, such as IFN-γ production from natural killer (NK) cells (42, 43). Interestingly, Gal-lectin and IFN-γ stimulate murine macrophages to produce NO (44). One group has shown that NO synthase inhibitors suppress IL-12 p40 mRNA expression, whereas NO generating compounds induce IL-12 p40 mRNA expression in murine macrophages (45).

This suggests a possible role for NO in Gal-lectin and IFN-γ-stimulated IL-12 production.

A long term goal of our laboratory is to develop a Gal-lectin-based subunit vaccine. Immunization of gerbils with the native Gal-lectin in Freund's adjuvant protected 67% of the animals from amoebic challenge (40). However, the gerbils that were not protected experienced exacerbated infections. A later study identified aa 1-436 of the Gal-lectin 170-kDa subunit as an immunosuppressive domain (39). In contrast, immunization of gerbils with recombinant portions of the cysteine-rich region of the 170-kDa subunit (aa 649-1202 and 758-1134) in adjuvant resulted in 71-81% protection with no adverse effects in the minority of unprotected animals (8, 9). The cysteine-rich region also contains multiple epitopes recognized by human T cells (46). Clearly, this region of the Gal-lectin is a strong candidate for use as a subunit vaccine.

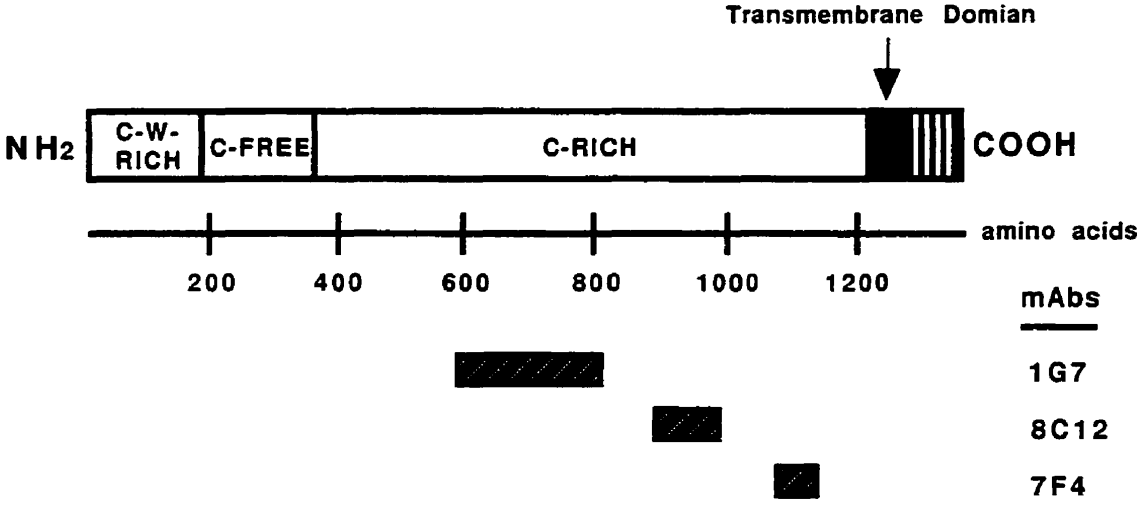

Figure 1. Localization of the epitopes recognized by mAbs reacting with the Gal-lectin 170-kDa subunit. The mapped epitopes are indicated by the hatched boxes below the 170-kDa subunit drawing. C= cysteine, W=tryptophan. This diagram has been adapted from reference 47.

The identification of the cysteine-rich region as the IL-12-inducing domain would further promote its vaccine potential. To this end, we examined the effects of a panel of anti-cysteine-rich region mAbs on Gal-lectin-induced IL-12 mRNA expression. The three mAbs we used mapped to non-overlapping epitopes within the cysteine-rich region (Figure

1). mAbs 1G7 (aa 596-818) and 8C12 (aa 895-998) both markedly inhibited IL-12 p40 mRNA expression in response to the Gal-lectin but not LPS (Manuscript III). In contrast, mAb 7F4 (aa 1082-1138) had no effect on Gal-lectin-stimulated IL-12 p40 mRNA expression. These results implicate aa 596-998 in the induction of IL-12 from macrophages. It may be possible to identify a smaller portion within this sequence that stimulates IL-12 production. However, a short peptide sequence would have fewer T cell epitopes and may not be recognized by the immune system of all individuals (39). The IL-12-inducing domain (aa 596-998) is present in the cysteine-rich regions already shown to be protective in gerbils (8, 9). In addition, this part of the cysteine-rich region is also responsible for macrophage TNF-α and NO production in response to Gal-lectin and IFN-γ (41, 44). Taken together, these findings strongly support the inclusion of aa 596-998 in a subunit vaccine to induce IL-12 and Th1-mediated immunity to *E. histolytica.*

DNA vectors and attenuated *Salmonella* spp. show great potential as delivery systems for vaccines against parasitic infections (48, 49). Successful immunization of animals with the Gal-lectin IL-12-inducing domain using one of these systems would be the next logical step towards the eventual development of a vaccine for use in humans. The available animal models for amoebiasis have limitations, though. Gerbils could be used to assess protection following vaccination. However, SCID mice, which have intact innate immune systems, may be more useful in demonstrating the potential for Gal-lectin to stimulate IL-12 production *in vivo.*

References

1. Salata R.A., Martinez-Palomo A., Murray H.W., Conales L., Trevino N., Segovia E., Murphy C.F., and Ravdin J.I. (1986) Patients treated for amebic liver abscess develop cell-mediated immune responses effective in vitro against *Entamoeba histolytica. J. Immunol.* 136: 2633-2639.

2. Denis M., and Chadee K. (1988) *In vitro* and *in vivo* studies of macrophage

functions in amebiasis. *Infect. Immun.* 56: 3126-3131.

3. Gill N.J., Ganguly N.K., Dilawari J.B., and Mahajan R.C. (1985) Lymphocyte subpopulations transformation studies in an experimental model of intestinal and hepatic amoebiasis. *Trans. R. Soc.Trop. Med.Hyg.* 79: 618-623.

4. Wang W., Keller K., and Chadee K. (1994) *Entamoeba histolytica* modulates the nitric oxide synthase gene and nitric oxide production by macrophages for cytotoxoicity against amoebae and tumour cells. *Immunology* 83: 601-610.

5. Perez-Tamayo R. (1986) Pathology of amebiasis. In *Amebiasis*. (Martinez-Palomo A., ed.). Elsevier, New York pp. 45-94.

6. Ghadirian E., Meerovitch E., and Hartman D-P. (1980) Protection against amebic liver abscess in hamsters by means of immunization with amebic antigen and some of its fractions. *Am.J. Trop. Med. Hyg.* 29: 779-784.

7. Soong C-J. G., Torian B.E., Abd-Alla M.D., Jackson T.F.H.G., Gatharim V., and Ravdin J.I. (1995) Protection of gerbils from amebic liver abscess by immunization with recombinant *Entamoeba histolytica* 29-Kilodalton antigen. *Infect. Immun.* 63: 472-477.

8. Zhang T., Cieslak P.R., and Stanley S.L. Jr. (1994) Protection of gerbils from amebic liver abscess by immunization with a recombinant *Entamoeba histolytica* antigen. *Infect. Immun.* 62: 1166-1170.

9. Soong C-J. G., Kain K.C., Abd-Alla M., Jackson T.F.H.G., and Ravdin J.I. (1995) A recombinant cysteine-rich section of the *Entamoeba histolytica* galactose-inhibitable lectin is efficacious as a subunit vaccine in the gerbil model of amebic liver abscess. *J. Infect. Dis.* 171: 645-651.

10. Chadee K., and Meerovitch E. (1984) The Mongolian gerbil (*Meriones unguiculatus*) as an experimental host for *Entamoeba histolytica. Am. J. Trop. Med. Hyg.* 33: 47-54.

11. Cieslak P.R., Virgin H.W., and Stanley S.L. Jr. (1992) A severe combined

immunodeficient (SCID) mouse model for infection with *Entamoeba histolytica. J. Exp. Med.* 176: 1605-1609.

12. Schain D.C., Salata R.A., and Ravdin J.I. (1992) Human T-lymphocyte proliferation, lymphokine production, and amebicidal activity elicited by the galactose-inhibitable adherence protein of *Entamoeba histolytica. Infect. Immun.* 60: 2143-2146.

13. Schain D.C., Salata R.A., and Ravdin J.I. (1995) Development of amebicidal cell-mediated immunity in gerbils (*Meriones unguiculatus*) immunized with the galactose-inhibitable adherence lectin of *Entamoeba histolytica. J. Parasitol.* 81: 563-568.

14. Armon P.J. (1978) Amoebiasis in pregnancy and the puerperium. *B. J. Ob. Gynecol.* 85: 264-269.

15. Lyche K.D., and Jensen W.A. (1997) Pleuropulmonary amebiasis. *Sem. Resp. Infect.* 12: 106-112.

16. Raghupathy R. (1997) Th1-type immunity is incompatible with successful pregnancy. *Immunol. Today* 18: 478-482.

17. Krishnan L., Guilbert L.J., Russell A.S., Wegmann T.G., Mosmann T.R., and Belosevic M. (1996) Pregnancy impairs resistance of C57BL/6 mice to *Leishmania major* infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. *J. Immunol.* 156: 644-652.

18. Krishnan L., Guilbert L.J., Wegman T.G., Belosevic M., and Mosmann T.R. (1996) T helper 1 response against *Leishmania major* in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. *J. Immunol.* 156: 653-662.

19. Mosmann T.R., and Sad S. (1998) The expanding universe of T cell subsets: Th1, Th2 and more. *Immunol. Today* 17: 138-146.

20. Salata R.A., Murray H.W., Rubin B.Y., and Ravdin J.I. (1987) The role of gamma

interferon in the generation of human macrophages cytotoxic for *Entamoeba histolytica* trophozoites. *Am. J. Trop. Med. Hyg.* 37: 72-78.

21. Denis M., and Chadee K. (1989) Cytokine activation of murine macrophages for *in vitro* killing of *Entamoeba histolytica* trophozoites. *Infect. Immun.* 57: 1750-1756.

22. Salata R.A., Martinez-Palomo A., Canales L., Murray H.W., Trevino N., and Ravdin J.I. (1990) Suppression of T-lymphocyte responses to *Entamoeba histolytica* antigen by immune sera. *Infect. Immun.* 58: 3941-3946.

23. Haque S., Khan I., Haque A., and Kasper L. (1994) Impairment of the cellular immune response in acute murine toxoplasmosis: Regulation of interleukin-2 production and macrophage-mediated inhibitory effects. *Infect. Immun.* 62: 2908-2916.

24. Majumder S., and Kierszenbaum F., (1996) Mechanisms of *Trypanosoma cruzi*-induced down-regulation of lymphocyte function. *J. Immunol.* 156: 3866-3874.

25. Hviid L., Theander T.G., Abu-Zeid Y.A., Abdulhadi N.H., Jakobsen P.H., Saeed B.O., Jepsen S., Bayoumi R.A.L., and Jensen J.B. (1991) Loss of cellular immune reactivity during acute *Plasmodium falciparum* malaria. *FEMS Microbiol. Immunol.* 76: 219-227.

26. Ahvazi B.C., Jacobs P., and Stevenson M.M. (1995) Role of macrophage-derived nitric oxide in suppression of lymphocyte proliferation during blood-stage malaria. *J. Leukoc. Biol.* 58: 23-31.

27. Candolfi E., Hunter C.A., and Remington J.S. (1995) Roles of gamma interferon and other cytokines in suppression of the spleen cell proliferative response to Concanavalin A and *Toxoplasma* antigen during acute toxoplasmosis. *Infect. Immun.* 63: 751-756.

28. Wang W., and Chadee K. (1992) *Entamoeba histolytica* alters arachidonic acid metabolism in macrophages *in vitro* and *in vivo*. *Immunology* 76: 242-250.

29. Wang W., and Chadee K. (1995) *Entamoeba histolytica* suppresses gamma

interferon-induced macrophage class II major histocompatibility complex Ia molecule and I-Aß mRNA expression by a prostaglandin E_2-dependent mechanism. *Infect. Immun.* 63: 1089-1094.

30. Talamás-Rohana P., Schlie-Guzman M.A., Hernández-Ramírez V.I., and Rosales-Encina J.L. (1995) T-cell suppression and selective in vivo activation of TH2 subpopulation by the *Entamoeba histolytica* 220-kilodalton lectin. *Infect. Immun.* 63: 3953-3958.

31. Nabors G.S., Afonso L.C.C., Farrell J. P., and Scott P. (1995) Switch from a type 2 to a type 1 T helper cell response and cure of established *Leishmania major* infection in mice is induced by combined therapy with interleukin-12 and Pentostam. *Proc. Natl. Acad. Sci. USA* 92: 3142-3146.

32. Zimmermann S., Egeter O., Hausmann S., Lipford G.B., Röcken M., Wagner H., and Heeg K. (1998) Cutting Edge: CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine Leishmaniasis. *J. Immunol.* 160: 3627-3630.

33. Grewe M., Czech W., Morita A., Werfel T., Klammer M., Kapp A., Ruzicka T., Schöpf E., and Krutmann J. (1998) Human eosinophils produce biologically active IL-12: Implications for control of T cell responses. *J. Immunol.* 161: 415-420.

34. van der Pouw Kraan T.C.T.M., Boeije L.C.M., Smeenk R.J.T., Wijdenes J., and Aarden L.A. (1995) Prostaglandin-E2 is a potent inhibitor of human interleukin-12 production. *J. Exp. Med.* 181: 775-779.

35. Rieser C., Böck G., Klocker H., Bartsch G., and Thurnher M. (1997) Prostaglandin E2 and tumor necrosis factor α cooperate to activate human dendritic cells: synergistic activation of interleukin-12 production. *J. Immunol.* 186: 1603-1608.

36. Romani L., Puccetti P., and Bistoni F. (1997) Interleukin-12 in infectious diseases. *Clin. Microbiol. Rev.* 10: 611-636.

37. Miller M.A., Skeen M.J., and Ziegler H.K. (1997) A synthetic peptide administered with IL-12 elicits immunity to *Listeria monocytogenes. J. Immunol.* 159: 3675-3679.

38. Silva R.A., Pais T.F., Appelberg R. (1998) Evaluation of IL-12 in immunotherapy and vaccine design in experimental *Mycobacterium avium* infections. *J. Immunol.* 161: 5578-5585.

39. Lotter H., Zhang T., Seydel K.B., Stanley S.L. Jr., and Tannich E. (1997) Identification of an epitope on the *Entamoeba histolytica* 170-kDa lectin conferring antibody-mediated protection against invasive amebiasis. *J. Exp. Med.* 185: 1793-1801.

40. Petri W.A. Jr., and Ravdin J.I. (1991) Protection of gerbils from amebic liver abscess by immunization with the galactose-specific adherence lectin of *Entamoeba histoyltica. Infect. Immun.* 59: 97-101.

41. Séguin R., Mann B.J., Keller K., and Chadee K. (1995) Identification of the galactose-adherence lectin epitopes of *Entamoeba histolytica* that stimulate tumor necrosis factor-alpha production by macrophages. *Proc. Natl. Acad. Sci. USA* 92: 12175-12179.

42. Hunter C.A., Chizzonite R., Remington J.S. (1995) IL-1ß is required for IL-12 to induce production of IFN-γ by NK cells. *J. Immunol.* 155: 4347-4354.

43. Tripp C.S., Wolf S.F., and Unanue E.R. (1993) Interleukin-12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency with listeriosis and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA* 90: 3725-3729.

44. Séguin R., Mann B.J., Keller K., and Chadee K. (1997) The tumor necrosis factor alpha-stimulating region of galactose-inhibitable lectin of *Entamoeba histolytica* activates gamma interferon-primed macrophages for amebicidal activity by nitric oxide. *Infect. Immun.* 65: 2522-2527.

45. Rothe H., Hartmann B., Geerlings P., and Kolb H. (1996) Interleukin-12 gene-expression of macrophages is regulated by nitric oxide. *Biochem. Biophys. Res. Commun.* 224: 159-163.

46. Velazquez C., Valette I., Cruz M., Labra M-L., Montes J., Stanley S.L. Jr., and

Calderon J. (1995) Identification of immunogenic epitopes of the 170-kDa subunit adhesin of *Entamoeba histolytica* in patients with invasive amebiasis. *J. Euk. Microbiol.* 42: 636-641.

47. Mann B.J., Chung C.Y., Dodson J.M., Ashley L.S., Braga L.L., and Snodgrass T.L. (1993) Neutralizing monoclonal antibody epitopes of the *Entamoeba histolytica* Galactose adhesin map to the cysteine-rich extracellular domain of the 170-kilodalton subunit. *Infect. Immun.* 61: 1772-1778.

48. Kalinna B.H. (1997) DNA vaccines for parasitic infections. *Immunol. Cell Biol.* 75: 370-375.

49. Chatfield S.W., Roberts M., Dougan G., Hormaeche C, and Khan C.M. (1995) The development of oral vaccines against parasitic diseases utilizing live attenuated *Salmonella. Parasitology* 110 suppl: s17-s25.