Mucosal Defense Mechanisms against Amebic Colitis

Zannatun Noor
Dhaka, Bangladesh

M.S., Biochemistry and Molecular Biology, University of Dhaka, 2006
B. S. with Honours, Biochemistry and Molecular Biology, University of Dhaka, 2005

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Abstract

Entamoeba histolytica is an enteric parasite, the causative agent of amebiasis, and a significant cause of diarrhea in infants in low income countries. *E. histolytica* adheres to host cells by a parasite Gal/GalNAc lectin and disrupts the mucosal barrier via a unique process named amebic trogocytosis, penetrating underlying tissue and destroying cells. Host responses at the site of infection are critical for resistance to the ameba.

` IL-25 is a cytokine that is produced by intestinal epithelial cells in response to the gut microbiome and is known to help maintain gut barrier function in colitis due to *Clostridium difficile*. We discovered that IL-25 expression is decreased in colon biopsy tissue from patients with amebic colitis. We also observed decreased IL-25 in the cecum during *E. histolytica* infection in the mouse model of amebic colitis. We hypothesized that IL-25 protects the intestinal epithelium from invasion by *E. histolytica*. To test this hypothesis we administrated recombinant IL-25 in mice infected with ameba. We found that rIL-25 treated mice had a significantly lower infection rate as measured via culture, ELISA and quantitative qPCR. Histologically, there was significantly less epithelial disruption in rIL-25 treated mice. We further found that IL-25 mediated protection was eosinophil dependent. When eosinophils were depleted with anti-Siglec-F, IL-25 administration was no longer protective.

In order to understand how eosinophils protect, we tested for the impact of IL-25 on TNFα. We found that patients with amebic colitis had a greater amount of TNFα in the intestine as measured by immunohistochemistry. rIL-25 administration suppressed TNFα induction in *E. histolytica* challenged mice. Depletion of TNFα with monoclonal antibodies in mice resulted in resistance to ameba infection. Therefore, our research

suggests that IL-25 may provide protection from amebiasis via two potential pathways, induction of protective Th2 responses, via eosinophils, and by suppression of inflammatory TNFα during infection.

We also considered whether antimicrobial peptide production was a mechanism of IL-25 mediated protection. Antimicrobial peptides play a crucial role in allowing epithelial cells to manage colonization with beneficial and pathogenic microorganisms. IL-25 is known to induce production of the antimicrobial peptide angiogenin-4. IL-25 mediated induction of angiogenin-4 was dependent upon IL-13 but not dependent on IL-22 or IL-17. During amebiasis, mice treated with IL-25 had increased IL-13. In these mice IL-13 neutralization abrogated angiogenin-4 production, however there was no change in susceptibility to amebiasis. We concluded that angiogenin-4 was not required for rIL-25 mediated protection against amebiasis.

In conclusion, this work identified a unique protection mechanism against amebiasis via IL-25 induced eosinophilia and TNFα suppression. Understanding how the host immune response influences the infection outcome of amebiasis promises to provide new avenues to the treatment or prevention of this parasitic cause of diarrhea.

List of abbreviations

qPCR Quantitive polymerase chain reaction

ELISA Enzyme-linked immunosorbent reaction

ILCs Innate lymphoid cells

IgA Immunoglobulin A

CDT *Clostridium difficile* transferase toxin

IEC Intestinal epithelial cells

rIL-25 Recombinant interleukin 25

Nos2 Inducible nitric oxide synthase

Chi3l1 Chitinase3-like 1

Epx Eosinophil peroxidase

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Dedication

This work is dedicated to my husband Mohammed Ahasanul Karim who has been a constant source of support and without him it was almost impossible for me to complete my thesis work.

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Chapter One

Introduction

1.1 *Entamoeba histolytica* prevalence

Diarrheal disease is one of the major causes of death in developing countries, particularly in young children. People exposed to poor sanitation and those who receive inadequate nutrition are particularly vulnerable. The enteric protozoal parasite *Entamoeba histolytica* is one of the leading causes of infectious diarrhea in the developing world, and also causes amebic colitis and liver abscess [1]. In two recent large multi-center studies, the Global Enteric Multicenter Study (GEMS) and the Malnutrition and Enteric Diseases study it was shown that *E. histolytica* was in the top 15 of microorganisms causing diarrhea in the critical first year of life in children in developing countries [2], [3]. While most infections are asymptomatic, 20% lead to the development of disease such as colitis, dysentery or liver abscess [4]. *E. histolytica* causes acute amoebic colitis and liver abscess all over the world, especially in Africa, Bangladesh, Southeast Asia, America and Egypt [1], [5]–[8]. In Dhaka, Bangladesh, 39% of children tested are infected within their first year of life with *E. histolytica* infection; 10% had diarrhea and 3% had dysentery [5]. *E. histolytica* also accounts for a significant proportion of diarrhea in travelers returning from endemic countries [9].

1.2 *E. histolytica* pathogenesis

Infection with the cyst form of the ameba occurs after ingestion of fecally contaminated food or water [10]–[12]. When the cysts reach the terminal ileum or colon, the ameba excyst into the trophozoite form, which can then adhere to the host epithelial cells with the Gal/GalNAc lectin. The trophozoites can re-encyst in the colon and are then excreted in the stool. Additionally, the trophozoites can induce contact-dependent killing of host cells, leading to invasion of the mucosa and submucosa, resulting in tissue destruction, secretory bloody diarrhea, and colitis[13]. Amebic invasion can also lead to the ulceration of the

intestinal mucosa, known as amebic colitis, which resembles inflammatory bowel disease [14]–[16].

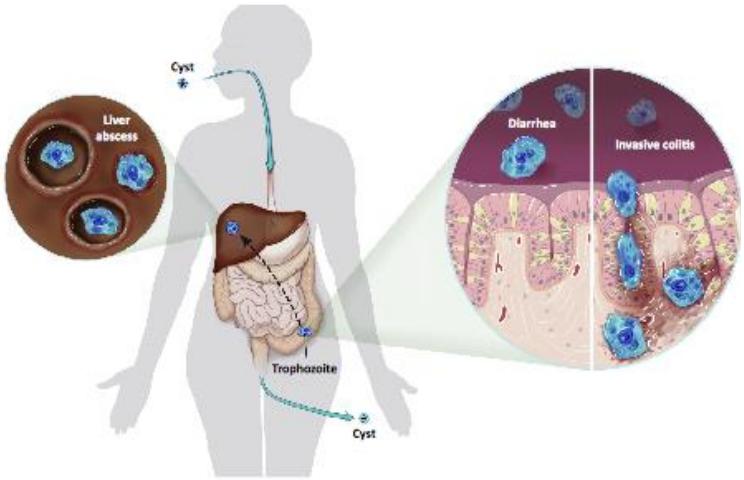
Petri et al., purified the Gal-lectin in 1987 [17]. The Gal/GalNac lectin is expressed on the plasma membrane of *E. histolytica* and has three subunits- the heavy subunit (hgl), light subunit (lgl), and the intermediate subunit (igl). Amebic trophozoites adhere to epithelial cells and colonic mucin with this lectin, which recognizes galactose and N-acetylgalactosamine (Gal/GalNAc) [17]. After adherence, *E. histolytica* degrades colonic mucin[18] and induces contact-dependent cytolysis of the host cell [19]**.** Contact dependent binding leads to apoptosis through caspase-3 activation and influx of Ca2+ ions of host cells [20]. Recently, Ralston et al. reported a novel mechanism of cell killing known as trogocytosis where *E. histolytica* trophozoites kill host cells by biting off and ingesting fragments of host cellular material [21].

1.3 Host Immunity against *E. histolytica* infection

i) Innate Immunity

Mucosal immunity is the first line defense mechanism against enteropathogens [22]**.** The initial host defense to *E. histolytica* comes from the host intestinal mucus layer, which prevents contact between trophozoites and host cells. Mucin has a high affinity-binding site for the Gal/GalNAc lectin of *E. histolytica,* and is thought to compete for trophozoite attachment by blocking access to the underlying epithelium to prevent host cell killing and parasite invasion. Mucin family members are an external barrier at the epithelial surface [23], [24]. STAT3-dependent leptin receptor signaling in intestinal epithelial cells (IEC) is also important for mediating protection against *E. histolytica* infection in a mouse model [25]. Leptin receptor signaling is also important in humans and a single **Figure 1.1**

Pathogenesis of *E. histolytica*. The cyst form of *E. histolytica* infects humans through contaminated food and water. When the cyst reaches to terminal ileum, excystation occurs and the ameba enter a trophozoite stage. Trophozoites can disrupt the mucosal barrier, penetrate underlying tissue and secretes enzyme that breaks down extracellular matrix and destroy cells. Image adapted from Katherine S. Ralston, Trends in Parasitology, September 2015 [21].


Cyst
Liver abscess
Diarrhea
Invasive colitis
Trophozoite
Cyst

amino acid polymorphism in leptin receptor (Q223R) was associated with susceptibility to *E. histolytica* infection in humans and caused susceptibility in a mouse model of amebic colitis [26]. C57BL/6J mice are otherwise highly resistant to *E. histolytica* infection, and the rapid clearance of trophozoites within hours after challenge suggests a form of innate resistance [27]. The resistance of C57BL/6J mice to *E. histolytica* infection is due to nonhematopoietic cells. The transfer of bone marrow from C57BL/6J mice to CBA/J mice did not protect the CBA/J mice, and bone marrow transfer from CBA/J mice to C57BL/6J mice did not diminish the resistance of the C57BL/6J mice to *E. histolytica* infection [28]. These data suggest a critical role for epithelial cells and innate immune responses to *E. histolytica* infections.

Intestinal epithelial cells (IEC) initiate the mucosal immune response through secretion of pro-inflammatory molecules and anti-apoptotic molecules in response to the pathogen and act as antigen presenting cells. In a co-culture system of epithelial cells with *E. histolytica*, IEC induced production of pro-inflammatory cytokines and chemokines [29]. From other studies, it has been shown that IEC can induce anti-inflammatory cytokines such as IL-25, IL-33 and TSLP, which have not been studied in amebiasis. IL-25 has both pathogenic and protective roles in the host. It is beneficial in helminth infections, but detrimental during asthma and allergic diseases [30] [31].

Another anti-inflammatory cytokine, IL-10, has been found to be crucial in amebiasis and is an important immunoregulator in the intestinal tract. It can block a pro-inflammatory immune response by inhibiting the inflammatory cytokine TNFα. IL-10 deficiency in C57BL/6J mice renders these animals susceptible to amebiasis [28]. Innate cells such as macrophages and neutrophils have been shown to be important in amebic

infection, and both cell types have been found to have amebicidal activity. Neutrophils are the first responders during amebic infection, and mice become more susceptible when neutrophils are depleted by anti-Gr-1 antibodies, though the possibility of other eosinophils or other granulocytes in contributing to protection has not been ruled out [27]. Macrophages are amebicidal through nitric oxide (NO) production, and they play an important role in the host response against amebic infection via pattern recognition receptor signaling [32]. In macrophages, nitric oxide synthase produces NO from L-arginine and inducible nitric oxide synthase (iNOS)-deficient mice were more susceptible to amebic liver abscess [33]. There is very little research investigating the role of eosinophils in amebic infection, although one study has shown that eosinophilia can reduce amebic liver abscess number and size in a gerbil model [34].

ii) Humoral immune response to *E. histolytica*

Humoral immunity is one of the other host defense systems to combat *E. histolytica* infection. Human studies in Bangladesh have shown that there is an association with pre-existing IgA and subsequent protection in children against *E. histolytica;* this study showed that children who had anti-lectin IgA had a 64% less chance of getting a new *E. histolytica* infection within the next 5 months of follow up [5]. Breast milk anti-lectin IgA also protects newborn children from *E. histolytica* infection [35]. Purified intestinal sIgA from rats immunized with the Gal-Gal/NAc lectin could inhibit trophozoite adherence *in vitro* [36]. One recent study in an immunized baboon model also showed there is an inverse correlation between IgA levels and *E. histolytica* DNA content after challenge with *E. histolytica* [37].

iii) Cell-mediated immune response to *E. histolytica*

Recently, cell-mediated immunity (CMI) has been shown to play a major role in vaccine-mediated protection against *E. histolytica* infection [25], [38]. Th1 responses in particular (IFNγ) provide protection against *E. histolytica* infection in immunized mice [39]. From adoptive transfer experiments, we know that LecA vaccine-mediated protection is transferable with $CD4^+$ and $CD8^+$ cells [38]. IFNγ is protective and inhibits *E. histolytica* infection [40]. Human studies showed the higher levels of IFNγ produced by peripheral blood mononuclear cells (PBMCs) in response to a soluble amoebic extract are associated with future susceptibility to symptomatic amebiasis [41]. IFNγ activates macrophages [42] to produce chemokines and mucosal defense molecules to give protection on the epithelium [43]**.** Neutrophils and macrophages kill *E. histolytica* after stimulation with IFNγ and TNFα *in vitro*[33], [44]. IFNγ positive $CD4^+$ T cells and IFNγ, IL-2, TNFα triple positive $CD4^+$ cells (multifunctional $CD4^+$ T cells) in blood are also correlated with protection in immunized mice [40]. TNFα kills *E. histolytica* trophozoites synergistically with IFNγ, and IL-2 induces resistance against reinfection after a liver abscess [45]. While TNFα induces macrophages and neutrophils to produce reactive oxygen species and nitric oxide to kill *E. histolytica*, excess amounts of TNFα can also damage tissue. One study has shown that higher levels of TNFα correlate with amebic diarrhea. In this study, TNFα levels were measured from soluble amebic extract (SAE) stimulated PBMCs of 138 non-related children, who were prospectively followed for 5 years. The study showed that over 5 years, diarrheal rate was 13% in children with low TNFα levels and 27% in children with higher TNFα levels [46]. In a severe-combined immunodeficient mouse-human intestinal xenograft model, intestinal damage and inflammation during amebic colitis was reduced by blocking TNFα [47].

In a CBA mouse model, IFNγ also promotes the trans-epithelial migration of neutrophils and Gr-1+ cells, which diminish both infection rate and inflammation [27]**.** Between $CD4^+$ and $CD8^+$ cells, $CD4^+$ T cells are the main source of IFNγ while $CD8^+$ produces IL-17 [38]. Both $CD4^+$ and $CD8^+$ cells showed amebicidal activity, though $CD8^+$ cells were more potent, capable of cytotoxicity at 100:1 (T cell: ameba) [38]. As well as IFNγ, other cytokines such as IL-17 also contribute to the protection, as demonstrated by neutralization studies in LecA/alum immunized mice [38]. $CD8^+$ T cell mediated protection is at least partly due to IL-17 production, though the protective mechanism of IL-17 has not been established. IL-25, a recently discovered member of the IL-17 cytokine family, functions differently than other IL-17 cytokines and has not been studied in amebiasis.

Development of vaccines against *E. histolytica*: Preventing *E. histolytica* infection by preventing adherence to cells would block parasite invasion and transmission[48]. Effective vaccination strategies against this parasite have the potential to save thousands of lives. *E. histolytica* is a gut pathogen, and directing the host protective responses to the site of infection would afford the most disease protection. Systemic vaccines often provide little protection at a mucosal site, so a vaccine should be designed with protection at mucosal site in mind by using either a mucosal delivery system or an adjuvant, which can program effector cells to mucosal site [49]**.** Vaccination with an amebic protein (LecA) has shown protection against *E. histolytica* infections in mice [40]. In the mouse model of amoebic colitis, his-tagged recombinant protein LecA with alum adjuvant has been shown to protect against *E. histolytica* infection with a protection rate of 62% [40].

1.4 *E. histolytica* and the microbiota

Interestingly, out of all individuals who become infected with *E. histolytica*, only 20% develop symptomatic disease. Host and parasite factors are both involved in the infectivity and resultant disease of the parasite. Very few studies have been done to find out the relationship between different components of the human intestinal microbiota and *E. histolytica* infection. Recently, it has been shown in a prospective study that diarrhea is associated with high parasite burden and expansion of a commensal bacterium *Prevotella copri*, which indicates a specific component of the microbiota could be associated with disease outcome as symptomatic or asymptomatic *E. histolytica* infection [50]. Another commensal bacteria, segmented filamentous bacteria (SFB), has shown to be protective against amebiasis in a mouse model. SFB can induce IL-17 and neutrophil recruitment, which has shown important in immunity against ameba [51].

1.5 IL-25 cytokine in immunity – role in gut barrier defense

The gastrointestinal tract is a major site for microbial entry and is continuously interacting with commensal and pathogenic bacteria [52]. Intestinal epithelial cells provide the first line of protection against pathogenic microbes via production of mucus and secretion of cytokines and antimicrobial peptides. Intestinal epithelial cells produce the cytokine IL-25 (originally called IL-17E) that has a role in epithelial barrier function [53][54].

IL-25 is a member of the Th17 cytokine family and is also known as IL-17E. The Th17 family consists of 6 family members, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F, where IL-17E has unique structure and function. IL-25 (IL-17E) belongs to the Th17 family of cytokines because of its sequence homology with the family, though its function is totally different from other family members. The sequence of IL-25 is 16-20% similar of IL17A. IL-25 is a 25 kDa protein containing 117 amino acid residues, and is

encoded in mouse chromosome 14 and human chromosome 114q11.2. It is known to induce a Th2 type response and control gut inflammation [55] [56].

IL-25 can act on innate immune cells (ILC2s) to produce anti-inflammatory responses [57] [58]. Intestinal epithelial cells and immune cells are both important for host protection against *E. histolytica*. The resistance to *E. histolytica* in C57BL/6J strain mice is primarily a result of non-hemopoietic cells, including epithelial cells [28]. For example, C57BL/6J mice lose resistance upon inducible knockout of the leptin receptor on gut epithelium [26]. Successful mucosal protection and homeostasis is dependent on the coordination of innate and adaptive immunity; IL-25 is a bridge between innate and adaptive immunity in the intestinal tract because it is produced from innate and adaptive sources and acts on both arms [55].

Barrier function is important for the prevention of systemic dissemination of commensal and pathogenic bacteria [59]. The downstream products of IL-25 signaling can protect intestinal barrier function by controlling pathogen invasion at the mucosal site, indicating a pivotal role of IL-25 in maintenance of normal homeostatis of the intestinal lining. IL-25 is important for modulation of tissue response and induces mucus production, and anti-microbial peptide production[55].

i) Sources of IL-25

Th2 cells were initially discovered as the source of IL-25. However, intestinal epithelial cells are also a major source of IL-25 [53]. Tuft cells constitutively produce IL-25 and are the sole source of IL-25 in the small intestine epithelium [60], [61]. Other sources of IL-

25 include Th2 cells, eosinophils, basophils, kidney cells, liver cells, lung cells, mast cells, macrophages, fibrobolast keratinocytes, NKT cells, and endothelial cells [31], [62]–[64].

ii) IL-25 signaling

The IL-25 receptor is a 50 KDa single transmembrane protein and a heterodimer receptor with two subunits: IL-17BR and IL-17B (Rh1) [65]–[67]. IL-25 has shown higher affinity for binding IL17BR than IL-17B [65]. Binding of IL25 to its receptor leads to induction and activation of transcription factors including STAT6, GATA3, NF-kβ, JUNNB, MAPK, and JNK [31], [55], [68], [69]. It also known that TNFR-associated factor (TRAF) 6 is important for IL-25R induced NFkB activation; however, IL-25 mediated MAPK activation is TRAF6 independent [70].

At first, IL-25 was known to induce type 2 cytokines from Th2 cells, but it was recently discovered that IL-25 also activates tissue resident group two innate lymphoid cells (ILC2) to induce IL-13 and IL-4 [60]. Therefore IL-25 can affect both innate and adaptive immune cell types. IL-25 is also known to act on mast cells, macrophages, dendritic cells, eosinophils, basophils, and monocytes. IL-25R is expressed on antigen-presenting cells, asthmatic lung tissue, airway smooth muscle cells, invariant NKT cells, intraepithelial lymphocytes, and epithelial cells [53], [68], [71]–[73].

iii) Regulation of IL-25

IL-25 is produced by intestinal epithelial cells in response to commensal bacteria, and germ-free mice produce less IL-25 than conventionally raised mice. IL-25 RNA expression is inhibited by TNFα in the brain capillary endothelial cell line, MBEC4 [74]. In human colonic explants, IL-25 production is enhanced with transforming growth factor

stimulation and also by anti-TNFα treatment. Therefore, in the human gut, TNFα negatively regulates IL-25 synthesis, where TGF β increases IL-25 production [75]. In other studies, it has been shown that the helminth *Heligmosomoides polygyrus bakeri* promotes host-derived IL-1β to suppress the IL-25 induced type 2 response to allow pathogen chronicity [76]. Recently it has been shown that tuft cells are a major source of IL-25 and tuft cell hyperplasia is dependent on IL-13 [60].

iv) Downstream effects of IL-25

IL-25 is known to induce type 2 responses and suppress anti-inflammatory responses. IL-25 can activate both innate and adaptive sources to produce type 2 responses. It is known to induce IL-4, IL-5, IL-9 and IL-13. IL-25 induces IL-4, which causes B cells to produce IgE [62]. IL-25 can dampen the inflammatory response by inhibiting inflammatory cytokines. IL-25 acts on macrophages and dendritic cells to suppress pro-inflammatory cytokine production such as IL-23, IL-22, IL-17, and TNFα [75]. One study has shown that commensal bacteria increase IL-25 production from epithelial intestinal cells and that elevated IL-25 results in decreased IL-23 and subsequently decreased IL-17 production. In the absence of commensal bacteria, such as in germ free mice, there is reduced IL-25 expression and increased IL-23 expression [77]. IL-25 acts on dendritic cells and suppresses Th17 cells via IL-13 production. IL-25 promotes accumulation of co-stimulatory molecules of CD80 and CD86 on DCs. IL-25 induces mastocytosis, eosinophilia and IgE from B cells [55][63].

IL-25 induces eosinophil infiltration as a downstream effector. Eosinophils are versatile cells that can play a role in host protection against various types of pathogens in the gut and also help in tissue remodeling and repair [78]. Activated eosinophils can

produce bactericidal pro-inflammatory cytokines and release granules containing cationic proteins to provide protection [78]. In mice, eosinophilic granule proteins major binding protein (MBP) and eosinophil peroxidase (EPO) are essential for protection against *Stongyloides stercoralis* and *Litomosoides sigmodontis* [79], [80]. Eosinophils can also release mitochondrial DNA, known as traps, which has toxic activity towards extracellular bacteria [81]. Eosinophils have been confirmed to protect against *Pseudomonas aeruginosa* infection [82]. In contrast, in an IBD-like colitis model, eosinophils have been shown to be pathogenic through GM-CSF production and tissue damage in the mouse intestine [83]. Antimicrobial peptides play an important role in control of the commensal bacteria in the gut, and provide defense against pathogens. IL-25 is a potent inducer of the antimicrobial peptide angiogenin-4, where it acts in an IL-13 dependent manner [84].

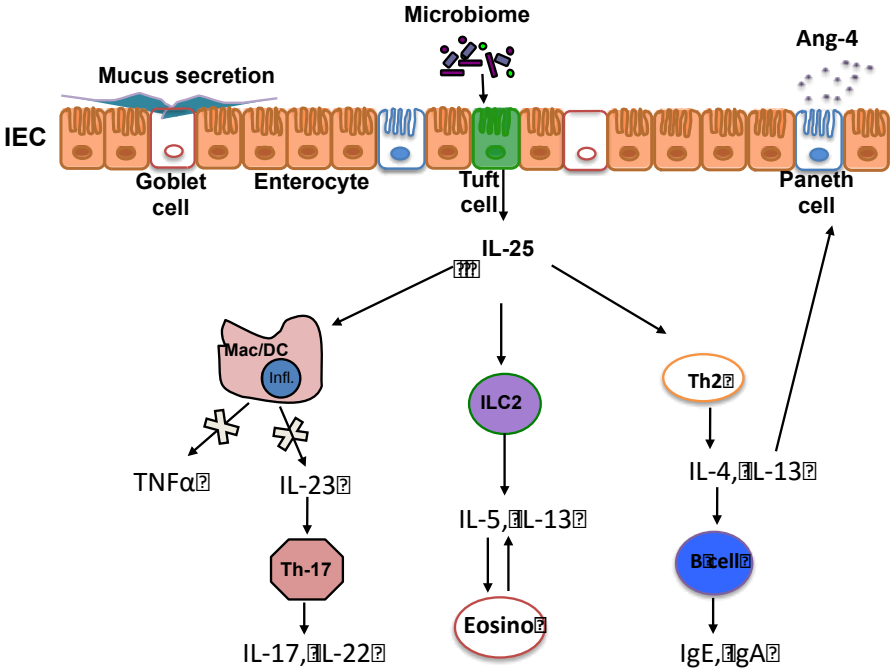
1.6 Role of IL-25 in infection and inflammation

i) Role of IL-25 in humans: The role of IL-25 in humans has not yet been studied extensively. IL-25 is constitutively produced in the human gut where it acts presumably to dampen intestinal inflammation as part of normal homeostasis. IBD patients have less IL-25 protein expression in the inflamed colon than healthy controls, and the same study also showed that IL-25 in human gut is expressed mostly by subepithelial macrophages [75][85]. Another study has shown that brain capillary endothelial cells have decreased IL-25 expression in multiple sclerosis lesion patients [74].

ii) IL-25 in asthma: Most research about IL-25 has been studied in an asthma and allergy model where IL-25 drives the pathogenesis. Misregulated IL-25 signaling leads to type 2

Figure 1.2 Role of IL-25 at homeostasis. IL-25 induces type 2 responses from both innate (i.e. Innate lymphoid cell 2) and adaptive sources (i.e. Th2 cells) and induces type 2

cytokines production like IL-4, IL-5, IL-9 and IL-13 [62]. At the same time IL-25 suppresses inflammatory responses to maintain gut homeostasis [77]. IL-25 acts on macrophages and suppress inflammatory cytokines IL-23, IL-22 and TNFα production.


Microbiome
Ang-4
Mucus secretion
IEC
Goblet cell
Enterocyte
Tuft cell
Paneth cell
IL-25
Mac/DC
Infl.
ILC2
Th2
TNFα
IL-23
IL-4, IL-13
IL-5, IL-13
Th-17
B cell
Eosino
IL-17, IL-22
IgE, IgA

responses to environmental antigens and is deleterious in many models of allergy and asthma [31]. IL-25 performs its pathogenic role by recruiting eosinophils, mast cells, and basophils, which are key modulators of disease [55]. Antigen-induced allergic airway inflammation is enhanced by IL-25 induction of Th2 cell dependent pathways [31]. IL-25 can worsen asthmatic pathology by inducing type 2 cytokines in steroid resistant IL-17RB myeloid populations [86]. IL-25 has been shown to be upregulated in asthma and promote angiogenesis [87]. Epithelial cell derived IL-25 is crucial for murine asthma, and IL-25 deficient mice revealed that airway inflammation is dependent on epithelial cells or eosinophil activation [88].

iii) IL-25 in helminth infection: Recent studies have revealed that IL-25 is an important cytokine responsible for protection against helminth infections by stimulating type 2 cytokine production from innate cell populations [89]. During *Trichuris muris* infection IL-25 plays a key role to promote type 2 cytokine dependent protection against *Trichuris muris* and at the same time also inhibits destructive inflammation in the intestine by inhibiting INFγ and IL-17 [90]. IL-25 has also been discovered to help expel helminths from gut by inducing IL-13, which then acts to stimulate mucus production from goblet cells [90].

iv) IL-25 in colitis: It has been revealed from different studies that IL-25 can inhibit Th1 and Th17 immunity and induce Th2 immunity. IL-25 administration can prevent experimental colitis in a mouse model [77][85]. However, IL-25 plays a pro-inflammatory role in oxazolone-induce ulcerative colitis model. Mucosal inflammation induced by oxazolone shows a clear type-2 inflammatory response and in this model IL-17BR+ IL-13 producing natural killer cells (NKT) and nuocytes drive intestinal inflammation. This study

showed that blocking IL-25 signaling has improved the disease clinical outcome by down-regulating type-2 cytokines and decreasing NKT cells [91].

v) IL-25 in autoimmunity: IL-25 regulates autoimmunity by suppressing Th17 responses and IL-25 knock out mice are highly susceptible to experimental autoimmune encephalomyelitis (EAE). IL-25 can induce IL-13 which causes Th17 suppression and required for IL-25 mediated protection from EAE [92].

vi) IL-25 and microbiota: IL-25 is known to maintain homeostasis by inhibiting the IL-23-IL-17 axis. Germ free mice have been shown to have undetectable levels of intestinal IL-25 and increased inflammation compared to conventional mice. In a *C. difficile* model, it has shown that alternation of microbiota by antibiotic treatment can suppress IL-25, which is further suppressed in the presence of *C. difficile*. The suppression of IL-25 contributes to pathogenicity of *C. difficile* by preventing an IL-25 induced protective eosinophil response in the gut (Buonomo EL, et al., Cell Reports, in press 2016).

1.7 Project goals

Mucosal defense is critical for protection against *E. histolytica* and intestinal epithelial cells provide the first line of defense against amebic infection. Once the intestinal epithelial barrier is broached, amebic infection is unchecked. However, it is unclear how mucosal defense mechanisms work during *E. histolytica* infections. The goal of this project was to develop an understanding of how mucosal immunity influences *E. histolytica* infections.

I hypothesized that IL-25 enhances the enteric epithelial barrier to provide protection against *E. histolytica* infection. The rationale for this hypothesis was that amebic

infection can lead to the ulceration of the intestinal mucosa and subsequent inflammatory responses such as TNFα that are deleterious. In contrast the cytokine IL-25 could act to suppress inflammatory conditions to maintain homeostasis. This would provide protection from infection by decreasing inflammation and protecting the epithelial barrier.

In this dissertation, by using human samples and a murine model for intestinal amebiasis, I have:

1) revealed that during *E. histolytica* infection the anti-inflammatory cytokine IL-25 is suppressed in humans and in the mouse model of amebic colitis;
2) tested the role of IL-25 by administrating recombinant IL-25 to mice during amebic colitis and identified the protective and gut maintaining role of IL-25;
3) demonstrated the protective mechanism of IL-25 during amebiasis which is eosinophil dependent.

Chapter Two

Eosinophil mediated protection during amebic colitis by interleukin-25 (IL-25).

2.1 Introduction

One of the leading causes of death in children under five years of age globally is diarrheal disease and one cause of severe diarrhea in developing countries is the intestinal parasite *Entamoeba histolytica* [93]. Infection is acquired fecal-orally from contaminated food and water. In two recent large multi-center studies, the Global Enteric Multicenter Study (GEMS) and the Malnutrition and Enteric Diseases study, it was reported that *E. histolytica* was among the top 15 microorganisms causing diarrhea in the critical first year of life in children in developing countries [2][3]. While most infections are asymptomatic, up to 20% of infections lead to the development of symptomatic disease such as colitis, dysentery or liver abscess [4]. The varied outcomes of *E. histolytica* infection are likely due to a combination of parasite, host and environmental factors [94].

E. histolytica disrupts the mucosal barrier in a sequential process of adherence to intestinal epithelial cells by a parasite Gal/GalNAc lectin, followed by killing of the epithelial cells in a nibbling process termed amebic trogocytosis, leading to penetration of the epithelium and destruction of underlying tissue.[14]–[16]. *E. histolytica* induces several inflammatory cytokines such as IL-23, IL-17 and TNFα [39]. TNFα induces macrophages and neutrophils to produce reactive oxygen species and nitric oxide to kill *E. histolytica*, however excess TNFα can also cause tissue damage [47]. *E. histolytica* also produces a homolog of the proinflammatory cytokine MIF, EhMIF, that can induce TNFα secretion [95]. In the setting of amebic colitis, it has been shown that blocking TNFα can reduce inflammation and intestinal damage [47].

Mucosal defense is critical for protection against *E. histolytica*. Intestinal epithelial cells provide the first line defense against amebic infection, however it is unclear how the

mucosal defense mechanisms work to prevent *E. histolytica* infection. STAT3-dependent leptin receptor signaling in intestinal epithelial cells (IEC) is important for protection against *E. histolytica* infection in a mouse model [25]. Epithelial cells produce the anti-inflammatory cytokine IL-25 (originally called IL-17E), which has been demonstrated to function as a pathogenic, protective, or homeostatic mediator in different contexts [53]. Intestinal epithelial tuft cells are major source of IL-25 [60]. It has also been shown that TNFα can negatively regulate IL-25 production in the human gut [75]. IL-25 is known to induce a type 2 response and suppress inflammatory responses. Innate lymphoid cells (ILC2s) respond to IL-25 with IL-13 and IL-5 production. IL-25 induced ILC2s has been shown to be protective against helminth infection [60] [57]. IL-25 can activate both innate and adaptive sources to produce type 2 responses, and induce type 2 cytokines, such as IL-4, IL-5, IL-9 and IL-13. IL-25 acts on macrophages and dampens the inflammatory response by decreasing IL-23, IL-22, IL-17 and TNFα [75]. One study has reported that commensal bacteria increase IL-25 production by epithelial intestinal cells and that elevated IL-25 resulted in decreased IL-23 and subsequent decreased IL-17 production [77]. IL-25 is also a potent inducer of the antimicrobial peptide angiogenin-4, and acts in an IL-13 dependent manner [84].

IL-25 induces eosinophil infiltration in the gut. Eosinophils are versatile cells that can play a role in host defense against various types of pathogens in the gut and also aid in tissue remodeling and repair [78]. A recent study has shown that eosinophils are effective in anti-parasite protection against a subset of parasites [96]. In mice, the eosinophilic granule proteins major basic protein (MBP) and eosinophil peroxidase (EPO) are essential for protection against *Stongyloides stercoralis* and *Litomosoides sigmodontis* [79] [80]. Microbiota regulated IL-25 protects against *C. difficile* infection via eosinophil infiltration

in the colon in a mouse model (Buonomo EL, et al., Cell Reports, in press 2016). *C. difficile* infection with strains containing CDT (*Clostridium difficile* transferase toxin) cause more severe pathology because they suppress the accumulation of eosinophils in the lamina propria of the colon (Cowardin CA, et al., Nature Microbiology, in press 2016). Eosinophils have also been reported to protect against *Pseudomonas aeruginosa* infection [82]. Eosinophilia was associated with reduced size and number of amoebic liver abscesses in the gerbil model [34].

Most research on IL-25 has been done in asthma and allergy models where deregulated IL-25 drives disease by recruiting eosinophils, mast cells, and basophils [55]. Recent studies have revealed that IL-25 plays an important role in protection against helminth infection [89]. During *Trichuris* infection IL-25 plays key role to promote type 2 cytokines, which protect against *Trichuris* and at the same time also inhibit destructive inflammation in the intestine [90].

Intestinal epithelial cells play a central role in protection against *E. histolytica* infection as does the gut microbiome. IL-25 is known to be regulated by the microbiota and to in turn regulate the maintenance of epithelial integrity during infection [51][50][77]. Therefore, we hypothesized that IL-25 provides one mechanism of epithelial defense against amebic colitis. We also hypothesized that IL-25 might also act to boost mucosal defense mechanisms by inducing immune cell recruitment and regulating cytokine production. In this study, we demonstrate that the anti-inflammatory cytokine IL-25 is suppressed during *E. histolytica* infection in humans and in a mouse model of amebic colitis. We tested the role of IL-25 by administering recombinant IL-25 to mice during amebic colitis and discovered that IL-25 has an important role in both protection from *E.*

histolytica and gut maintenance. Finally we demonstrated that this IL-25-mediated protection is eosinophil dependent. In summary, this study identifies a unique epithelial cell mediated innate immune protection mechanism against amebiasis, which sheds light on the varied infection outcome in amebiasis.

2.2 Methods

Mice

Six-week-old male CBA/J mice and C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific pathogen–free facility in microisolator cages and provided autoclaved food (Lab diet 5010) and water ad libitum. The University of Virginia Institutional Animal Care and Use Committee approved all procedures.

Recombinant IL-25 treatment

Mice were injected intraperitoneally with 0.5 μg recombinant IL-25 (R&D Systems) or 100 μl PBS each day for 4 days before and through 4 days after *E. histolytica* challenge.

E. histolytica challenge

The trophozoites used for the *E. histoytica* challenge were initially originated from lab-derived strain HM1:IMSS (ATCC, VA) and sequentially passaged in vivo by injection into the CBA/J mouse cecum. Cecal contents were collected and cultured in trypsin-yeast-iron (TYI-S-33) medium with bovine serum (Sigma-Aldrich, St Louis, MO), Diamond Vitamins (JRH Biosciences, Lenexa, KS), and 100 units per ml penicillin with 100 microgram per ml streptomycin. Trophozoites were grown to log phase and laparotomy was used to challenge mice intracecally with two million trophozoites in 150 μl of media.

E. histolytica infection evaluation

Mice were harvested 7 days after *E. histolytica* challenge and cecal contents were collected to evaluate infection by culture, *E. histolytica* antigen detection and *E. histolytica* DNA detection. 300 µl of cecal contents were cultured in complete TYI-S-33 medium with supplemental antibiotics for 3 days at 37 °C. 200 µl of cecal contents were used for *E. histolytica* antigen detection using *E. histolytica* II ELISA kit (Techlab, Blacksburg, VA). 200 µl of cecal contents were used for DNA isolation using the Qiagen DNA extraction kit (Qiagen, Hilden, Germany) and a qRT-PCR assay was used for *E. histolytica* DNA quantification. For quantification, standards were generated by isolating DNA from 10^6 *E. histolytica* trophozoites in culture and then serially dilution. Sample concentration was calculated using standard curve-generated Threshold Cycle (Ct) values and known trophozoite number in standard serial dilutions. The primer and probe sequences were as follows: forward primer Eh-f, AAC AGT AAT AGT TTC TTT GGT TAG TAA AA; reverse primer Eh-r, CTT AGA ATG TCA TTT CTC AAT TCA T; probe Eh-YYT, ATT AGT ACA AAA TGG CCA ATT CAT TCA-dark quencher. Primers and probe were purchased from Integrated Technologies, Coralville, IA, USA.

Cecal tissue processing

Cecal tissue was cut into three pieces: one was placed in Bowman's fixative (Sigma-Aldrich), one was rinsed with phosphate-buffered saline (PBS) and stored in RNA later (Ambion, Foster City, CA) for quantitative RT-PCR analysis and the third piece was stored at -80^{o}C for cytokine measurement by ELISA.

Cytokine measurement from cecal tissue and cecal content

Cecal tissue was processed for cytokine measurement by ELISA. Cecal tissue was homogenized by bead-beating with buffer consisting of 1M HEPES and HALT protease inhibitor cocktail (Thermo-Fisher Scientific Inc., Rockford, IL) and then kept on ice for 30 minutes with buffer containing Triton X 100, HEPES and HALT protease inhibitor cocktail. The homogenate was then spun at 10,000 x *g* for 10 minutes and the supernatant was collected for cytokine protein measurement. Cytokines measured by ELISA (R&D systems) included IL-4, IL-5, IL-9, IL-23, IL-17, IL-22 and TNFα. Samples were run according to the manufacturer's protocol and measured as pg/ml of supernatant. Cytokine concentrations were normalized to total protein concentration obtained from the Pierce BCA Protein Assay (ThermoFisher). For cytokine measurement from mouse cecal content, 100 µl of cecal content was mixed in PBS containing protease inhibitor cocktail (Roche) and used for cytokine measurement by ELISA as per the manufacturers' instructions.

Mouse hematoxylin and eosin (H&E) staining

Tissue in Bowman's fixative was cut into cross-sections and paraffin embedded, and then stained with hematoxylin and eosin (H&E) by the Histology Core facility at the University of Virginia. Blindly, three independent readers scored epithelial disruption (Buonomo EL, et al., Cell Reports, in press 2016). The scale was between 0-5. If more than 80% of epithelial layer in one field was disrupted then it was scored as 5. If 61-80% of epithelial layer disrupted the score was 4. If 41-60% of epithelial layer disrupted then 3. If 21-40% of epithelial layer disrupted the score was 2. If 1-20% of epithelial layer disrupted then the score was 1. If the epithelial layer was intact the score was 0. Two different fields were chosen randomly to score from each sample.

RNA extraction and quantitative RT-qPCR

Cecal tissue from RNA later was flushed with sterile PBS and total RNA was extracted from the cecal tissue using the RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. For reverse transcription, total RNA was transcribed using SuperScript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). qRT-PCR was performed on reverse-transcribed cDNA using an iQ SYBR green supermix (Bio-Rad Laboratories) in the iCycler iQ System (Bio-Rad Laboratories). The following primers were published previously and used in this study: βActin; βActin forward: 5-AGCCATGTACGTAGCCATCC-3, βActin reverse: 5-CTCTCAGCTGTGGTGGTGAA-3; glyceraldehyde- 3-phosphate dehydrogenase, (GAPDH); GAPDH forward: 5-TGCACCACCAACTGCTTAGC -3, GAPDH reverse: 5-GGCATGGACTGTGGTCATGAG -3; iNOS, iNOS forward: 5-CTGGAGGAGCTCCTGCCTCATG -3, iNOS reverse: 5- GCAGCATC CCCTCTGATGGTG-3; Arginase-1, Arginase-1 forward: 5- GCT CCA AGC CAA AGT CCT TAG AGA T-3, Arginase-1 reverse: 5- AGG AGC TGT CAT TAG GGA CAT CAA C -3. Primers were purchased from Integrated DNA Technologies Coralville, Iowa, USA. Chitinase 3-like 1 primer (RT² qPCR Primer Assay for Mouse Chi3l1), IL-13 primer (RT² qPCR Primer Assay for Mouse Il13) and eosinophil peroxidase primer (Mm_Epx_1_SG QuantiTect Primer Assay) were purchased from Qiagen (Hilden, Germany). The calculated relative quantity of the cytokine mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and βActin mRNA.

Cytokine treatment and neutralization

CBA/J mice were treated with 0.5 μg of recombinant IL-25 each day for a total of 8 doses (4 days prior through 4 days post-challenge with *E. histolytica)* and control mice received PBS. rIL-25 treated mice received 40 μg anti-Siglec-F (clone 238047, R&D Systems) or

IgG2a isotype (clone 54447, R&D systems) at day -1, day 1 and day 3 post-infection. Mice were euthanized after 7 days of infection. For TNFα neutralization, mice were treated with 500 μg of anti-TNFα mAb (clone XT3.11, BioXcel) or control rat IgG1 (clone HRPN, BioXcel) intraperitoneally at day -1, day 1 and day 3 post-infection. The mice were euthanized at day 7 post-infection. In order to inhibit IL-1β receptor signaling, two doses of anakinra (SOBI) (17mg/kg body weight per dose) were given to mice in 8-hour intervals prior to infection with *E. histolytica.* After infection, mice were given three doses per day for three days. Mice were euthanized on day 4 post-infection. For IL-13 cytokine depletion, mice were treated with 200 μg anti-IL-13 antibody (262A-5-1, Genentech) or isotype control (clone GP120.9709, Genentech) on day -1, on day 1 and on day 3. Mice were challenged with *E. histolytica* intracecally by laparotomy and euthanized after 7 days of infection.

Human colon biopsy immunohistochemistry

Amebic colitis patients' colon biopsies were from deidentified patients at the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b) with consent. Human biopsy tissues for the control group were obtained from the Biorepository and Tissue Research Facility at the University of Virginia and confirmed negative for tissue pathology upon histological examination. The Biorepository and Tissue Research Facility of University of Virginia performed immunochemistry staining for IL-25 and TNFα. Paraffin embedded sections of cecum were cut into 4 μm thick histologic sections, placed on negatively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Slides were deparaffinized and antigen retrieval was performed in a PT Link instrument (Dako, Glostrup, Denmark) at 97° C for 20 minutes in low pH antigen retrieval solution.

Immunohistochemistry was done on a robotic platform (Autostainer, Dako). Endogenous peroxidases were blocked using Peroxidase and Alkaline Phosphatase Blocking Reagent (Dako). Polyclonal rabbit antibody to IL-25 (Cat.#MAB 1258, R&D systems) and TNFα (Cat# SC 52746, Santa Cruz Biotechnology) were diluted at 1:400 and 1:75, and applied at ambient temperature for 30 and 60 minutes, respectively. Antibody binding was visualized by incubation with Envision^{TM} Rabbit Link (Dako) and then incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB+). All the slides were counterstained with hematoxylin; subsequently they were dehydrated, cleared and mounted for the assessment. IL-25 or TNFα staining was scored in intestinal epithelial cells and lamina propria. Scoring was based on intensity and abundance of IL-25 or TNFα and was done blindly by three independent scorers (Buonomo EL, et al., Cell Reports, in press 2016). The staining scale was between 0-5. The percent of visual field that had intense brown staining within one or two villi was scored. If this occurred in 81-100%, of the field then it was scored as 5. If this occurred in 61--80%, of the field then it was scored as 4. If this occurred in 41-60%, of the field then it was scored as 3. If this occurred in 21-40%, of the field then it was scored as 2. If this occurred in 1-20%, of the field then it was scored as 1. The samples that had no staining scored as 0. There were randomly two different fields chosen from each sample.

Human stool cytokines

Stool samples were collected from children in a prospective study of amebiasis in Mirpur, an urban slum area in Dhaka, Bangladesh. The study was approved by Research Review Committee (RRC) and Ethical Review Committee (ERC) of the icddr,b and the Institutional Review Board (IRB) of the University of Virginia. Stool samples were collected monthly from children with informed consent from parents or guardians of the

children. Stool was collected from 30 children during amebic diarrhea and intestinal amebiasis was confirmed by *E. histolytica* antigen detection using the *E. histolytica* II ELISA kit (Techlab, Blacksburg, VA). Control stool was collected from the same group of children 1 month before and 1 month after amebic diarrhea and tested negative for *E. histolytica* by antigen detection using *E. histolytica* II ELISA kit (Techlab, Blacksburg, VA). Stool was mixed in PBS containing protease inhibitor cocktail (Roche) and spun at 900 x *g* for 10 min followed by 15,800 x *g* for 10 min at 4° C to remove insoluble material. IL-1β and TNFα cytokines were measured using Human IL-1beta High Sensitivity ELISA kit (ebioscience, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis

Student's t-test or Mann-Whitney U non-parametric test were used for comparisons between two groups. Paired t- test was used for the children's stool cytokine analysis. P values of less than 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism, GraphPad Software, San Diego California, USA. All experiments are representative of at least three independent replicates.

2.3 Results

IL-25 is suppressed during *E. histolytica* infection. Because of the importance of the microbiome and intestinal epithelium in defense against *E. histolytica* colitis, we hypothesized that IL-25 would be protective against amebic infection in cecum. In fact, IL-25 has been shown to play a role in epithelial protection during colitis from another intestinal pathogen, *C. difficile* (Buonomo et al, Cell Reports). Human colon biopsy samples from control and amebic colitis patients were stained for IL-25 by

immunohistochemistry (Fig. 2.1A) (descriptive data of the patients and controls is shown in Table 1). IL-25 staining was present both in the intestinal epithelium and in cells in the lamina propria. IL-25 was less abundant in amebic colitis patients (Fig. 2.1).

We utilized the mouse model of amebic colitis to test the importance of IL-25 in defense from *E. histolytica* invasion. CBA/J mice were challenged with *E. histolytica* (by laparotomy and intracecal injection of trophozoites) and compared to control mice that received a laparotomy but not *E. histolytica.* IL-25 was decreased at day 1 and day 2 in both groups, but remained depressed after day 3 solely in *E. histolytica* challenged mice. Sham challenged mice returned to baseline levels of IL-25 after day 3 (Fig. 2.2). Therefore, we concluded that IL-25 was suppressed in *E. histolytica* infection in both humans and in the mouse model. Interestingly, there was a non-statistically significant trend that *E. histolytica* – challenged mice that cleared *E. histolytica* infection had higher IL-25 (closed blue circles) than the infected mice (closed red circles).

IL-25 has a protective role against *E. histolytica* colitis in mouse model. In order to see if IL-25 would protect mice against amebiasis, we injected into the peritoneum 0.5 µg of rIL-25 or PBS daily for 4 days prior to, and 4 days after *E. histolytica* challenge. We found that the *E. histolytica* infection rate and parasite burden in the cecum were decreased in the rIL-25 treated group compared to the PBS treated group (Fig. 2.4A-C). These data supported a protective role for IL-25 against *E. histolytica* infection. To test if IL-25 was protective at the level of the gut epithelial cells during amebic infection, we looked at epithelial disruption during *E. histolytica* challenge. There was less epithelial disruption in the rIL-25 treated group than in the PBS treated group at 7 days after *E. histolytica* challenge (Fig. 2.5). Therefore we concluded that IL-25 acted to reduce *E. histolytica*

Figure 2.1: IL-25 is suppressed during *E. histolytica* infection in humans. (A) Representative IL-25 immunohistochemical staining of human biopsy samples taken from the colon of control and amebic colitis patient. (B) Histological scoring for IL-25 in the human colon biopsies. Control patients included patients with diarrhea, polyps, and Crohn's disease. *P value less than 0.05.

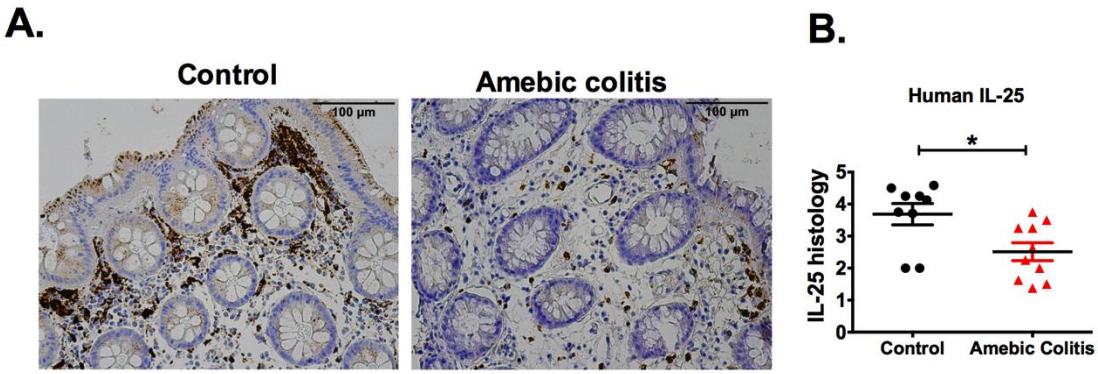

A.
Control
Amebic colitis
100 µm
100 µm
B.
Human IL-25
*
IL-25 histology
0
1
2
3
4
5
Control
Amebic Colitis

Table 1: Descriptive data on amebic colitis and healthy control group for biopsies.

ID	*E. histolytica*	Reason for biopsy
52595F258	Positive	Amebic colitis
52675F260	Positive	Amebic colitis
54255F408	Positive	Amebic colitis
54255F408	Positive	Amebic colitis
57371F435	Positive	Amebic colitis
57371F317	Positive	Amebic colitis
57371F317	Positive	Amebic colitis
57371F317	Positive	Amebic colitis
57371F434	Positive	Amebic colitis
57382F310	Positive	Amebic colitis
57382F311	Positive	Amebic colitis
57382F436	Positive	Amebic colitis
57881F481	Positive	Ambic colitis
WAP9-06	Negative	Rectal colitis
WAP9-07	Negative	Crohn's disease
WAP9-12	Negative	Polyp
WAP9-13	Negative	Diarrhea
WAP9-14	Negative	Crohn’s disease
WAP9-15	Negative	Dysplasia
WAP9-16	Negative	Diarrhea
WAP9-17	Negative	diarrhea
WAP9-18	Negative	diarrhea

Figure 2.2: IL-25 is suppressed during *E. histolytica* infection in the mouse model. IL-25 protein levels were measured in cecal tissue lysates of mice before (day 0) and after *E. histolytica* cecal challenge (closed black circle is sham challenged, closed red circle is *E. histolytica* challenged and infected, closed blue circle is *E. histolytica* challenged and cleared mice). *P value less than 0.05.

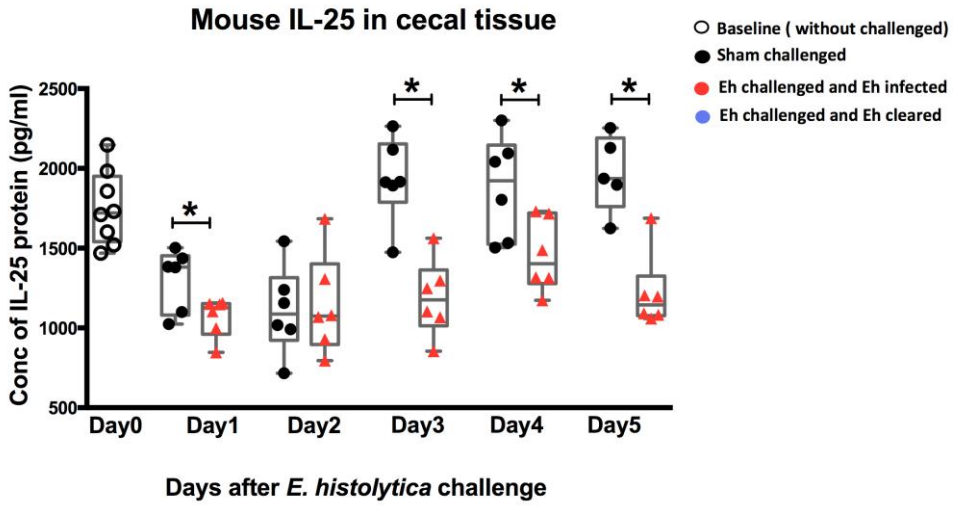

Mouse IL-25 in cecal tissue
Baseline (without challenged)
Sham challenged
Eh challenged and Eh infected
Eh challenged and Eh cleared
Conc of IL-25 protein (pg/ml)
2500
2000
1500
1000
500
*
*
*
*
Day0
Day1
Day2
Day3
Day4
Day5
Days after E. histolytica challenge

Figure 2.3: Time course of rIL-25 treatment and *E. histolytica* infection. Mice were injected intraperitoneally with 0.5 µg recombinant IL-25 or PBS each day for total 8 days and were *E. histolytica* challenged after 4 doses of rIL-25. Cecal contents were harvested at 7 days after *E. histolytica* challenge.

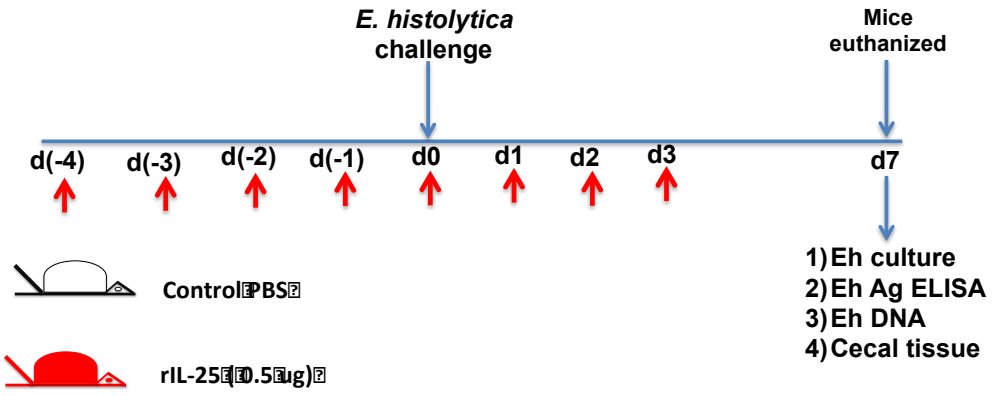
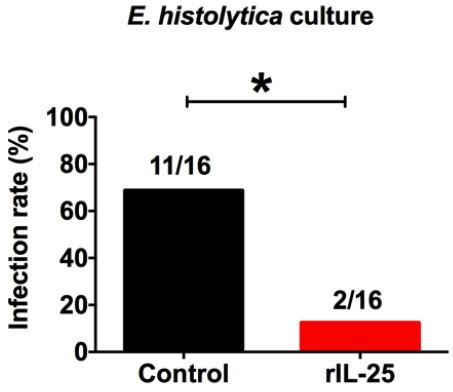
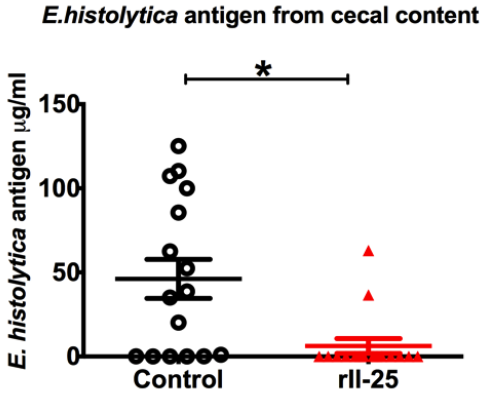

E. histolytica
challenge
Mice
euthanized
d(-4)
d(-3)
d(-2)
d(-1)
d0
d1
d2
d3
d7
1) Eh culture
2) Eh Ag ELISA
3) Eh DNA
4) Cecal tissue
Control (PBS)
rIL-25 (0.5 ug)

Figure 2.4: IL-25 prevents *E. histolytica* colonization during colitis in mouse model. Mice were injected intraperitoneally with 0.5 μg recombinant IL-25 (red bar or closed red triangle) or PBS (black bar or open circle) each day for 8 days and were challenged with *E. histolytica* on day 5. Cecal contents were harvested 7 days after *E. histolytica* challenge (day 12) and parasite infection was evaluated by (A) culture (B) *E. histolytica* DNA detection and (C) *E. histolytica* antigen detection. *P value less than 0.05.

A.


E. histolytica culture
*
100
80
60
40
20
0
Infection rate (%)
11/16
2/16
Control
rIL-25

B.


E.histolytica antigen from cecal content
*
150
100
50
0
E. histolytica antigen μg/ml
Control
rIl-25

C.

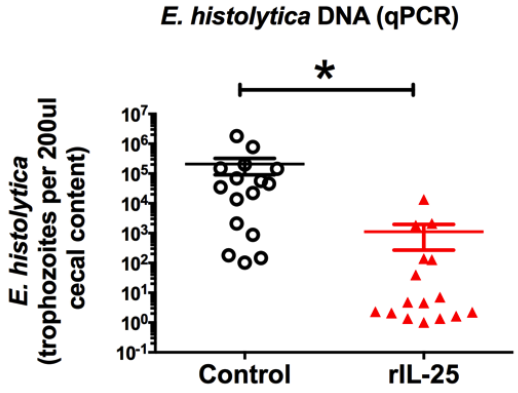
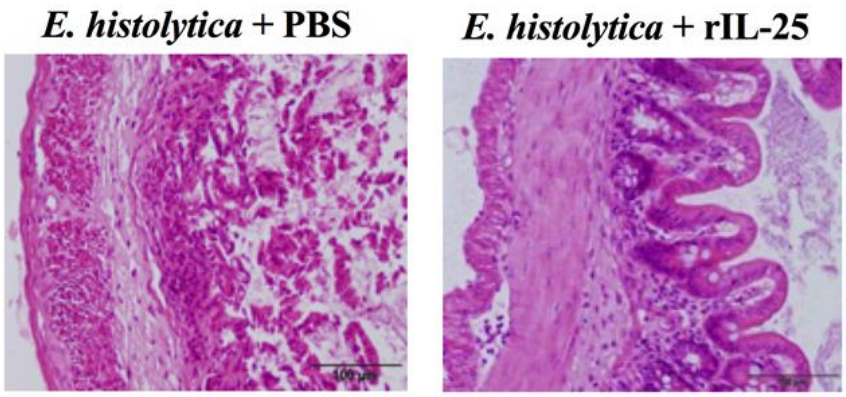
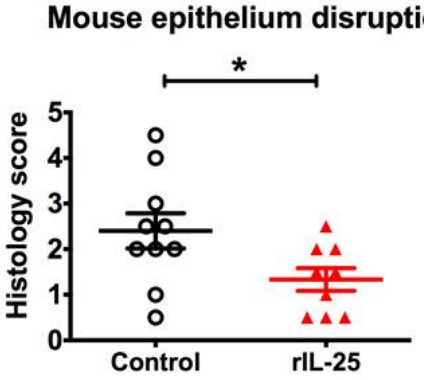

E. histolytica DNA (qPCR)
*
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
E. histolytica (trophozoites per 200ul cecal content)
Control
rIL-25

Figure 2.5: IL-25 prevents intestinal damage during *E. histolytica* colitis in mouse model. Mice were injected intraperitoneally with 0.5 µg recombinant IL-25 (closed red triangle) or PBS (open black circle) each day for 8 days and were challenged with *E. histolytica* on day 5. Mice were euthanized 7 days after *E. histolytica* challenge (day 12). (A) Representative cecal histopathology of *E. histolytica* challenged mice with or without IL-25 treatment. (B) Epithelial disruption score in *E. histolytica* challenged with or without rIL-25. *P value less than 0.05.

A.


E. histolytica + PBS
E. histolytica + rIL-25

B.


Mouse epithelium disruption
*
Histology score
0
1
2
3
4
5
Control
rIL-25

burden and maintain the gut barrier during amebic infection.

rIL-25 administration induced Th2 type responses and suppressed inflammatory responses. It was shown in previous studies that IL-25 regulates type 2 immunity as a downstream mechanism of tissue healing during colitis of other etiologies [62]. In order to understand the shape of immune response induced by IL-25 during amebiasis, we measured type 2 cytokines. IL-4, IL-5 and IL-9 were assessed by ELISA from cecal tissue lysates of mice after *E. histolytica* challenge with or without rIL-25 treatment. We found that IL-4 and IL-5 were elevated in rIL-25 treated mice compared to control (Fig. 2.6A, 2.6B), whereas the IL-9 level was not different between the 2 groups (Fig. 2.6C). Also, we found that inducible nitric oxide synthase encoding mRNA (*Nos2*) was decreased in the presence of rIL-25 (Fig 2.6D). On the other hand, the amount of mRNA encoding chitinase3-like 1 (*Chi3l1*) and eosinophil peroxidase (*Epx*) was upregulated in the presence of rIL-25 (Fig 2.6E-F). These data suggested that rIL-25 induces type 2 responses during *E. histolytica* infection. We also measured the inflammatory cytokines IL-23, IL-17 and TNFα which are known to be suppressed by IL-25. These cytokines were decreased in the cecal tissue lysate and cecal contents of rIL-25 treated mice (Fig. 2.7 A-F).

Eosinophils were important for IL-25 mediated protection against amebiasis. IL-25 is known to induce eosinophilia, and IL-25 induced eosinophilia is the key modulator to protect from pathogenesis in *C. difficile* colitis (Buonomo EL, et al., Cell Reports, in press 2016). The change in eosinophil peroxidase (*Epx*) mRNA expression with IL-25 during amebiasis suggested that eosinophils could be important for protection against amebic colitis (Fig 2.6E). We tested the importance of eosinophils in protection against amebic colitis by depleting them with anti-Siglec-F. Anti-Siglec-F monoclonal antibody or an IgG2a isotype control antibody was administered to IL-25 treated mice.

Figure 2.6: rIL-25 administration increased type 2 cytokines and type 2 responses in *E. histolytica* challenged mice. Cecal tissue was collected from rIL-25 or PBS treated mice 7 days after *E. histolytica* challenge and the cytokines IL-4, IL-5 and IL-9 measured by ELISA (A, B, C). Cecal tissue was collected from rIL-25 or PBS treated mice 1 day after *E. histolytica* challenge and Inducible nitric oxide synthase (*Nos2*), Chitinase3-like 1 (*Chi3l1*) and eosinophil peroxidase (*Epx*) mRNA were measured (D, E, F). *P value less than 0.05.

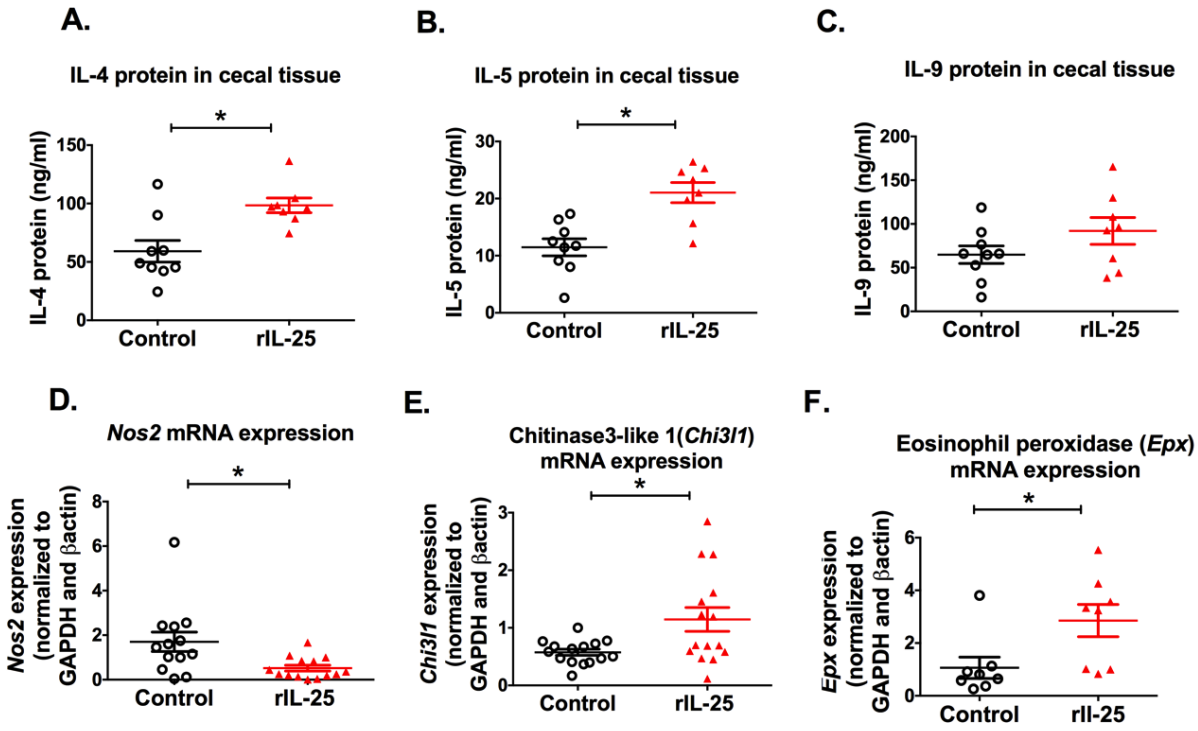
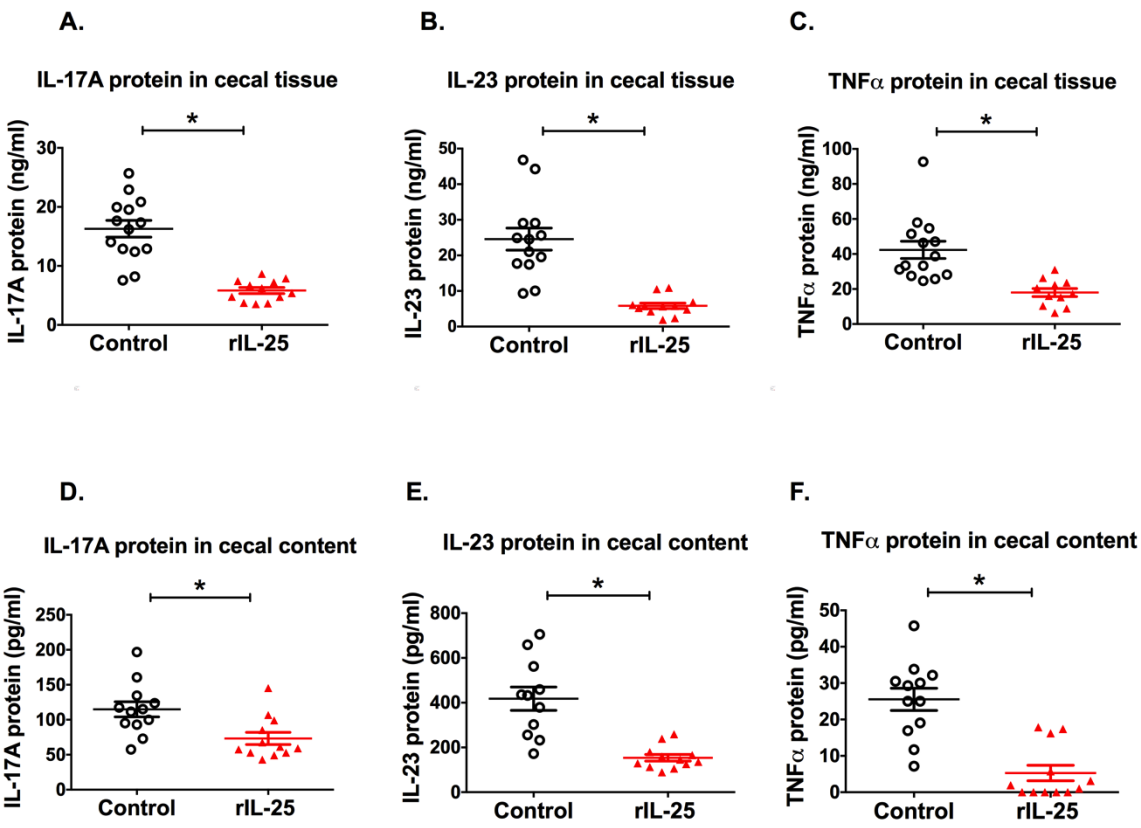

A.
IL-4 protein in cecal tissue
IL-4 protein (ng/ml)
150
100
50
0
*
Control
rIL-25
B.
IL-5 protein in cecal tissue
IL-5 protein (ng/ml)
30
20
10
0
*
Control
rIL-25
C.
IL-9 protein in cecal tissue
IL-9 protein (ng/ml)
200
150
100
50
0
Control
rIL-25
D.
Nos2 mRNA expression
Nos2 expression (normalized to GAPDH and βactin)
8
6
4
2
0
*
Control
rIL-25
E.
Chitinase3-like 1(Chi3l1) mRNA expression
Chi3l1 expression (normalized to GAPDH and βactin)
3
2
1
0
*
Control
rIL-25
F.
Eosinophil peroxidase (Epx) mRNA expression
Epx expression (normalized to GAPDH and βactin)
6
4
2
0
*
Control
rIl-25

Figure 2.7: rIL-25 administration suppressed inflammatory responses in *E. histolytica* challenged mice. Cecal tissue was collected from rIL-25 or PBS treated mice 7 days after *E. histolytica* challenge and the cytokines IL-17A, IL-23 and TNFα (A, B, C) measured by ELISA. Cecal content was collected from rIL-25 or PBS treated mice after 7 days of *E. histolytica* challenge and the amount of IL-17, IL-23 and TNFα was measured by ELISA (D, E, F). *, P value is less than 0.05.


A.
IL-17A protein in cecal tissue
IL-17A protein (ng/ml)
30
20
10
0
*
Control
rIL-25
B.
IL-23 protein in cecal tissue
IL-23 protein (ng/ml)
50
40
30
20
10
0
*
Control
rIL-25
C.
TNFα protein in cecal tissue
TNFα protein (ng/ml)
100
80
60
40
20
0
*
Control
rIL-25
D.
IL-17A protein in cecal content
IL-17A protein (pg/ml)
250
200
150
100
50
0
*
Control
rIL-25
E.
IL-23 protein in cecal content
IL-23 protein (pg/ml)
800
600
400
200
0
*
Control
rIL-25
F.
TNFα protein in cecal content
TNFα protein (pg/ml)
50
40
30
20
10
0
*
Control
rIL-25

The anti-*E. histolytica* effect of rIL-25 was diminished by the depletion of eosinophils (Fig 2.9A-B), which suggested that recruitment of eosinophils is essential for IL-25 induced protection. Eosinophils are known as a major source of IL-4, which plays a role in tissue healing mechanism [97][98]. We found that IL-4 was elevated by rIL-25 treatment, and was diminished by anti-Siglec-F treatment (Fig 2.9C). These findings suggest that eosinophils were a major source of IL-4 induced by IL-25.

TNFα is upregulated during amebic colitis in humans. It is known that TNFα can suppress IL-25 production in the human gut during autoimmune inflammation. Also, the amount of TNFα was increased in the absence of IL-25 in the amebic colitis mouse model [75][92]. In the present study, we found that rIL-25 treatment reduced inflammatory TNFα (Fig 2.7 C, Fig 2.7 F) during amebiasis in mouse. We therefore assessed the amount of TNFα in colon biopsy samples collected from amebic colitis and control patients by immunohistochemistry (Fig 2.10) (patients' characteristics are presented in Table 1). We found that the amount of TNFα protein was higher in amebic colitis patients as assessed by immunohistochemistry. From these results, we hypothesized that suppression of TNFα expression is one possible mechanism by which IL-25 mediates protection against amebiasis.

IL-25 mediated protection was detected on days 4 and 7 after *E. histolytica* challenge and was accompanied with decreased TNFα in mouse model. To find out when phenotypic changes induced by IL-25 happened during *E. histolytica* infection, we treated mice with rIL-25 or PBS and euthanized mice on different days after *E. histolytica* challenge. There was no difference in *E. histolytica* DNA levels in cecal content at 1 day

Figure 2.8: Time course of rIL-25 and anti-Siglec-F intraperitoneal administration and *E. histolytica* challenge in CBA/J mice. Mice were injected daily intraperitoneally with 0.5 µg recombinant IL-25 starting 4 days prior to challenge for a total 8 days. 20 µg anti-Siglec-F or an isotype control mAb was administered intraperitoneally on alternate days from 1 day prior to *E. histolyti*ca challenge to 3 days after challenge (3 doses). Mice were euthanized 7 days after *E. histolytica* challenge.

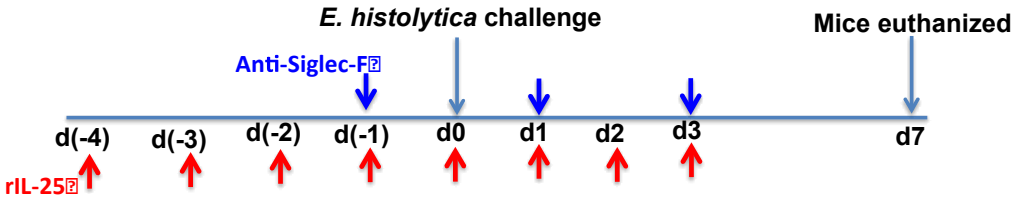
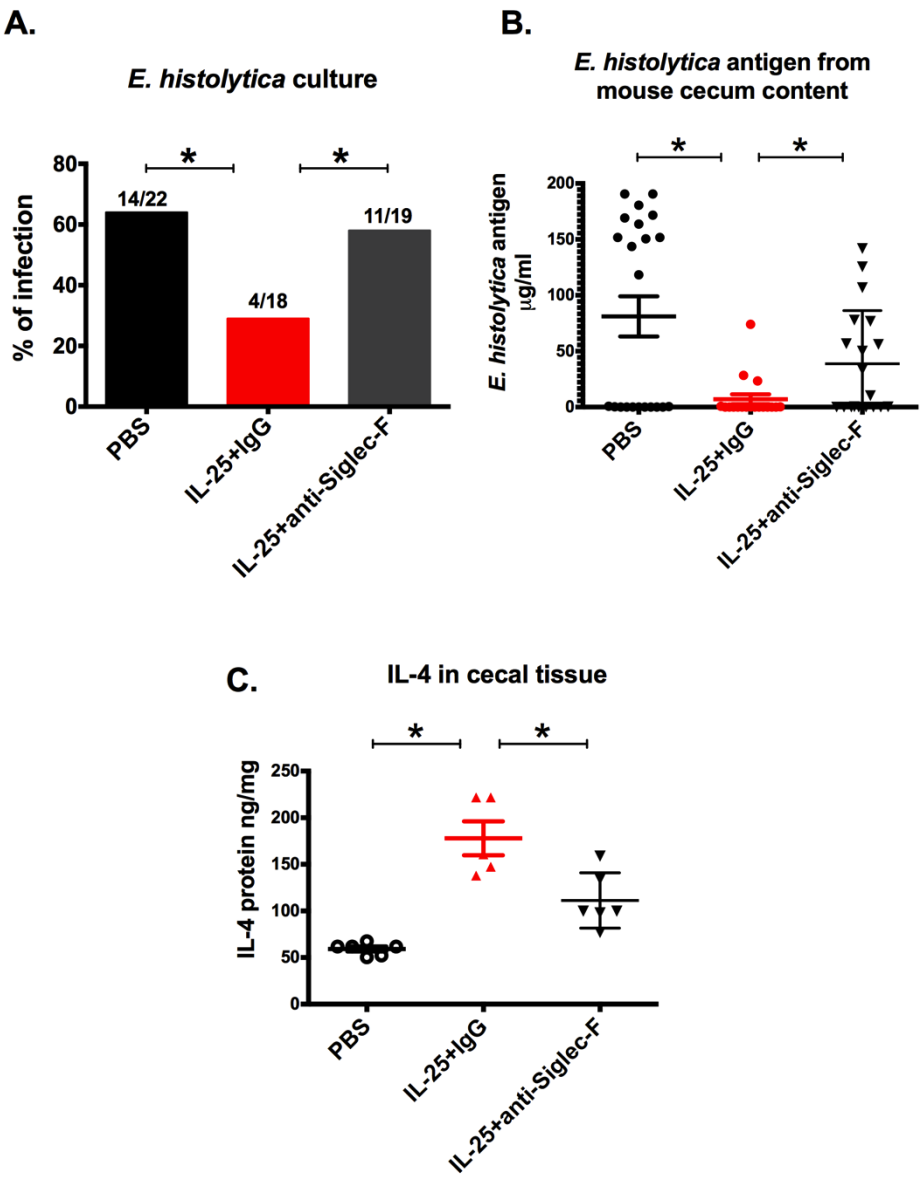

E. histolytica challenge
Mice euthanized
Anti-Siglec-F
d(-4)
d(-3)
d(-2)
d(-1)
d0
d1
d2
d3
d7
rIL-25

Figure 2.9: IL-25 dependent protection against amebiasis was eosinophil-dependent. Mice were injected intraperitoneally with 0.5 µg recombinant IL-25 daily starting 4 days prior to challenge for a total 8 days. 20 µg anti-Siglec-F or an isotype control mAb was administered intraperitoneally on alternate days from 1 day prior to *E. histolyti*ca challenge to 3 days after challenge (3 doses). Mice were euthanized 7 days after *E. histolytica* challenge (day 12) and cecal contents were harvested to evaluate infection by (A) culture and (B) *E. histolytica* antigen detection. (C) IL-4 level measured from cecal tissue lysate with or without eosinophil depletion.*P value less than 0.05.


A.
E. histolytica culture
% of infection
80
60
40
20
0
*
*
14/22
4/18
11/19
PBS
IL-25+IgG
IL-25+anti-Siglec-F
B.
E. histolytica antigen from mouse cecum content
E. histolytica antigen µg/ml
200
150
100
50
0
*
*
PBS
IL-25+IgG
IL-25+anti-Siglec-F
C.
IL-4 in cecal tissue
IL-4 protein ng/mg
250
200
150
100
50
0
*
*
PBS
IL-25+IgG
IL-25+anti-Siglec-F

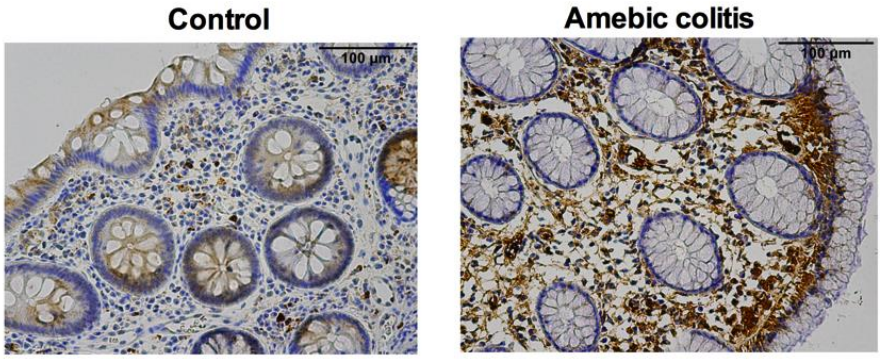
E. histolytica challenge between rIL-25 treated and PBS treated mice. However, rIL-25 treated mice contained lower amounts of *E. histolytica* DNA on days 4 and 7 after *E. histolytica* challenge (Fig 2.11A). TNFα was decreased in both day 4 and day 7 in the presence of rIL-25 (Fig 2.11B).

Anti-TNFα protected mice from amebiasis. We hypothesized that IL-25 induced suppression of TNFα was one possible mechanism of protection against amebiasis in the mouse model. To test this hypothesis, we neutralized TNFα by use of a monoclonal antibody in CBA/J mice and then infected them with *E. histolytica* intracecally. We confirmed lower infection rate by culture (Fig 2.13A) and lower antigen load by ELISA (Fig 2.13B) in mice treated with anti-TNFα antibody compared to mice without treatment. These findings (Fig 2.10, Fig 2.11 and Fig 2.13) suggest that inhibition of TNFα protected mice from amebic colitis and that IL-25 suppression of TNFα could be one potential mechanism of the protective action of IL-25.

IL-1β was upregulated in *E. histolytica* infection in humans and in the mouse model. IL-1β has been reported to suppress IL-25 during infection with the helminth *Heligmosomoides polygyrus* [76]. From in vitro tests, we know that co-culture of epithelial cell lines with *E. histolytica* results in the production of IL-1β [35] [36]. We measured the amount of IL-1β in stool collected from children in Bangladesh with amebic diarrhea and also from the stool collected two months before diarrhea and from the stool two months after the diarrhea in their healthy condition without amebic infection. IL-1β was higher during diarrhea (Fig 2.14).

Figure 2.10: TNFα increased during *E. histolytica* infection in humans. (A) Representative photomicrographs of TNFα immunohistochemical staining from human biopsy samples taken from the colon of control and amebic colitis patients. (B) Histological scoring for TNFα in human colon biopsies. Control patients included patients with diarrhea, polyps, and Crohn's disease. *P value less than 0.05.

A.


Control
Amebic colitis
100 µm
100 µm

B.

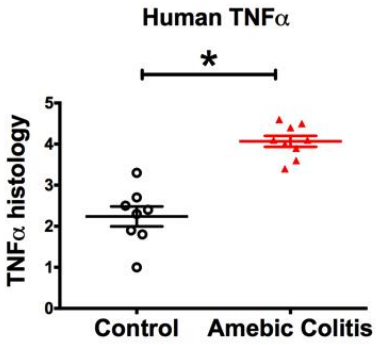
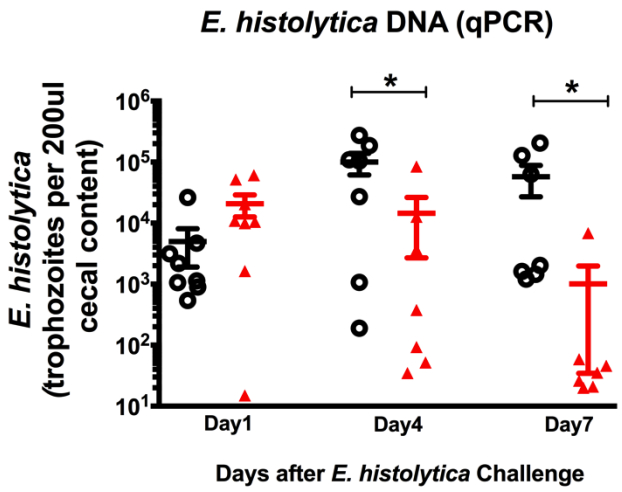

Human TNFα
*
TNFα histology
0
1
2
3
4
5
Control
Amebic Colitis

Figure 2.11: IL-25 mediated protection against amebic colitis was associated with suppression of TNFα in the cecum in the CBA/J mouse model. Mice were injected intraperitoneally with 0.5 µg recombinant IL-25 (closed red triangle) or PBS (open black circle) each day for 4 days prior to infection and 4 days after infection. Mice were euthanized 1, 4 or 7 days after *E. histolytica* challenge. *E. histolytica* DNA detection from cecal content (A) and TNFα measured from cecal tissue lysate (B). *P value less than 0.05.

A.


E. histolytica DNA (qPCR)
E. histolytica (trophozoites per 200ul cecal content)
10^6
10^5
10^4
10^3
10^2
10^1
*
*
Day1
Day4
Day7
Days after E. histolytica Challenge

B.

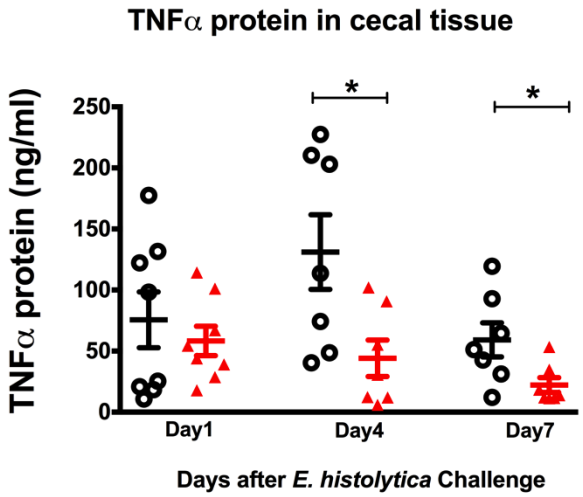

TNFα protein in cecal tissue
TNFα protein (ng/ml)
250
200
150
100
50
0
*
*
Day1
Day4
Day7
Days after E. histolytica Challenge

Figure 2.12: Time course of rIL-25 or anti-TNFα intraperitoneal administration and *E. histolytica* challenge in CBA/J mice. Mice were injected intraperitoneally with an IgG1 isotype control antibody, with 0.5 μg recombinant IL-25 daily, or 0.5 μg anti-TNFα intraperitoneally on alternate days from 1 day prior to *E. histolytic*a challenge to 3 days after challenge (3 doses).

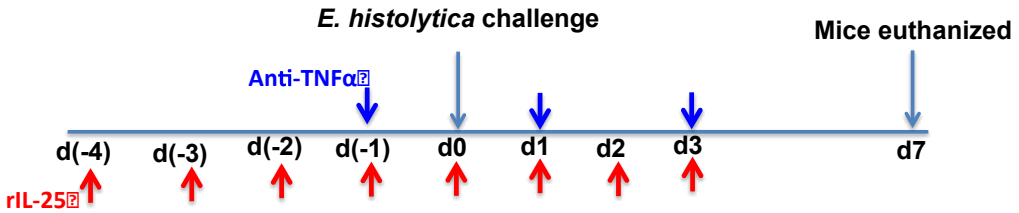
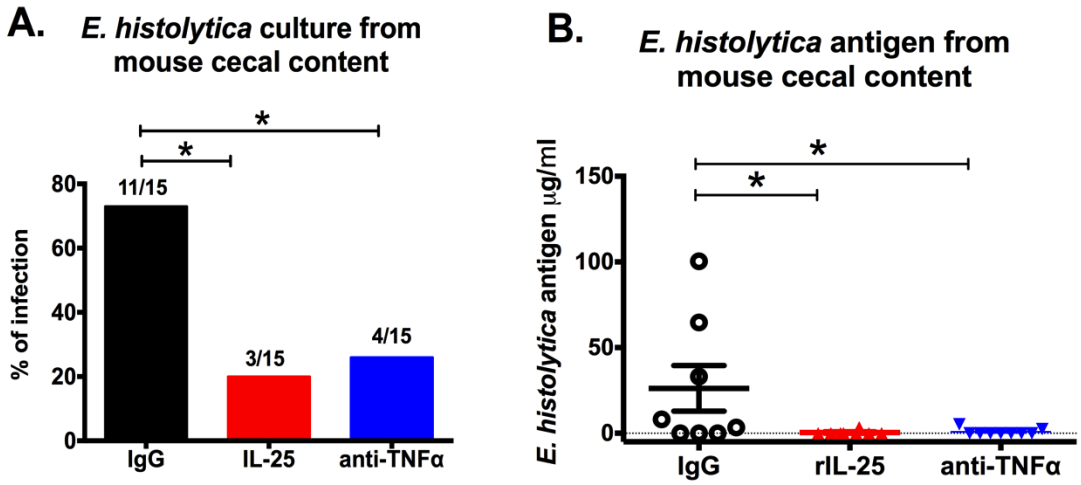

E. histolytica challenge
Mice euthanized
Anti-TNFα
d(-4)
d(-3)
d(-2)
d(-1)
d0
d1
d2
d3
d7
rIL-25

Figure 2.13: Anti-TNF treatment protected mice from amebiasis. Mice were injected intraperitoneally with an IgG1 isotype control antibody, 0.5 μg recombinant IL-25 daily, or 0.5 μg anti-TNFα intraperitoneally on alternate days from 1 day prior to *E. histolytic*a challenge to 3 days after challenge (3 doses). Mice were euthanized 7 days after *E. histolytica* challenge. (A) *E. histolytica* culture positivity and (B) *E. histolytica* antigen in cecal content. *P value less than 0.05.


A.
E. histolytica culture from mouse cecal content
% of infection
80
60
40
20
0
11/15
3/15
4/15
IgG
IL-25
anti-TNFα
*
*
B.
E. histolytica antigen from mouse cecal content
E. histolytica antigen μg/ml
150
100
50
0
IgG
rIL-25
anti-TNFα
*
*

IL-1β level was also increased in *E. histolytica* challenged mice (Fig 2.16 A). We tested for a correlation of IL-25 and IL-1β level in the mouse cecum in *E. histolytica* challenged or sham challenged mice and observed an inverse correlation (Fig 2.16 B). Then we investigated if there was any causality behind this inverse correlation. To test this, we blocked the IL-1β receptor signaling pathway using the IL-1β receptor antagonist anakinra. The IL-25 level from cecal tissue was unchanged by ankinra treatment. This was consistent with IL-1β not being responsible for the decrease in IL-25 observed during amebic infection (Fig 2.16 C).

***E. histolytica* resistant C57BL/6J mice had elevated IL-25 mRNA in cecal tissue compared to susceptible CBA/J mice.** In the mouse model, C57BL/6J mice are resistant and CBA/J mice are susceptible to *E. histolytica* infection. To see if IL-25 production correlated with susceptibility, we measured the amount of IL-25 mRNA in these two genetic backgrounds and found that the expression of IL-25 mRNA was higher in C57BL/6J mice than in CBA/J mice. When CBA/J mice were challenged with *E. histolytica* the IL-25 level decreased in *E. histolytica* infected mice as we also have seen in Figure 2.2. However in C57BL/6J mice, the IL-25 level did not decrease after *E. histolytica* challenge (Fig 2.17A). Our data suggested higher level of IL-25 in C57BL/6J mice could make mice resistant to amebic infection. Recently it has been reported that intestinal epithelial tuft cells are a major source of IL-25 and that tuft cell differentiation is induced by IL-13 [60]. We measured the expression of IL-13 mRNA in cecal tissue in these two genetic backgrounds and found down-regulation of IL-13 in CBA/J mice compared to C57BL/6J mice (Fig 2.17B). It could be that the higher level of IL-13 in C57BL/6J mice induced tuft cell expansion and resulted in higher amount of IL-25 expression in C57BL/6J mice than in CBA/J mice.

Figure 2.14: IL-Iβ is elevated in symptomatic amebiasis in humans. The amount of IL-1β protein measured by ELISA in stool collected from 26 children at 2 months before diarrhea (*E. histolytica* negative), during diarrhea (*E. histolytica* positive) and 2 months after diarrhea (*E. histolytica* negative). *P value less than 0.05.

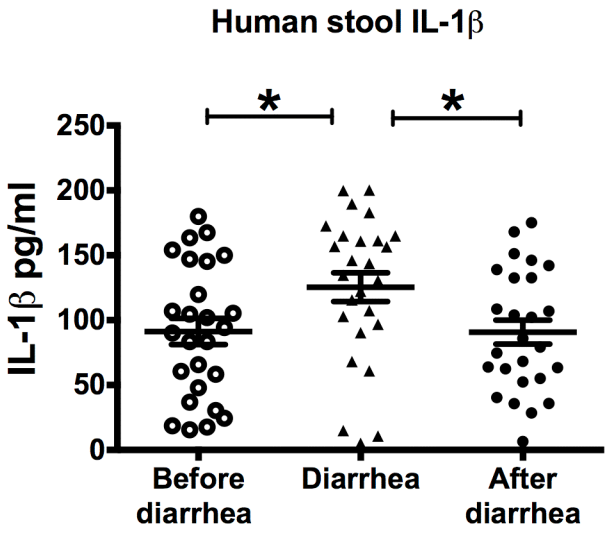

Human stool IL-1β
*
*
250
200
150
100
50
0
IL-1β pg/ml
Before diarrhea
Diarrhea
After diarrhea

Figure 2.15: Time course of IL-1β receptor antagonist anakinra intraperitoneal administration and *E. histolytica* challenge in CBA/J mice. Mice were treated with anakira 50 mg/kg body weight per day and doses were given every 8 hours. Two doses were given prior to *E. histolytica* infection and doses continued until day 4 after infection. Mice were euthanized after 4 days of infection.

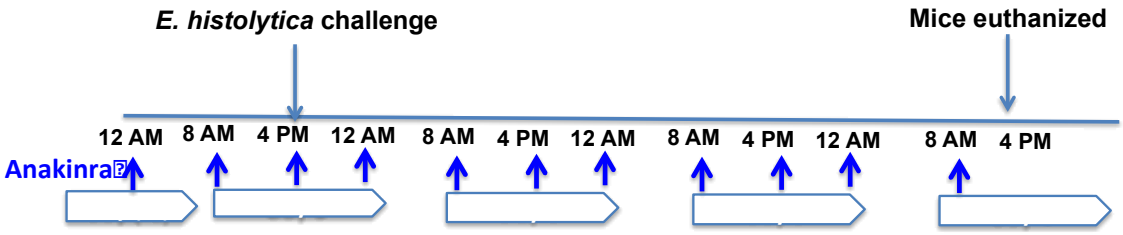
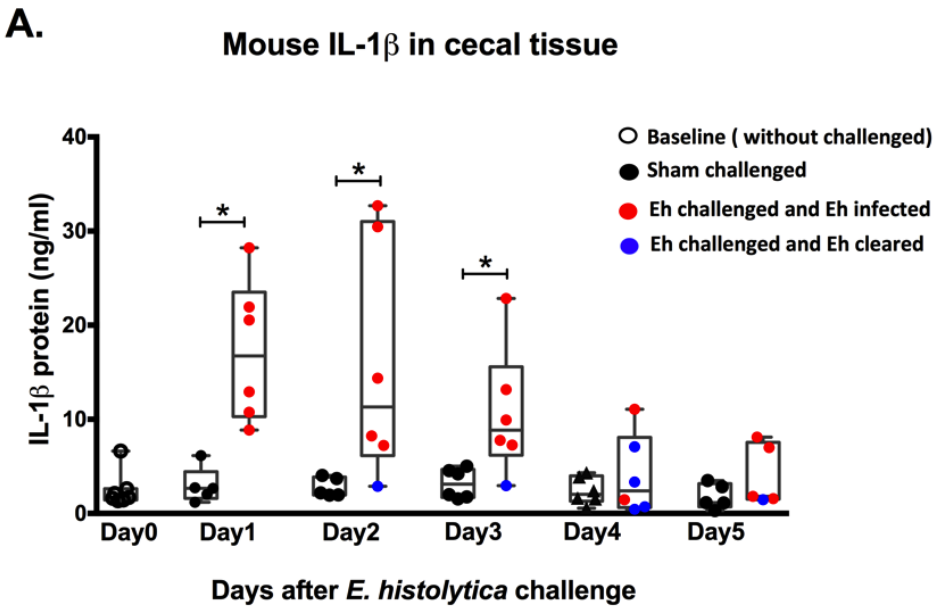
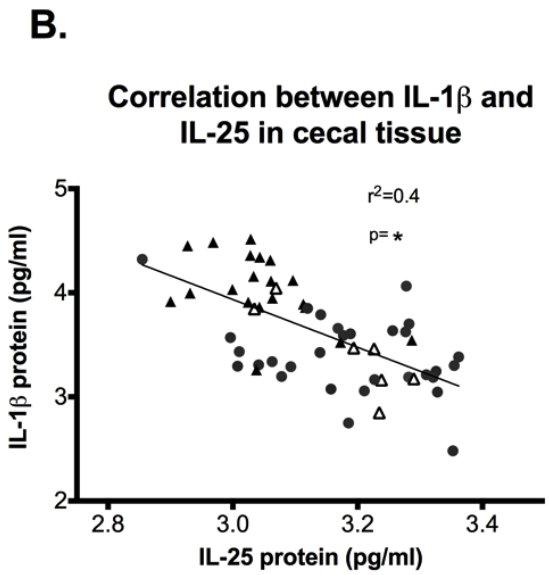

E. histolytica challenge
Mice euthanized
12 AM
8 AM
4 PM
12 AM
8 AM
4 PM
12 AM
8 AM
4 PM
12 AM
8 AM
4 PM
Anakinra

Figure 2.16: IL-Iβ is upregulated in symptomatic infection in mouse model. (A) IL-1β protein measured in cecal tissue lysates of mice before (day 0) and after *E. histolytica* cecal challenge (closed black circle is sham challenged, closed red circle is *E. histolytica* challenged and infected, closed blue circle is *E. histolytica* challenged and cleared mice). (B) Correlation between the amounts of IL-1β and IL-25 in cecal tissue in mice (closed triangle is E. *histolytica* challenged and infected mice, open triangle is *E. histolytica* challenged and uninfected mice, and closed circle is sham challenged mice). (C) Mice were treated with anakira (50 mg/kg body weight per day) every 8 hours. Two doses were given prior to *E. histolytica* infection and doses continued until day 4 after infection. Mice were euthanized after 4 days of infection and the amount of IL-25 was measured from cecal tissue by ELISA. *P value less than 0.05.


A.
Mouse IL-1β in cecal tissue
IL-1β protein (ng/ml)
40
30
20
10
0
Day0
Day1
Day2
Day3
Day4
Day5
Days after E. histolytica challenge
Baseline (without challenged)
Sham challenged
Eh challenged and Eh infected
Eh challenged and Eh cleared
*
*
*


B.
Correlation between IL-1β and IL-25 in cecal tissue
IL-1β protein (pg/ml)
5
4
3
2
2.8
3.0
3.2
3.4
IL-25 protein (pg/ml)
r2=0.4
p= *

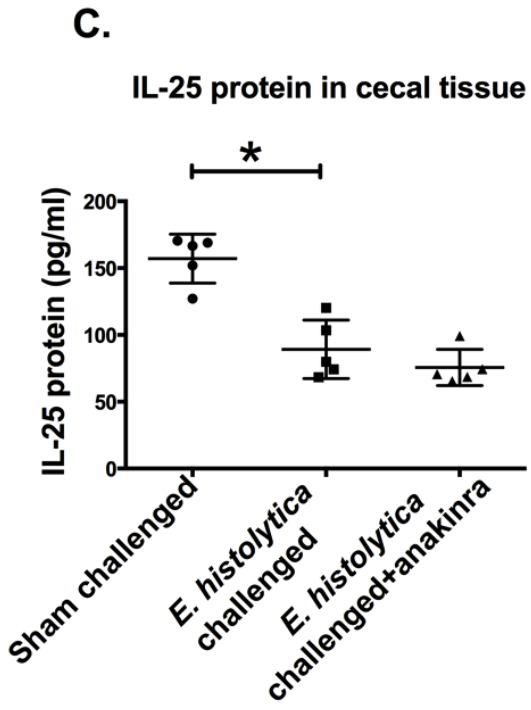
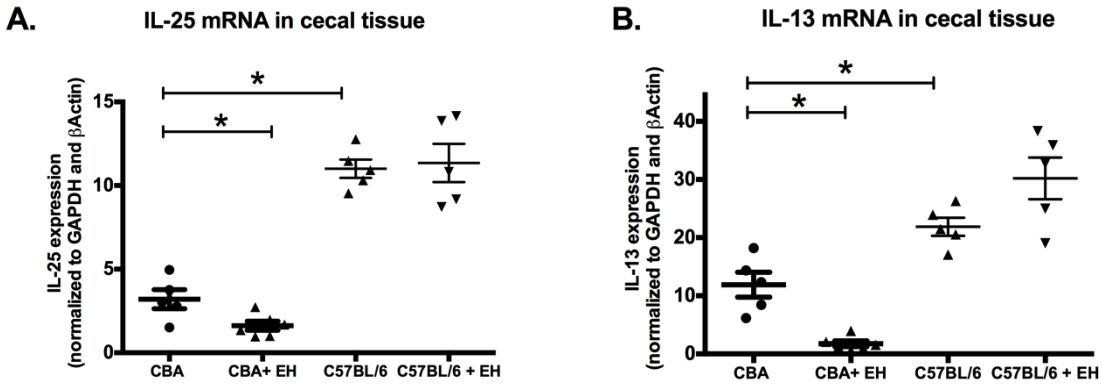

C.
IL-25 protein in cecal tissue
IL-25 protein (pg/ml)
200
150
100
50
0
*
Sham challenged
E. histolytica challenged
E. histolytica challenged+anakinra

Figure 2.17: *E. histolytica* resistant C57BL/6J mice had higher cecal IL-25 and IL-13 than susceptible CBA/J mice. IL-25 and IL-13 expression was measured in cecal tissue in *E. histolytica* challenged and sham challenged mice at day 4 in different genetic backgrounds. *P value less than 0.05.


A.
IL-25 mRNA in cecal tissue
IL-25 expression
(normalized to GAPDH and βActin)
15
10
5
0
*
*
CBA
CBA+ EH
C57BL/6
C57BL/6 + EH
B.
IL-13 mRNA in cecal tissue
IL-13 expression
(normalized to GAPDH and βActin)
40
30
20
10
0
*
*
CBA
CBA+ EH
C57BL/6
C57BL/6 + EH

2.4 Discussion

We have found that IL-25 in the gut is protective against amebiasis. It has also been reported that microbiota regulated IL-25 had a protective role against *Clostridium difficile* infection, and that IL-25 mediated protection required eosinophils (Buonomo EL, et al., Cell Reports, in press 2016). In agreement with this finding we discovered that IL-25 protected mice from amebiasis via eosinophils. IL-25 reduced the number *E. histolytica* cells and antigen load in the cecum after challenge. In contrast, in the *C. difficile* model reported by Buonomo et al., rIL-25 administration provided protection by acting on host tissue, not on bacterial burden. Thus IL-25 induced eosinophils may act differently in defense against *E. histolytica* than *C. difficile*.

We have observed that IL-25 is suppressed in humans and mice with amebiasis. It was previously reported that IL-25 is also suppressed in C. *difficile* infection and in germ-free mice (Buonomo EL, et al., Cell Reports, in press 2016) [77]. While not formally demonstrated in our studies, the mechanism of IL-25 suppression may be via *E. histolytica* mediated disruption of the microbiome since IL-25 is regulated in part by the microbiome [77]. Another mechanism of suppression could be via IL-1β. During helminth infection, it was reported that IL-1β maintains *Heligmosomoides polygyrus bakeri* chronicity by suppressing IL-25 and IL-33 [76]. We previously demonstrated in in vitro experiments that *E. histolytica* rapidly induced IL-1β secretion from intestinal epithelial cells (IECs) [100]. Therefore, we hypothesized that *E. histolytica* induces IL-1β secretion resulting in suppression of IL-25 secretion. We detected increased IL-1β levels in cecal tissue in *E. histolytica* challenged mice when compared to sham challenged mice. Also, we demonstrated that IL-25 levels were inversely correlated with IL-1β levels in mouse cecal tissue. However, no differences were detected in IL-25 levels by disrupting the IL-1β

signaling pathway using the IL-1β receptor antagonist anakinra, suggesting that IL-1β is not the mechanism of IL-25 suppression.

In the present study, depletion of eosinophils from rIL-25 treated mice made mice as susceptible to *E. histolytica* infection as untreated mice, which suggested that eosinophils play a central role in the cellular mechanism of IL-25 mediated protection. However, it is not clear how eosinophils provide protection against amebic colitis. In the DSS colitis model, eosinophils protect against intestinal pathology by decreasing inflammatory mediators via attenuated neutrophil infiltration [101]. Eosinophils also are capable of inducing rapid wound healing responses by pathogen disruption [96]. However, further experiments are required to explore the mechanism of how eosinophils provide protection in the IL-25 pathway.

We hypothesized that IL-25 cause an influx of eosinophils that ultimately function to suppress inflammation to provide protection against amebic colitis. IL-25 mediated protection in amebiasis was associated with a shift from a proinflammatory response to type 2 immunity. We have found that rIL-25 administration in mice suppressed expression of inflammatory cytokines (IL-23, IL-17 and TNFα) and induced type 2 cytokines (IL-4, IL-5). The proinflammatory cytokine TNFα plays a crucial role in intestinal inflammation during amebic colitis. In the present study, we found that TNFα level assessed in human colon biopsy specimens was higher in amebic colitis compare to control patients. Also, administration of rIL-25 decreased inflammatory cytokine levels, including TNFα, in the mouse model during amebic colitis. TNFα depletion by monoclonal antibody made mice resistant to *E. histolytica* infection. Peterson *et. al*., has reported that amebic diarrheal episodes are positively correlated with an increased amount of TNFα levels in the blood

[46]. In a study of experimental autoimmune encephalomyelitis, it was reported that IL-25 knockout mice had increased amounts of IFNγ, IL-17 and TNFα [92]. Our results suggest eosinophil suppression of TNFα expression is a possible mechanism of IL-25 mediated protection.

In conclusion, we have found that IL-25 is suppressed during amebic colitis through an apparently IL-1β independent pathway. Administration of IL-25 in a mouse model reduced the number of *E. histolytica* trophozoites and epithelial disruption in the cecum. From these results, we concluded that IL-25 mediated protection was eosinophil dependent. We have also demonstrated that eosinophils may protect by suppressing TNFα, as IL-25 suppressed TNFα levels, and neutralization of TNFα prevented amebic colitis.

Chapter Three:

Interleukin-25 mediated induction of angiogenin-4 is interleukin-13 dependent

Part of this chapter has been adapted from "Interleukin-25 Mediated Induction of Angiogenin-4 Is Interleukin-13 Dependent". Noor Z, Burgess SL, Watanabe K, Petri WA Jr. PLoS One. 2016;11:e0153572. doi: 10.1371/journal.pone.0153572.

3.1 Introduction

The intestinal epithelial layer provides a physical barrier that separates commensal and pathogenic microorganisms from submucosal tissue. It maintains homeostatic relationships between the host and commensal microorganisms by means of limiting antigenic and pathogenic exposure. Epithelial cells play an important role in this intestinal homeostasis by secreting cytokines, mucus and antimicrobial peptides. Interleukin-25 is a Th2 associated cytokine often produced alongside IL-4, IL-5, IL-13 and IL-9 [62], [102] . IL-25 is secreted from gut epithelial cells following stimulation by commensal bacteria, and IL-25 suppresses the IL-23-IL-17 axis to control gut inflammation [77]. However, the role and mechanism of IL-25 in induction of antimicrobial peptides has not been clearly defined. Antimicrobial peptides play an important role in control of the commensal bacteria in the gut, and provide defense against pathogens. IL-22, which is induced by IL-23, is well known to trigger the secretion of antimicrobial peptides from Paneth cells [103]. However, it is unlikely that IL-25 acts via IL-23, as IL-23 secretion is suppressed by IL-25 [77]. Previous studies have reported that the Th2 cytokine IL-13 induces Paneth and goblet cells to produce an antimicrobial peptide, angiogenin-4 [104]. Here we show that IL-25 is a potent inducer of the antimicrobial peptide angiogenin-4, and acts in an IL-13 dependent manner. This work investigated the role of angiogenin-4 to protect mice from amebic colitis in IL-25 induced pathway.

Angiogenin-4 induces blood vessel formation and is a member of the ribonuclease family of proteins. Its activity as an antimicrobial peptide is more recently characterized [105]. During *Salmonella* challenge, IL-23 induces IL-22 production which triggers Paneth cells to produce angiogenin-4 [106]. During *Trichuris muris* infection, angiogenin-4 expression is correlated with worm expulsion [107]. During *Trichinella spiralis* infection, worm expulsion is accompanied by IL-25 mediated host protection and IL-25 induces

angiogenin-4 expression [108]. Angiogenin-4 is well known as a Paneth cell-derived antimicrobial peptide, however it is also known that it is produced by goblet cells during *Trichuris muris* infection under control of IL-13 [104]. However, there is not clear evidence that explains how IL-25 induces angiogenin-4 production. Here, we demonstrate that IL-25 induces angiogenin-4 production in an IL-13 dependent manner, rather than via IL-22 or IL-17.

3.2 Materials and Methods

Mice

Six-week-old male CBA/J mice (Jackson Laboratories) were housed in a specific pathogen–free facility in micro isolator cages and provided autoclaved food (Lab diet 5010) and water ad libitum. The University of Virginia Institutional Animal Care and Use Committee approved all procedures.

Recombinant IL-25 or rIL-13 treatment and cecal tissue collection

Mice were injected intraperitoneally with 0.5 micrograms of recombinant IL-25 (RnD system) or PBS in a 100 microliter volume each day for 4-10 days. Recombinant IL-13 was injected each day for a total of four doses. Mice were harvested to collect cecal tissue.

E. histolytica challenge and infection evaluation

The trophozoites used for the *E. histoytica* challenge were initially originated from lab-derived strain HM1:IMSS (ATCC, VA) and sequentially passaged in vivo by injection into the CBA mouse cecum. Trophozoites were grown to log phase and laparotomy was used to challenge mice intracecally with two million trophozoites in 150 µl of media. Mice were harvested 7 days after *E. histolytica* challenge and cecal contents were collected to evaluate

infection by culture, *E. histolytica* antigen detection and *E. histolytica* DNA detection. 300 µl of cecal contents were cultured in complete TYI-S-33 medium with supplemental antibiotics for 3 days at 37 °C.

Quantitative real-time RT-PCR

Total RNA was isolated from cecal tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was generated using the tetro cDNA synthesis kit (Bioline USA Inc. USA). Mouse angiogenin-4 and IL-13 gene expression was measured by real-time PCR using Sybr green with normalization to expression of the mouse house keeping genes βActin and GAPDH. IL-13 primers were purchased from Qiagen (Hilden, Germany). Angiogenin-4 primer sequences were: Angiogenin-4 forward: 5'-TTGGCTTGGCATCATAGT -3', Angiogenin-4 reverse: 5'-CCAGCTTTGGAATCACTG -3', Data were normalized with house keeping gene βActin; βActin Forward: 5'- AGCCATGTACGTAGCCATCC-3', βActin Reverse: 5'-CTCTCAGCTGTGGTGGTGAA -3', and GAPDH; GAPDH Forward: 5'-TGCACCACCAACTGCTTAGC -3', GAPDH Reverse: 5'-GGCATGGACTGTGGTCATGAG -3'. Primers were purchased from Integrated DNA Technologies Coralville, Iowa, USA.

Immunohistochemistry and scoring

Cecum tissue were fixed with Bouin's solution (Sigma-ALDRICH, St. Louis, MO) and paraffin embedded sections of cecum were cut into four micron histologic sections, placed on charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Then slides were deparaffinized and antigen retrieval was performed in PT Link instrument (Dako,

Glostrup, Denmark) at 97 ^{o}C for 20 minutes in low pH antigen retrieval solution. Immunohistochemistry was done on a robotic platform (Autostainer, Dako). Endogenous peroxidases were blocked using Peroxidase and Alkaline Phosphatase Blocking Reagent (Dako). Polyclonal rabbit antibody to Angiotensin 4 (obtained from Dr. Lora Hooper, Univ. Texas Southwestern Medical Center, Dallas, TX) was diluted 1:2,000, and applied at ambient temperature for 60 minutes. Antibody binding was visualized by incubation with Envision^{TM} Rabbit Link (Dako) and then incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB+). All the slides were counterstained with hematoxylin subsequently; they were dehydrated, cleared and mounted for the assessment. Scoring was based on intensity and abundance of angiogenin-4 staining and was done blindly by three independent scorers. The staining scale was between 0-5.We considered the percent of visual field that had intense brown staining within one or two villi. If this occurred in 81-100%, of the field then it was scored as 5. If this occurred in 61--80%, of the field then it was scored as 4. If this occurred in 41-60%, of the field then it was scored as 3. If this occurred in 21-40%, of the field then it was scored as 2. If this occurred in 1-20%, of the field then it was scored as 1. The samples that have no staining scored as 0. There are randomly two different fields chosen from each sample.

Antibody Neutralization

6 week old male CBA/J mice were treated with 0.5 micrograms of recombinant IL-25 each day for a total of 7 doses and control mice received PBS. Recombinant IL-25 treated mice received 200μg anti IL-17 (Amgen) or 200μg anti IL-22 antibody (Genentech) or 200μg anti-IL-13 antibody (Genentech) or isotype control on day 3, on day 5 and on day 7. Mice were euthanized one day after the last injection.

Statistical analysis

Student's *t*-test or Mann-Whitney non-parametric *t*-test was used for comparisons between two groups. *P* values of less than 0.05 were considered significant. Statistical analysis was presented using GraphPad Prism, GraphPad Software, San Diego California, USA. All experiments are representative of at least two independent replicates.

3.3 Results

rIL-25 administration induces angiogenin-4 expression in a dose dependent manner. In order to test if IL-25 induces angiogenin-4 production, we treated CBA/J mice with recombinant IL-25 (rIL-25), and then we measured angiogenin-4 encoding mRNA expression in mouse ceca by qPCR. We found that angiogenin-4 expression was more than 100-fold higher in rIL-25 treated mice than in PBS treated mice (Fig 3.1A). We confirmed this pattern of expression by performing immunohistochemistry for angiogenin-4 in cecal tissue. Angiogenin-4 protein expression was highly upregulated in rIL-25 treated mice with expression observed in intestinal epithelial cells in the crypts and villi (Figs 3.1B-3.1D). We then tested for a dose dependent induction of angiogenin-4 by rIL-25. We found that 4 vs 8 doses of rIL-25 induced a 7-fold and 218-fold increase in angiogenin-4 compared to the PBS control (Fig 3.2). We concluded that IL-25 induced angiogenin-4 expression in the cecal intestinal epithelium.

rIL-25 induces angiogenin-4 in an IL-13 dependent manner. IL-25 is known to induce Th2 cytokines, including IL-13. Therefore, the role of IL-13 in the ability of IL-25 to induce angiogenin-4 was examined. CBA/J mice were treated with rIL-25 (closed square, n=8) and control mice received PBS (closed circle, n=7). IL-13 encoding mRNA relative

Figure 3.1: rIL-25 administration induces angiogenin-4 expression. CBA/J mice were treated with 0.5 micrograms of recombinant IL-25 (triangle, n=11) each day for a total of 10 doses over 10 days. Control mice received PBS (open circle, n=11). (A) *Ang4* mRNA expression was measured from mouse cecal tissue and relative to to *Gapdh* and *Actb* mRNA. (B) Histological scoring (1 to 5; low to high) for Angiogenin-4 in cecum from mice treated with PBS or rIL-25. Representative IHC staining for angiogenin-4 in samples of cecum tissue from PBS treated (C) or rIL-25 treated mice (D).

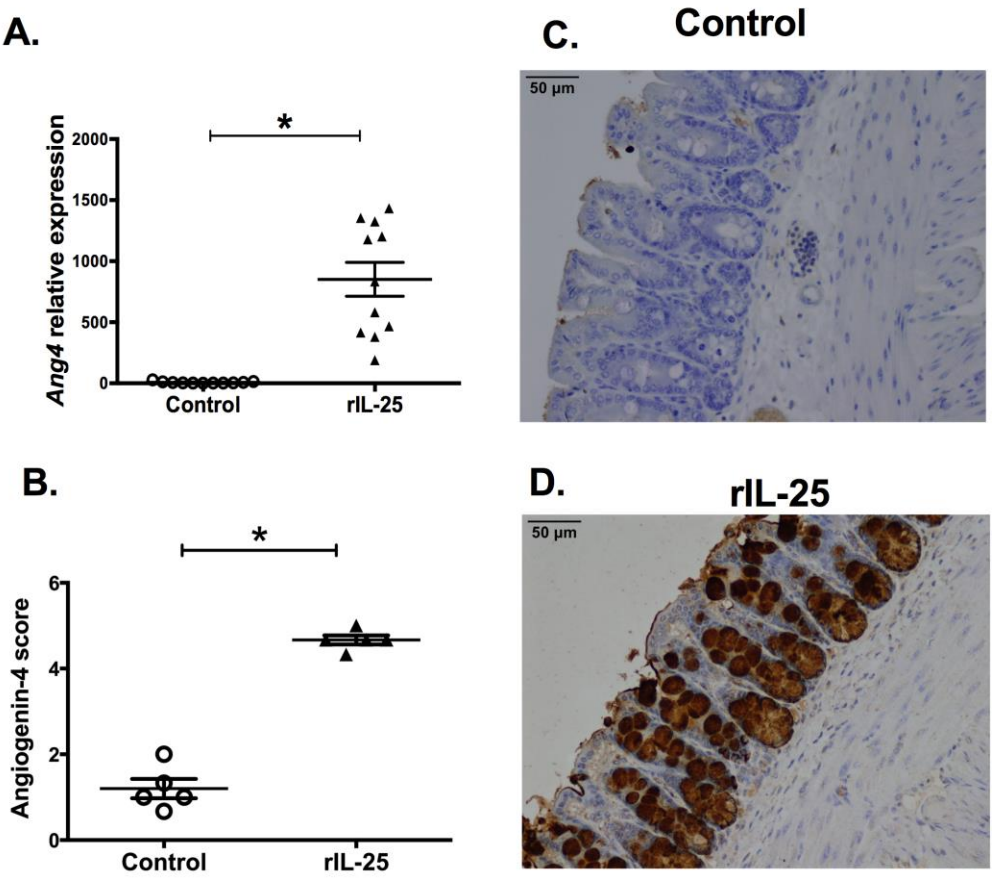

A.
*
Ang4 relative expression
2000
1500
1000
500
0
Control
rIL-25
B.
*
Angiogenin-4 score
6
4
2
0
Control
rIL-25
C.
Control
50 µm
D.
rIL-25
50 µm

Fig 3.2: rIL-25 administration increases angiogenin-4 expression in a dose dependent manner. CBA/J mice were treated with 0.5 micrograms of rIL-25 each day for a total 4 doses (triangle, n=7) or 8 doses (inverse triangle, n=5) and control mice received 4 or 8 doses of PBS (open circle, n= 7 for 4 doses and n=5 for 8 doses). *Ang4* mRNA expression was measured from mouse cecal tissue and relative to to *Gapdh* and *Actb* mRNA.

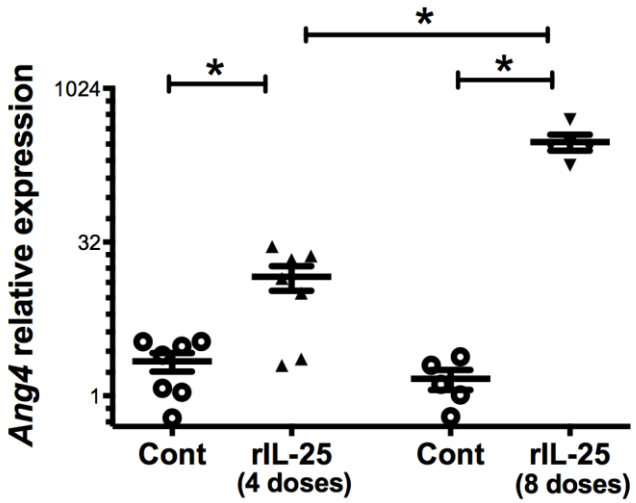

*
*
*
Ang4 relative expression
1024
32
1
Cont
rIL-25
(4 doses)
Cont
rIL-25
(8 doses)

Fig 3.3: rIL-25 administration induces IL-13 expression. CBA/J mice were treated with 0.5 micrograms of recombinant IL-25 (closed square, n=8) each day for a total of 5 doses and control mice received PBS (open circle, n=7). *Il13* mRNA expression was measured from mouse cecal tissue and relative to to *Gapdh* and *Actb* mRNA.

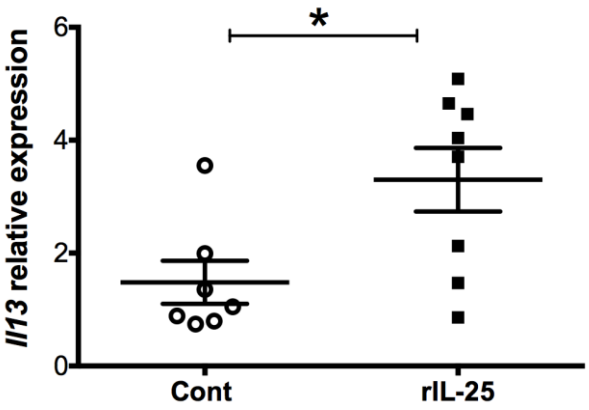

*
6
4
2
0
Il13 relative expression
Cont
rIL-25

Fig 3.4: rIL-13 administration induces angiogenin-4 expression. CBA/J mice were treated with 0.5 micrograms of recombinant IL-13 (triangle, n=5) on each day for total 4 doses. Control mice received PBS (open circle, n=5). (A) *Ang4* mRNA expression was measured from mouse cecal tissue and relative to to *Gapdh* and *Actb* mRNA. Angiogenin-4 relative expression was measured from mouse cecal tissue and normalized with house-keeping gene GAPDH and β actin. (B) Histological scoring (1 to 5; low to high) for Angiogenin-4 in mouse cecum from PBS or rIL-13 treated mice. Representative IHC staining for angiogenin-4 in samples of cecum tissue from PBS treated (C) or rIL-13 treated mice (D).

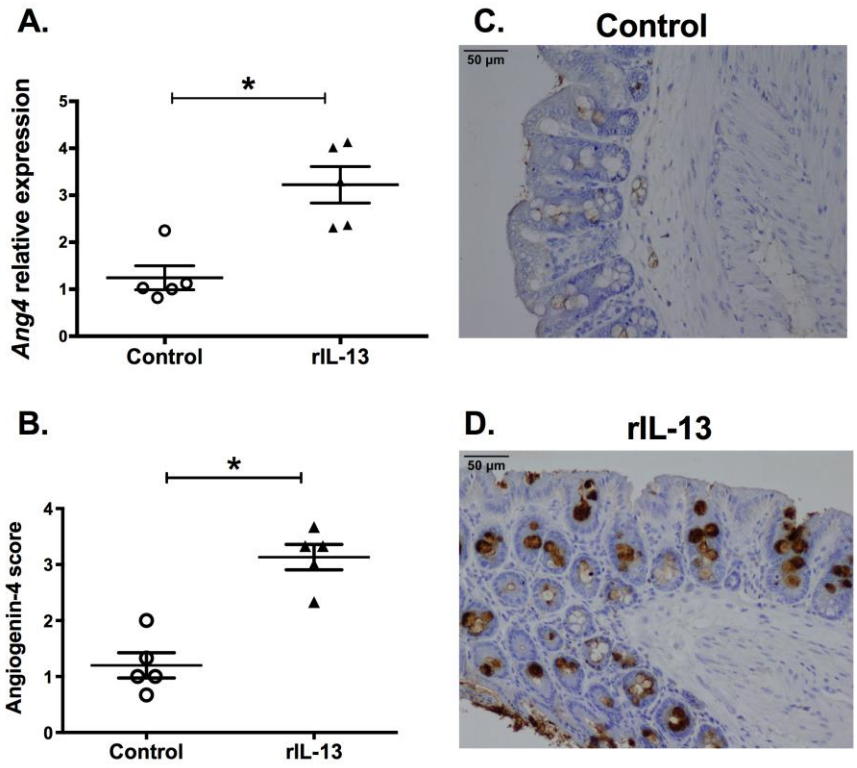

A.
Ang4 relative expression
*
5
4
3
2
1
0
Control
rIL-13
B.
Angiogenin-4 score
*
4
3
2
1
0
Control
rIL-13
C.
Control
50 µm
D.
rIL-13
50 µm

Fig 3.5: Depletion of IL-13 abrogates rIL-25 induction of angiogenin-4. CBA/J mice were treated with 0.5 micrograms of recombinant IL-25 each day for a total 6 doses. Control mice received PBS. Recombinant IL-25 treated mice received 200μg anti-IL-13 antibody or isotype control on day 3 and on day 5. (A) *Ang4* mRNA expression was measured from mouse cecal tissue and relative to to *Gapdh* and *Actb* mRNA. (B) Histological scoring (1 to 5; low to high) for Angiogenin-4 in cecum from mice treated with PBS or rIL-25 with isotype control or rIL-25 with anti-IL-13. Representative IHC staining for angiogenin-4 in samples of cecum tissue from PBS treated (C) or rIL-25 with isotype control (D) or rIL-25 with anti-IL-13 treated mice (E).

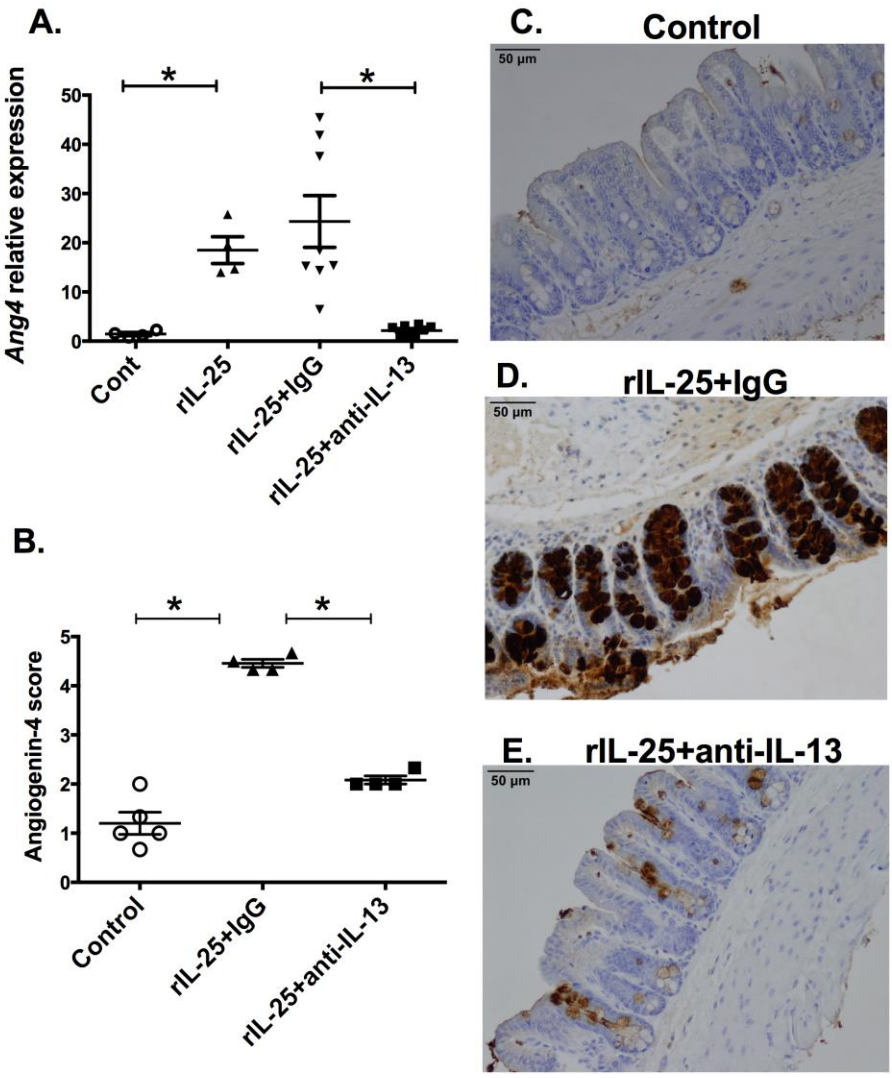
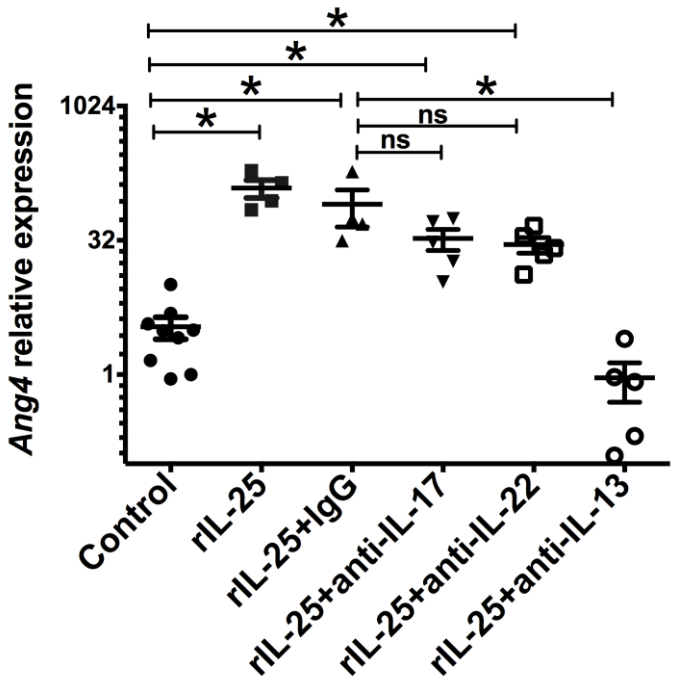

A.
Ang4 relative expression
50
40
30
20
10
0
*
*
Cont
rIL-25
rIL-25+IgG
rIL-25+anti-IL-13
B.
Angiogenin-4 score
5
4
3
2
1
0
*
*
Control
rIL-25+IgG
rIL-25+anti-IL-13
C.
Control
50 µm
D.
rIL-25+IgG
50 µm
E.
rIL-25+anti-IL-13
50 µm

Figure 3.6: rIL-25 induced angiogenin-4 production is not significantly influenced by IL-17 or IL-22 blockade. CBA/J mice were treated with 0.5 micrograms of recombinant IL-25 each day for total 7 doses and control mice received PBS. rIL-25 treated mice received 200µg anti IL-17 antibody (inversed triangle) or 200µg anti IL-22 antibody (open square) or 200µg anti-IL-13 antibody (open circle), or isotype control on day 3, on day 5 and on day 7 . *Ang4* mRNA expression was measured from mouse cecal tissue and relative to to *Gapdh* and *Actb* mRNA.

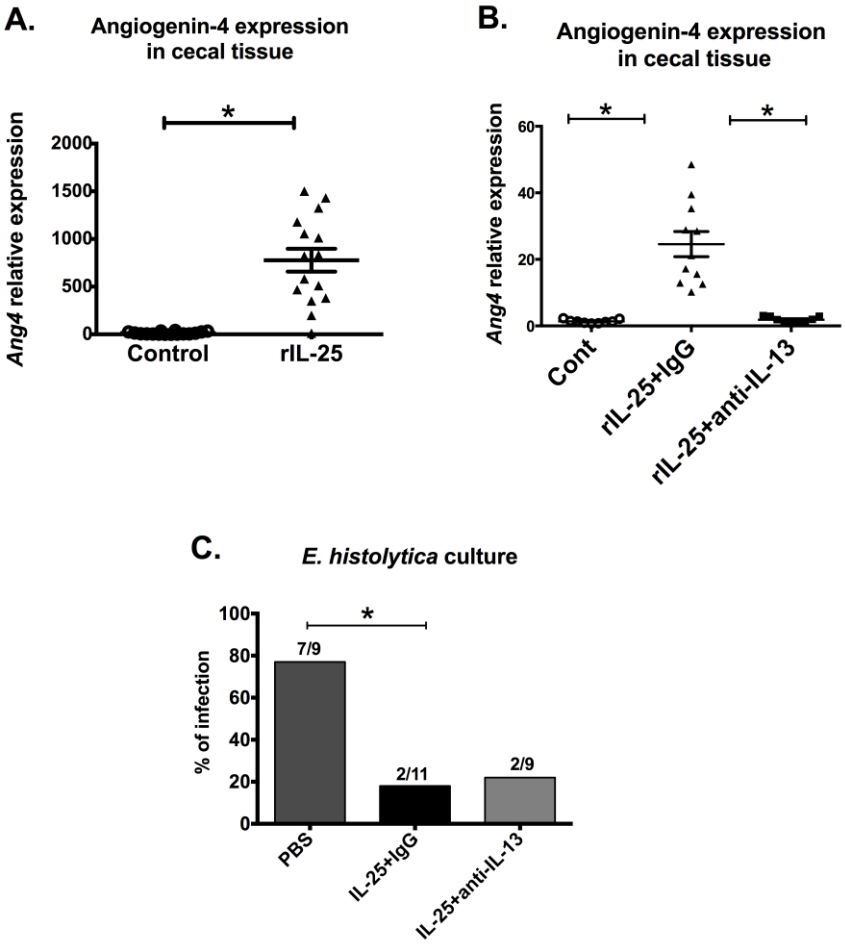

*
*
*
*
*
ns
ns
1024
32
1
Ang4 relative expression
Control
rIL-25
rIL-25+IgG
rIL-25+anti-IL-17
rIL-25+anti-IL-22
rIL-25+anti-IL-13

expression was measured from mouse cecal tissue. We found that there was significantly higher expression of IL-13 in rIL-25 treated mice than that in control mice (Fig 3.3). Previous studies have reported that IL-13 can induce Paneth cell degranulation and trigger the release of the antimicrobial peptide angiogenin-4. We confirmed that IL-13 induced angiogenin-4 encoding mRNA expression in cecal tissue (Fig 3.4A) and angiogenin-4 protein expression in cecal epithelial cells (Figs 3.4B-3.4D). IL-25 mediated angiogenin-4 encoding mRNA expression was abrogated when IL-13 was depleted by neutralizing antibodies (Fig. 3.5A). This result was confirmed via immunohistochemistry of angiogenin-4 as before (Figs 3.5B-3.5E). These results indicated that IL-25 induced angiogenin-4 expression was mediated via cytokine IL-13.

IL-17 and IL-22 do not play major roles in IL-25 mediated angiogenin-4 induction. IL-17 and IL-22 are both known to be potent inducers of angiogenin-4 from Paneth cells during infection. We therefore tested the requirement of IL-22 or IL-17 in IL-25 mediated angiogenin-4 production. We depleted IL-22 or IL-17 by monoclonal antibodies in rIL-25 treated mice. We found that angiogenin-4 encoding mRNA expression was partially decreased by the depletion of either IL-22 or IL-17 in rIL-25 treated mice, whereas IL-13 neutralization completely abrogated IL-25 induced angiogenin-4 expression (Fig 3.6). We conclude that IL-25 induced angiogenin-4 encoding mRNA expression largely depends on induction of IL-13, rather than IL-22 or IL-17, by IL-25.

Angiogenin-4 is likely not the protective factor in IL-25 mediated resistance to amebiasis. We have found that IL-25 induces a robust angiogenin-4 expression during amebic infection (Fig 3.7 A) and that IL-25 mediated angiogenin-4 expression during amebisis was IL-13 dependent. Mice treated with anti-IL-13 did not express angiogenin-4

Figure 3.7: IL-25 induced angiogenin-4 production and protection against amebiasis. A) Angiogenin-4 gene expressionin mice treated with PBS control (open circle) or rIL-25 (closed triangle) 7 days post *E. histolytica* challenge. Angiogenin-4 expression(C) and culture positivity (D) in mice treated with PBS or IL-25 with or without IL-13 neutralization, measured at day 7 post *E. histolytica* infection.


A.
Angiogenin-4 expression in cecal tissue
Ang4 relative expression
2000
1500
1000
500
0
*
Control
rIL-25
B.
Angiogenin-4 expression in cecal tissue
Ang4 relative expression
60
40
20
0
*
*
Cont
rIL-25+IgG
rIL-25+anti-IL-13
C.
E. histolytica culture
% of infection
100
80
60
40
20
0
*
7/9
2/11
2/9
PBS
IL-25+IgG
IL-25+anti-IL-13

during infection (Fig3.7 B). We wanted to know if higher level of angiogenin-4 production play role in IL-25 mediated protection against amebiasis. We have depleted angiogenin-4 production by anti-IL-13 treatment in rIL-25 treated group in *E. histolytica* challenged mice. We have found that IL-25 induce angiogenin-4 encoding mRNA expression has abrogated in the absence of IL-13 but no change in susceptibility (Fig-D). We did not detect a difference in culture positivity in mice lacking angiogenin-4 caused by anti-IL-13 treatment, suggesting angiogenin-4 is not responsible for protection from *E. histolytica*.

3.4 Discussion

IL-25 is known to have a protective role against helminth infections through the induction of a Th2 response. However, the role of IL-25 in antimicrobial peptide induction, which can also play a role in gut barrier protection, has not been well studied. In the present study, we found that both IL-25 and IL-13 induce angiogenin-4 expression in the intestinal epithelium. Antibody-mediated neutralization of IL-13 blocked IL-25-mediated angiogenin-4 induction, demonstrating that IL-13 was acting downstream of IL-25.

A previous report suggested that IL-13 is a key mediator of angiogein-4 production: both IL-13 and IL-4 trigger degranulation of Paneth cells to release antimicrobial peptides [109]. The antimicrobial peptide angiogenin-4 is known to be induced by IL-9 in an IL-13 dependent way. It has also been shown that IL-13 knock out mice with suppressed angiogenin-4 failed to expel *T. muris* [104]. Other cytokines related to IL-25 that are known to induce antimicrobial peptides, include IL-22 and IL-17A [106], [110].

Therefore, we looked to see if angiogenin-4 expression by induced by IL-25 is controlled by IL-22 or IL-17. When we measured angiogenin-4 expression in IL-25 treated and IL-22 or IL-17 neutralized mice, we found that IL-25 induced angiogenin-4 expression

was not decreased. Therefore we concluded IL-25 induced angiogenin-4 encoding mRNA expression mostly depends on induction of IL-13, rather than IL-22 or IL-17.

IL-13 has been shown to play important roles in triggering secretion of antimicrobial peptides from both Paneth cells and goblet cells [104], [109], and both cells are present in the cecum. Therefore, it is possible that IL-25 induces angiogenin-4 production from both cell types, and that IL-13 is needed for this process. Future studies may examine this possibility.

The role of antimicrobial peptides during amebiasis is incompletely understood. A recent study showed the antimicrobial peptide cathelicidin was upregulated during amebic colitis in a murine model. However, *E. histolytica* was resistant to killing by this antimicrobial peptide, perhaps because *E. histolytica* released a cysteine protease that could cleave cathelicidin [111]. In our study, we have observed that rIL-25 induced angiogenin-4 expression during amebic infection and this was IL-13 dependent. In rIL-25 treated mice with IL-13 neutralization angiogenin-4 production was attenuated, but this had no effect on susceptibility. These findings suggest that angiogenin-4 might not have antimicrobial effects on the parasite or, as in the case of cathelicidin, the parasite might have developed ways to degrade the angiogenin-4 protein and evade the immune system. Investigating how the *E. histolytica* parasite avoids killing by antimicrobial peptides would be an interesting area of future study with the potential of identifying novel therapeutic targets.

In conclusion, work described in this section, has identified IL-25 as an inducer of the antimicrobial peptide angiogenin-4 in an IL13-dependent manner. However, rIL-25-treated mice, that did not produce angiogenin-4, were still protected from amebiasis, indicating

that angiogenin-4 is not a significant protective factor in IL-25 mediated resistance to amebiasis. That said, this work enhances our knowledge on the sequence of events that underlies induction of angiogenin-4 by IL-25. The understanding of IL-25 regulation of the antimicrobial peptide angiogenin-4 may contribute to the understanding of its role in intestinal barrier protection, and in the development of therapeutic applications of IL-25 in the treatment of enteric diseases.

Chapter Four

Discussion and future prospectives

4.1 Discussion

The most important finding from our study is that IL-25 – elicited eosinophils protect against amebic colitis. Recently, Buonomo et al., have reported that microbiota regulated IL-25 plays a protective role against *Clostridium difficile* infection through eosinophils (Buonomo EL, et al., Cell Reports, in press 2016). Additionally, Cowardin et al., showed *C. difficile* infection with strains containing CDT (*Clostridium difficile* transferase toxin) cause more severe pathology because they suppress the accumulation of eosinophils in the lamina propria of the colon (Cowardin CA, et al., Nature Microbiology, in press 2016). Consistent with these findings in a bacterial colitis, we have discovered that IL-25 provides protection from amebic colitis in an eosinophil-dependent process. An unexpected aspect therefore in defense from infectious colitis may be protection via type 2 innate immunity. One difference between *C. difficile* and amebic colitis is that *C. difficile* IL-25 acts by reducing host inflammation and does not reduce pathogen burden, whereas for *E. histolytica* IL-25 reduces *E. histolytica* parasites from the cecal lumen. These disparate observations indicate that there may be differences in how eosinophils induced by type 2 immunity act to protect from amebiasis and *C. difficile*.

From Buonomo et al. and Zaph et al., it is well known that the microbiota regulates IL-25 protein expression (Buonomo EL, et al., Cell Reports, in press 2016) [77]. Buonomo et al., has shown that depression of the microbiota with antibiotic treatment suppressed IL-25 production which was followed by further suppression by *C. difficile* infection in the mouse gut. In line with this finding, the microbiota has been shown to be very important in amebiasis, both with the ability to worsen or protect from amebiasis. Alteration of the microbiota by the addition of the commensal *Clostridia*, segmented filamentous bacteria

(SFB), protected mice from amebiasis. This protection could be transferred with bone marrow-derived dendritic cells from an SFB-infected mouse into a naïve mouse and was neutralized by anti-IL17A [51]. The mechanism of SFB-mediated protection therefore appeared to not involve IL-25 but instead a type 17 immune response. In humans, Gilchrist et al., has shown that expansion in the gut microbiome of the bacterium *Prevotella copri* was associated with amebic diarrhea [50]. Prevotella has been demonstrated in other studies to induce both Th1 and Th17-responses, and one could envision that these responses could suppress IL-25 and increase TNFα (see below), and therefore increase susceptibility to amebic diarrhea. From these seemingly contradictory results, a tentative conclusion can still be drawn that there is evidence for the microbiota inducing both type 2 and type 17 immune responses to amebiasis, with evidence for protective roles of both. One could envision that type 17 immune-induced neutrophils act by killing the parasite, while type 2 induced eosinophils act not only by parasite killing but through tissue repair via inhibition of TNFα and by the actions of alternatively activated macrophages.

IL-25 as expected induced type 2 responses in the gut. We found that rIL-25 administration in mice suppressed inflammatory cytokines (IL-23, IL-17 and TNFα) and induced type 2 cytokines (IL-4, IL-5). However, we have not determined whether suppression of inflammatory cytokines was a direct effect of rIL-25. In an indirect manner, it could be possible that a thicker mucous layer induced by rIL-25 protects from tissue invasion by *E. histolytica* which in turn resulted in lower levels of inflammatory cytokines in cecal tissue [6]. Induction of type 2 cytokines and suppression of inflammatory cytokines could also result from conversion of classical macrophages to alternative ones. IL-25 administration caused downregulation of nitric oxide synthase two (*Nos2*), which suggests the presence of type 1 classical macrophages, and upregulation of Chitinase3-like 1

(*Chi3l1*) which suggests the presence of type 2 alternative macrophages. However gene expression of another type 2 macrophage gene, arginase-1 was unchanged. This is consistent with previous studies showing that IL-25 induces the secretion of type 2 cytokines via switching classically activated macrophages to alternatively activated macrophages [112]. However, our study has the limitation that we have assessed these cytokines from cecal tissue samples, not from selectively isolated macrophages. The hypothesis that IL-25 acts to protect from amebiasis through conversion of type 1 to type 2 macrophages therefore remains to be tested.

Intestinal epithelial cells (IECs) play a critical role in defense from *E. histolytica* infection. Epithelial cells are a major source of IL-25 production in the mouse small intestine [60]. In the murine model of *E. histolytica* infection, susceptibility is strain-dependent. Long lasting infection with chronic inflammation is established in susceptible strains (CBA/J and C3H/HeJ), whereas *E. histolytica* is rapidly cleared in resistant strains (C57BL/6, BALB/c and others) [27]. Hamano et al., showed that bone marrow transfer from a CBA/J to a C57BL/6J mouse did not make the C57BL/6J mouse susceptible, indicating that resistance of C57BL/6J mice against *E. histolytica* infection is due to non-hematopoietic cells [28]. From these results, we hypothesized that the resistance of C57BL/6J mice could be because their epithelial cells produce more IL-25 than those of CBA/J mice. In support of this hypothesis, we found that gut IL-25 is higher in C57BL/6J mice than CBA/J mice at steady state. Future work will be required to test if this increase in epithelial IL-25 is responsible for the intrinsic resistance of C57BL/6J mice to amebiasis.

An apparently separate pathway of defense against amebic colitis is STAT3-dependent leptin receptor signaling [25]. This protection acts at the level of the leptin

receptor on intestinal epithelia, as demonstrated by IEC-specific deletion of the receptor, and requires neutrophil recruitment to the colon. Future work will be required to better delineate the roles of these two pathways of granulocyte-mediated protection, eosinophils and neutrophils. What is clear at this time is that depletion of either eosinophils or neutrophils renders animals susceptible to amebiasis.

We discovered that IL-25 is repressed in humans with amebic colitis and in the mouse model. It is also suppressed in C. *difficile* infection and in germ free mice. The mechanism of IL-25 suppression in amebiasis is not known. The mechanism of IL-25 suppression may be via *E. histolytica* disruption of the microbiome. Another mechanism of suppression could be via IL-1β. During helminth infection, it was reported that IL-1β maintains *Heligmosomoides polygyrus bakeri* chronicity by suppressing IL-25 and IL-33 [76]. From *in vitro* tests, we previously demonstrated that *E. histolytica* rapidly induced IL-1β secretion from intestinal epithelial cells (IECs) [29]. Therefore, we hypothesized that *E. histolytica* induces IL-1β secretion resulting in suppression of IL-25 secretion. In fact we observed increased IL-1β levels in cecal tissue in *E. histolytica* challenged mice when compared to sham challenged mice. Also, we demonstrated that IL-25 levels were inversely correlated with IL-1β levels in mouse cecal tissue. However, we failed to observe any differences in IL-25 levels by disrupting the IL-1β signaling pathway using the IL-1β receptor antagonist anakinra, suggesting that IL-1β is not the mechanism of IL-25 suppression, suggesting another still to be identified mechanism of IL-25 suppresssion.

Depletion of eosinophils with anti-Siglec-F abrogated IL-25-dependent protection against amebiasis. The role of eosinophils in amebiasis had not been studied intensively [113]. There was one report where induction of eosinophils, by use of *Toxocara canis*

antigen, reduced amebic liver abscess size and number. Also supporting a role for eosinophils in protection is the observation that degenerated eosinophil products (Charcot-Leiden crystals) are present along with trophozoites in the stool of patients with amebiasis [114]. The eosinophil granule protein eosinophil peroxidase (EPO) is capable of killing *Trichinella spiralis* in vitro, and could be tested in future studies for its amebicidal properties [80] [79]. The mechanism underlying how eosinophils decreased *E. histolytica* burden and maintained barrier function remains unclear. It is suggested that eosinophils protect the host through repair of mucosal tissue [96]. Eosinophils are known as a major source of IL-4, which plays a role in tissue healing mechanism [97][98]. In our study, we found that IL-25 induction of IL-4 levels was blocked when eosinophils were depleted with monoclonal antibodies. Considered together, these findings uggested that eosinophilia following IL-25 secretion activated tissue remodeling pathways through IL-4 during the early stage of disease, which resulted in reduction of parasite load. Although it has not been studied whether IL-25 acts directly on eosinophils, direct stimulation by IL-25 may be possible as the IL-25 receptor IL-25RB has been reported to be expressed on human eosinophils [115][116]. Future investigation is required to understand the cellular and molecular mechanism of the IL-25-eosinophil pathway during amebiasis.

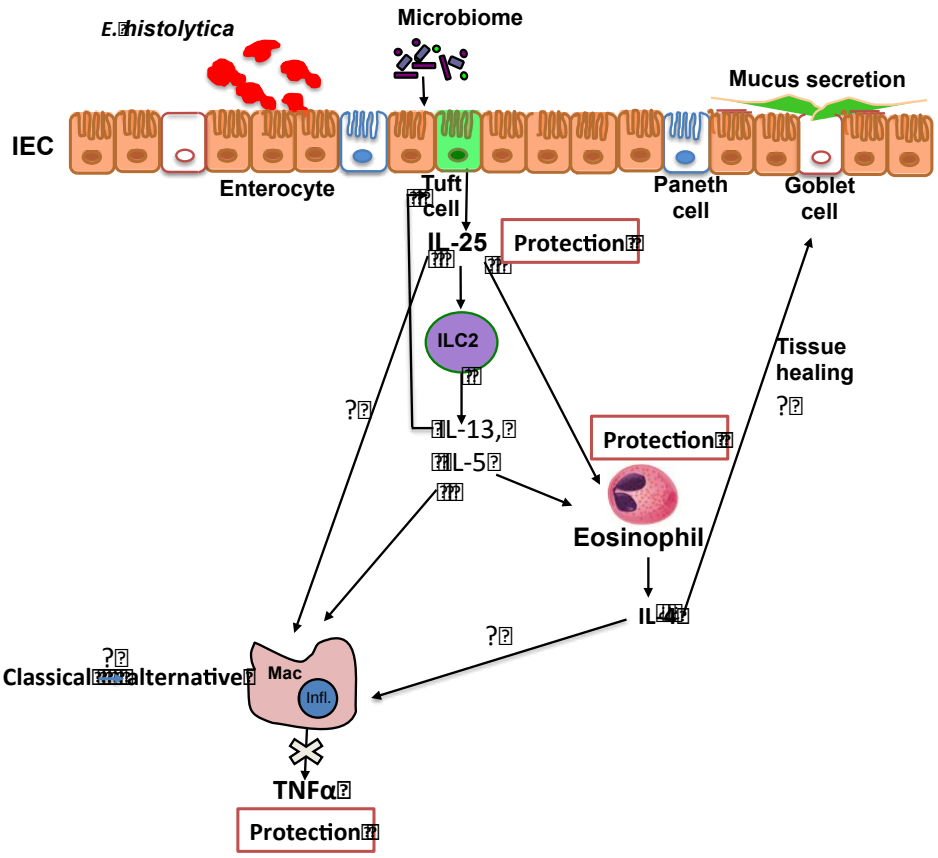
The proinflammatory cytokine TNFα plays a crucial role in intestinal inflammation during amebic colitis. In the present study, we found that the TNFα level detected in colon biopsy specimen was higher in amebic colitis. Also, administration of rIL-25 decreased inflammatory cytokine levels, including TNFα, in the mouse model during amebic colitis. TNFα depletion by monoclonal antibody also made mice resistant to *E. histolytica* infection. Peterson *et. al*., has shown that amebic diarrheal episodes are positively correlated with TNFα levels in the blood [46]. In vitro experiments have identified a

chemotactic effect of TNFα on *E. histolytica* and showed distribution displacement of the *E. histolytica* up the gradient towards TNFα. They reported in the absence of chemoattractant [117]. In a study of experimental autoimmune encephalomyelitis, it was reported that IL-25 knockout mice had increased IFNγ, IL-17 and TNFα compared to wild type mice [92]. Our results suggest eosinophil suppression of TNFα amount is a possible mechanism of IL-25 mediated protection, although the detailed interaction between TNFα and IL-25 has not been studied in the present study.

Very recent studies have shown that tuft cells are expanded by worm infections in the mouse gut and these cells produce the majority of IL-25. IL-25 in turn acts in part by stimulating innate lymphoid cells 2 (ILC2) to produce IL-13 and IL-5. Tuft cell differentiation is dependent on IL-13, and IL4-Rα (common receptor subunit for IL-13 and IL-4) signaling is sufficient to induce expansion of tuft cells. Innate lymphoid cells (ILC2s) respond to IL-25 with IL-13 and IL-5 production. ILC2s are regulators of innate immunity and tissue remodeling and have also been reported to be important to provide protection against helminth infection, such as *Nippostongylus brasiliensis* [60]. In our amebic colitis study, IL-13 neutralization in rIL-25 treated mice did not inhibit the ability of IL-25 to protect. IL-13 induced tuft cells can produce IL-25, and IL-25 can induce ILC2s to produce IL-13. Therefore IL-13 can act both upstream and downstream of IL-25. It could be possible that IL-13 is important upstream of IL-25, which would act to expand tuft cells number to increase IL-25 production. In contrast, administration of rIL-25 maintained the downstream protective mechanism, such as induction of type 2

Figure 4.1: IL-25 mediated protection from amebic colitis. *E. histolytica* suppresses the amount of IL-25. IL-25 administration provides protection against amebic colitis through

eosinophils**.** IL-25 suppresses inflammatory cytokines (IL-23, IL-17 and TNFα) and induces type 2 cytokines (IL-4, IL-5). Neutralization of TNFα prevent amebic colitis. Eosinophil mediated protection may be through the ability to suppress TNFα amount.


E. histolytica
Microbiome
Mucus secretion
IEC
Enterocyte
Tuft cell
Paneth cell
Goblet cell
IL-25
Protection
ILC2
Tissue healing
?
IL-13,
IL-5
Protection
Eosinophil
IL-4
?
Classical
Alternative
Mac
Infl.
TNFα
Protection

responses through ILC2 or other possible downstream mechanism. We concluded that IL-25 induction of IL-13 was not required for protection. These results suggested a specific type 2 response is required to play the protective role against amebic colitis.

Epithelial cells are also a key source of both IL-33 and thymic stromal lymphopoietin (TSLP) that have similar ability to induce type 2 responses. We do not know whether IL-33 or TSLP also act in the same pathway as IL-25. IL-25 stimulation of ILC2 can result in secretion of IL-5 that could control eosinophil homeostasis [118].

In conclusion, we have found that IL-25 is suppressed during amebic colitis through an apparently IL-1β independent pathway. Repletion of IL-25 in a mouse model reduced *E. histolytica* trophozoite number, antigen load, and epithelial disruption in the cecum. From these results, we conclude that IL-25 mediated protection against amebic colitis is eosinophil dependent. We have also demonstrated that eosinophils may protect by suppressing TNFα production, as IL-25 suppressed TNFα levels in the cecum, and neutralization of TNFα prevented amebic colitis.

4.2 Future prospectives:

The insights provided by this dissertation lead to several important questions:

1) How is IL-25 suppressed during amebiasis?

An interesting query from this study is how protein expression of IL-25 is suppressed during amebic infection. Intestinal epithelial tuft cells are a major source of IL-25 and

tuft cell hyperplasia is dependent upon IL-13. IL-25 can induce IL-13 by acting on ILC2s. Therefore, IL-13, tuft cells, IL-25 and ILC2s make a circuit that induces type 2 responses. Does amebiasis suppress IL-25 secretion by suppressing the ability of tuft cells to produce IL-25? We have found that IL-13 is suppressed during amebic infection. This raises the possibility that IL-25 suppression is due to inhibition of IL-13, which could be tested by treatment of *E. histolytica* infected mice with IL-13. It is also possible that there is a direct inhibitory effect of the parasite on IL-25 production by tuft cells, or indirectly via changes in the microbiome.

2) Is there any role of microbiota in regulation of IL-25?

From our lab, Watanabe and Petri (unpublished) have found that dysbiosis from antibiotic treatment causes normally resistant C57BL/6J mice to become susceptible to *E. histolytica* and that these antibiotic treated mice have less IL-25 than control (without antibiotic treatment) mice. This supports the notion that IL-25 production in response to the gut microbiome is an important determinant of the outcome of an infectious challenge with *E. histolytica*. We have found in the current study that resistant C57BL/6J mice have higher IL-25 levels than susceptible CBA/J mice. It would be interesting to investigate the extent to which the higher intestinal IL-25 production by C57BL/6J mice is due to the gut microbiome, the mouse genetic background or both. One could test the role of the microbiome in *E. histolytica*-induced IL-25 suppression by fecal transplant from wild-type uninfected to infected mice.

3) How do eosinophils protect from amebic colitis?

The mechanism by which eosinophils are involved in IL-25 mediated protection against amebiasis is unknown. Eosinophils are a major source of IL-4, and IL-4 is well known to act in a tissue-healing mechanism via alternatively activated macrophages and to be protective against helminths, in part through production of intestinal mucin glycoproteins. We have hypothesized that IL-25 induced eosinophils could play a protective role against amebiasis via IL-4. We have seen that eosinophil depletion abrogates IL-25 induced IL-4 production. However before concluding that eosinophils are a major source of gut IL-4 it will be necessary to measure intracellular IL-4 in eosinophils during amebic infection using flow cytometry. One could then test for a role of IL-4 through blockade of the IL4 receptor or with anti-IL-4.

Another possible mechanism of eosinophil protection is through suppression of inflammatory TNFα. It would be interesting to know if M1 macrophages (classically activated) are the major source of TNFα during amebic infection. We hypothesize that eosinophil-induced IL-4 has an effect on class shifting of macrophages from M1 (classical) to M2 (alternative), with M2 macrophages suppressing TNFα production. It would be interesting to test if eosinophil depletion can reverse the IL-25 mediated suppression of TNFα. One could then explore if TNFα is acting to promote amebic colitis via *E. histolytica* chemotaxis in the gut or indirectly through TNFα – mediated inflammation.

It is also known from the literature that IL-25 can act directly on ILC2s to produce IL-5 and IL-13 [60]. IL-5 also enhances eosinophil recruitment. It would be important to know if protection from *E. histolytica* requires that eosinophils be primed by IL-25 directly or if IL-5 would be able to induce eosinophil protection. We can test if rIL-5 administration can induce eosinophil infiltration to protect mice from amebic colitis. It may be also that IL-25

primes eosinophils to protect in a way that IL-5 does not. Discovering this would then give one a tool to explore the protection afforded by eosinophils, by testing how an IL-25 priming changes eosinophil gene expression and/or function.

4) What is the role of ILC2 during amebiasis?

ILC2s have the IL-25 receptor and their proliferation and activation are supported by IL-25. IL-25 induced ILC2s are protective against helminth infection. It is still unknown what the role of ILC2s is in amebiasis. It would be rational to study the role of ILC2 cells in the IL-25 induced protective pathway during amebic colitis. First, we would like to know the abundance of ILC2s during amebic infection with or without IL-25 treatment. It would also be interesting to know whether these cells act via production of IL-5. A related question (see above) is whether IL-5 can induce protective eosinophils during amebiasis?

5) Do macrophages shift from M1 (classical) to M2 (alternative) by rIL-25 induction during amebiasis?

We have seen type 2 responses up-regulated during amebiasis by rIL-25 treatment, although we do not know if M2 macrophages are involved. We identified gene expression in cecal tissue representative of M2 macrophages. To confirm this, it would be necessary to purify macrophages by electronic cell (FACS) sorting and then assess M1 vs M2 characteristics such as arginase, chitinase and NOS2.

Also, we have hypothesized M1 macrophages enhance inflammation during *E. histolytica* infections, leading to *E. histolytica* disease, whereas M2 macrophages provide protection by reducing inflammatory responses, such as suppressing TNFα. In order to test this hypothesis, it will be important to test if M2 macrophages can suppress TNFα and it would

be worthwhile to know whether M2 macrophages are a source of protective IL-10 in *E. histolytica* infection.

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