Early interactions between *Entamoeba histolytica* and mucosal cells

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ABSTRACT

The pathogenesis of the enteric protozoan parasite *Entamoeba histolytica* remains poorly understood. Moreover, the host responses during the early periods of interaction in the gut remain to be clarified. In this study I investigated the cell specific responses to the parasite and the importance of cross talk between epithelial-immune cells that could potentially influence the outcome of infection, with a central focus on Nuclear factor (NF)-κB. NF-κB is a ubiquitous transcription factor that plays a critical role in mucosal inflammation and its regulation by *E. histolytica* has not been studied so far. Gal-lectin is a well characterized parasite virulence factor and vaccine candidate. I first characterized the interactions between Gal-lectin and macrophages and found that several proinflammatory genes are upregulated as early as 2h. The Gal-lectin activated NF-κB and up-regulated Toll like receptor-2 expression in an NF-κB- and p38 Mitogen Activated Protein (MAP) kinase-dependent manner. As intestinal epithelial cells (IEC) form the first line of active host defense against mucosal pathogens, I determined the interaction between ameba soluble proteins and naive IEC. I observed that the parasite could elicit a chemokine response via activation of PI3 kinase and phosphorylation of p65 subunit to induce monocyte chemoattractant protein-1. The consequent recruitment of immune cells could be responsible for colonic inflammation. Finally, I made the novel observation that in macrophage-primed IEC, ameba proteins elicited a cytoprotective stress response. Using a combination of siRNA and over expression studies, I demonstrated that amebic proteins increased the expression and phosphorylation of Heat shock protein (Hsp) 27 thereby enhancing its association with and subsequent inhibition of Inhibitory κB kinase (IKK). The resulting inhibition of NF-κB could be a potential mechanism that explains the absence of inflammation in the majority of infected individuals. Taken together, the findings of this study open up a new facet in the pathogenesis of amebiasis and unravel a novel paradigm to study host-parasite interactions in the gut.

ABREGE

La pathogénèse du parasite protozoaire *Entamoeba histolytica* demeure inconnue. De plus, la response immunitaire de l'hôte durant les premières intéractions dans l'intestin doit être clarifié. Dans cette etude, j'ai investigé la response cellulaire spécifique au parasite et l'importance de la diaphonie entre les cellules épithéliales et immunitaires qui pourrait influencer l'infection,spécifiquement au niveau de NF-κB. NF-κB est un facteur de transcription omniprésent qui joue un rôle critique dans l'inflammation mucosale. Jusqu'à date la régulation de NF-κB par *E. histolytica* n'a pas été étudié. La Gal-lectin est un facteur de virulence du parasite bien charactérisé et une protéine candidate pour un vaccin contre l'amibiase. J'ai premièrement charactérisé les intéractions entre la Gal-lectin et les macrophages et j'ai trouvé que l'expression de plusieurs gènes proinflammatoires augmente après deux heures. La Gal-lectin a activé NF-κB et augmenté l'expression du récepteur Toll-2 de façon dépendente de NF-κB et des MAP kinases. Puisque les cellules intestinales épithéliales (CIE) forment une première ligne de défense contre les pathogènes intestinaux, j'ai déterminé l'intéraction entre les protéines solubles de l'amibe et des CIE naïves. J'ai observé que le parasite pouvait induire l'expression de chimokines (MCP-1) par l'activation de PI3 kinase et par la phosphorylation la sub-unité p65. Le recrutement des cellules immunitaires pourraient donc être responsable pour l'inflammation colonique. Finalement j'ai fait la nouvelle observation, que dans les CIE premièrement exposées aux macrophages, les protéines solubles amibiennes peuvent provoquer une response stress cytoprotectrice. En utilisant la siARN et étudiant l'expression des gènes, j'ai démontré que les protéines amibiennes augmentent l'expression et la phosphorylation de la protéine de choc thermique, Hsp27. Cette activation augmente l'association d' Hsp27 avec IKK résultant dans l'inhibition de IKK. L'inhibition de NF-κB pourrait être un mécanisme qui expliquerait l'absence d'une response inflammatoire dans la majorité des cas d'infections. Les résultats de cette étude permettent d'explorer d'autres aspects dans la pathogenèse de l'amibiase et permettent d'introduire un nouveau paradigme pour étudier les intéractions entre les parasites intestinaux et l'hôte.

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LIST OF ABBREVIATIONS

Ab-	Antibody
ADH-	Alcohol dehydrogenase
APC-	Antigen presenting cell
ATP-	Adenosine triphosphate
BCL-	B cell lymphoma protein
CD-	Complementarity differentiation
CO-	Carbon monoxide
COX-	Cyclo oxygenase
DNA-	Deoxy ribonucleic acid
Eh-	*Entamoeba histolytica*
ELISA-	Enzyme linked immunosorbent assay
ENA-	Epithelial-neutrophil activating peptide
ERK-	Extracellular signal regulated kinase
Gal/GalNAc lectin-	Galactose/ N-acetyl galactosamine –inhibitable lectin
GM-CSF-	Granulocyte-macrophage colony stimulating factor
GRO-	Growth-regulated oncogene
HEK-	Human embryonic kidney
Hsp-	Heat shock protein
IFN-	Interferon
Ig-	Immunoglobulin
IKK-	Inhibitory kappa B kinase
IL-	Interleukin
iNOS-	Inducible nitric oxide synthase
IRAK-	Interleukin-1 receptor-associated kinase
IRF-	Interferon regulatory factor
JNK-	Jun- NH_2 (amino) terminal kinase
kDa-	Kilo Dalton
MAb-	Monoclonal antibody
MAL-	MyD88 adapter-like protein

MAP kinase-	Mitogen activated protein kinase
MCP-	Monocyte chemotactic protein
MIP-	Macrophage inflammatory protein
MyD-	Myeloid differentiation
NADPH-	Nicotinamide adenine dinucleotide phosphate
NF-κB-	Nuclear factor-κ B
NO-	Nitric oxide
NOD-	Nucleotide oligomerization domain
PCR-	Polymerase chain reaction
PI3 kinase-	Phosphatidyl inositol 3 kinase
PKC-	Protein kinase C
RAG-	Recombinase activator gene
RNA-	Ribonucleotide acid
ROS-	Reactive oxygen species
SAP-	Soluble amebic proteins
SCID-	Severe Combined Immuno Deficiency
SIGIRR-	Single immunoglobulin IL-1R-related molecule
SOCS-	Suppressor of cytokine signaling
TICAM-	TIR-containing adapter molecule
TIR-	Toll/ IL-1 receptor
TIRAP-	TIR-domain containing adapter protein
TGF-β-	Transforming growth factor β
TLR-	Toll like receptor
TNBS-	Tinitrobenzene sulfonic acid
TNF-	Tumor necrosis factor
TRAF-	Tumor necrosis factor receptor-associated factor
TRAM-	TRIF-related adaptor molecule
TRIF-	Toll/IL-1 receptor domain-containing adaptor inducing IFN-β

THESIS OFFICE STATEMENT

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

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

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

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (1) a table of contents (2) a brief abstract in both English and French (3) an introduction which clearly states the rational and objectives of the research (4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper) (5) a final conclusion and summary (6) a thorough bibliography and (7) an appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the

accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

STATEMENT OF ORIGINALITY

The following aspects of this thesis are considered contributions of original knowledge:

Manuscript I

This manuscript describes up regulation of Toll like receptor-2 expression by *E. histolytica* Gal/GalNAc lectin. This is the first report of modulation of Toll like receptor by a parasitic molecule. Also, the signaling intermediates activated by this important amebic molecule have been identified. These observations are of immense help in characterizing Gal-lectin as a potential vaccine candidate and also in understanding the role of this molecule during pathogenesis.

Manuscript II

This study shows that amebic components can induce the macrophage chemokine, MCP-1 via post-translational modification of NF-κB p65 in intestinal epithelial cells (IEC). The observation that activation by *E. histolytica* leads to NF-κB p65 phosphorylation via PI3 kinase/Akt leading to a chemokine production is the first report of this unique signaling pathway in IEC. This study further justifies the role of IEC as initiators of colonic inflammation in amebic infection.

Manuscript III

This paper, describing ameba induced stress response inhibiting NF-κB in macrophage conditioned intestinal epithelial cells, is riddled with several novel observations. This is the first report showing that (1) macrophage secretions prime intestinal epithelial cells for stress response, (2) parasite induced stress response inhibits an inflammatory mediator, (3) amebic components have a beneficial effect on gut and, (4) phosphorylated Hsp27 binds to and negatively regulates IKK activity in intestinal epithelial cells

STATEMENT OF AUTHORSHIP

All the experiments described in Manuscripts I-III were designed and carried out by myself. I am also responsible for the analysis and interpretation of all findings. Dr. Kris Chadee, the co-author in all manuscripts was the thesis supervisor and provided financial support for the study, advice with regard to study designs and help in the preparation of manuscripts and thesis. Dr. Barbara Mann provided purified Gal-lectin and Ms. Lisa Dutil helped with immunoflourescent studies in the first manuscript.

SECTION I: LITERATURE REVIEW

Introduction

Amebiasis is an acute or chronic intestinal disease caused by the protozoan parasite *Entamoeba histolytica*. The disease affects 1% of the world population and is widely prevalent in tropical developing countries such as India, Bangladesh, Mexico and South Africa (1). The intestinal form of the disease is characterized by colonic inflammation, dysentery with severe abdominal pain and discomfort.

Pathogenesis of amebic colitis is poorly understood. *In vivo* and *in vitro* studies suggest two different modes of initiation of inflammation. One theory proposes that parasite contact with intestinal epithelial cells is required to initiate tissue destruction while the other school of thought suggests that soluble amebic factors can initiate an abnormal host response in the absence of contact. These processes may not necessarily be mutually exclusive but might operate simultaneously during infection. Both the concepts suggest that host inflammatory response plays a major role in tissue destruction and the ensuing symptoms such as colonic ulceration and bloody diarrhea. A major hindrance in our understanding of how the disease occurs stems from the lack of a suitable animal model that mirrors the human disease. The SCID-HU- Xenograft model attempted to address this and the *in vivo* observations made from this model has contributed significantly to our understanding of the host responses during amebic infection (2, 3). However, these studies are not mechanistic and have drawbacks such as species specificity. Consequently, despite few novel observations during the last decade, unfortunately the pathogenesis of intestinal amebiasis still remains a puzzle. For example, the host and parasite factors that mediate inflammation have not been elucidated fully; also it is not clear if host cells, in particular, intestinal epithelial cells play an active role in the initiation of inflammation and finally, it is not known if the cross talk between different cells of the mucosa can potentially modulate the outcome of infection.

The parasite has three well-defined virulence factors: Galactose/N-acetyl Galactosamine inhibitable lectin (Gal-lectin), Cysteine proteases and amebapores (4). Gal-lectin is a surface adhering protein (adhesin) essential for binding to the host cells and subsequent cell death. It is also highly immunogenic and is a candidate vaccine molecule. It has previously been shown to induce TNF-α (5) and IL-12 (6) from mouse and human macrophages. Apart from this, its potential to activate innate defenses and role in the pathogenesis have not been studied well. Toll like receptors (TLR) are a recently discovered group of pattern recognition receptors of the innate immune system that are increasingly being shown to play a major role during infection and inflammation (7). All TLRs discovered to date, activate the transcription factor NF-κB which is a key regulator of the pro-inflammatory response. It has been previously shown that inhibiting this transcription factor significantly reduced inflammation in the mouse xenograft model (2). However, the parasite molecules that induce NF-κB and the significance of its induction or suppression in amebic infection are not known.

Intestinal epithelial cells form a single layer of mucosal barrier that respond to pathogens by secreting various mediators such as chemokines and cytokines and by increased surface expression of receptors (8). Thus, they are increasingly being demonstrated to play an active role in signaling the immune system and modulate the disease process. Given the extensive cellular infiltration observed during amebic colitis, it is of great interest to understand the mechanism of immune cell homing that could potentially aggravate tissue damage. While previous studies implicate IL-8 as a potent mediator for neutrophil infiltration during amebic infection (9), the chemokines responsible for homing of other cell types have not been explored.

Recently the intestinal epithelial cell responses to pathogens or commensals have been shown to be altered by immune cells (10). The stress response is a universal response to various stressors such as thermal, chemical, oxidative or toxic stimuli. It is characterized by expression of heat shock proteins which act as chaperones and prevent the cellular proteins from denaturation, ultimately conferring protection to cells. This stress response has been shown to suppress the cellular inflammatory responses (11) and its role in

intestinal amebiasis has been hitherto unexplored. Moreover a frank amebiasis is seen only in 10% of infected people suggesting that some sort of anti-inflammatory mechanisms operate in majority of individuals.

With this background, the current study was taken up with the following specific objectives.

1. To study the regulation of Toll like receptors by Gal-lectin in macrophages
2. To understand the mechanism of induction of monocyte chemotactic protein-1 by amebic components from naive intestinal epithelial cells
3. To check the stress response induced by amebic components and its modulation of NF-κB activation in macrophage conditioned intestinal epithelial cells.

Our studies showed that Gal-lectin induces a global pro-inflammatory response in macrophages and also up regulates TLR-2 expression via NF-κB and p38 MAP kinase. This could have significant effect both in the pathogenesis of amebiasis and also during vaccination with Gal-lectin molecule. For the first time we also observed that soluble amebic factors could differentially activate NF-κB in naive and macrophage conditioned intestinal epithelial cells. The significance of this observation is 2-fold; in naive epithelial cells the activation of NF-κB via PI3 kinase/P65 pathway results in the secretion of chemokine MCP-1, which could play a role in the initiation of inflammation. On the other hand, our observation of suppressed NF-κB activation via the stress response in macrophage conditioned epithelial cells could explain the absence of inflammation in majority of individuals infected with the parasite.

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CHAPTER 1: *ENTAMOEBA HISTOLYTICA* AND AMEBIASIS

1.1 *E. histolytica* - Epidemiology and life cycle

Entamoeba histolytica is a unicellular parasite belonging to the Phylum Protozoa, Class Lobosea, Order Amoebida and Family Entamoebidae. It is the causative agent of human amebiasis which is found to affect 50 million people around the world causing 40,000-100,000 thousand deaths per year (1). The disease is the fourth leading parasitic cause of mortality behind malaria, Chagas' disease and Schistosomiasis. Amebiasis is widely prevalent in developing countries in Asia, Africa, Middle East and South America. Susceptible factors include poor personal hygiene, unsanitary conditions, and overcrowding. Incidence in non-endemic countries such as the United States and Canada is around 1-2%. Increased cases in developed countries are observed in institutionalized populations (2, 3), male homosexuals (4) and international travelers (5). While in the majority of infected people the parasite resides in the large intestine as a non-harmful commensal, in 10 % of people, it invades the mucosa and cause amebic dysentery with severe abdominal discomfort (6). Rarely, the parasite penetrates deep into the submucosa and reaches different organs such as the liver, lung or even the brain via the blood circulation. Amebic Liver Abscess (ALA) is a severe complication with high mortality in 1% of amebiasis patients and is found more often in men (10 male to 1 female) (7). However, severe cases are found in young children, pregnant women and elderly people (8). Invasive amebiasis has also been reported as an emerging parasitic disease in AIDS patients (9).

Earlier studies of the parasite and pathogenesis of disease were confusing because of the presence of morphologically indistinguishable but non-pathogenic *E.dispar*. Recent genetic and biochemical advancements have been successful in differentiating these organisms as two distinct species and lead to a shift in research to identify how these differences define the pathogenic or otherwise nature of these two amebae. Immunological tests such as ELISA, genetic tests as PCR and restriction length polymorphism are now available that can reliably identify these two species (10-12).

The life cycle of *E. histolytica* is simple and straightforward with humans as the only natural hosts and no secondary or intermediate hosts. Infection occurs through ingestion of quadrinucleated cyst (8-20 μM) through food and water contaminated by feces of infected hosts. Excystation takes place in the terminal ileum releasing eight motile vegetative forms, trophozoites (10-60 μM) that colonize the large intestine, primarily the colon. During this phase, the parasite can invade the mucosa and cause disease in 10% of individuals. Trophozoites feed on the gut flora, multiply by binary fission and under unknown environmental cues, form cysts which are passed out through feces to continue the life cycle. **To summarize, amebiasis, caused by the enteric protozoan parasite *E. histolytica*, is a significant health problem around the world and poor sanitation and personal hygiene are risk factors for the disease.**

1.2 Pathogenesis of intestinal amebiasis

Both host and parasite factors play a role in the pathogenesis of amebiasis which involves three distinct stages; colonization, mucus depletion and destruction of host epithelial and immune cells. The first stage is the colonization of trophozoites to the mucosal surface of large intestine. Trophozoites adhere to the mucins via surface Gal-lectin molecule and various inhibitors such as Galactose, N-acetyl Galactosamine, monoclonal antibodies to Gal-lectin and secretory IgA can effectively prevent colonization and also binding to target cells. In the next stage, the parasite disrupts the mucous barrier by a host of enzymes, particularly, the cysteine proteases. Finally, amebae comes in contact with epithelial cells and kills them through a yet unknown mechanism, most probably involving amebapores. Following destruction of epithelial layer, trophozoites penetrate further and kill immune cells whose products contribute to tissue destruction and aggravate inflammation. At this stage, parasite might get into blood vessels and be carried to different organs such as liver, lung or even brain. Amebic liver abscess is the most frequent extra-intestinal manifestation. While 90% of intestinal amebiasis cases are manifested in the form of bloody diarrhea/dysentery, other forms such as fulminating colitis, appendicitis and ameboma of colon are not uncommon.

Colonization is the first and essential step for both establishing the infection and for pathogenesis of invasive disease. As mentioned, this is mediated by the parasite surface molecule, Gal-lectin which binds to the Galactose and N-acetyl D-Galactosamine moieties of the colonic mucin (13, 14). *E. histolytica* Gal-lectin is a hetero-dimeric protein consisting of a 170 kDa heavy subunit linked to a 31/35 kDa light subunit via disulphide bond. Recently, the light chain was found to associate non-covalently with a 150 kDa intermediate subunit (15). Gal-lectin binds to the exposed terminal sugars of mucins and the target cells like bacteria and host epithelial/immune cells. Owing to their high oligosaccharide content, mucins bind to amebae and prevent them from making contact with host epithelial cells (16-18). Intestinal microbiota also competes with amebae for binding to the mucins and thus reducing the trophozoite colonization (19). Amebae have been shown to bind purified rat and human colonic mucins with high affinity (Kd=8.2X 10^{-11} M^{-1}) (14). In addition, mucins, by their gel forming properties, trap the pathogens and protect the underlying epithelial layer. Thus mucins serve two different purposes; on one hand they help in establishing the amebic infection while at the same time trap the parasite and prevent its invasion. However, paradoxically it has been observed that while purified rat colonic mucins can effectively bind amebae trophozoites, the parasite fails to colonize the rat gut, suggesting involvement of additional host and / or parasitic factors that determine the success or otherwise of parasite colonization (13).

In the second phase, trophozoites overcome the mucous barrier by two purported mechanisms; depletion and destruction. The precise mechanism of mucous depletion is not known. Studies in the gerbil model of invasive disease suggest that *E. histolytica* trophozoites release a mucous secretagogue that induces hypersecretion and eventual depletion of mucous from goblet cells (20, 21). The exact nature of this secretagogue has not been identified. Recent studies suggest this could be the prostaglandin E_2 (PGE_2). PGE_2 has a potent mucous secretogogue effect on rat and human colonic epithelial cells (22) and *E. histolytica* has been shown to contain a cyclooxygenase (COX)-like enzyme (23). The enzyme has been isolated from the parasite, purified and shown to catalyze the conversion of arachidonic acid to PGE_2. Amebic trophozoites also evoke massive mucous secretion in human colonic cells in a PKC-dependent manner (24). In contrast to

the mucous depletion, mechanisms of mucous destruction have been recently elucidated well. Cysteine proteases (CP) are key players in the destruction of the mucous layer while glycosidases might also be important. Amebae secreted products have been shown to degrade purified mucins from human colonic epithelial cells and the degraded mucins were less efficient in binding to amebae trophozoites (25). Specifically, suppressing *E. histolytica* cysteine protease 5 (EhCP5) expression makes the parasite less virulent (26). Interestingly, this particular CP is absent in the non-virulent species *E. dispar*. In addition to CPs, ameba also produce glycosidases such as β-N-acetylhexosaminidase, α-D-glucosidase, β-L-fucosidase, β-D-galactosidase and β-D-glucosidase (27, 28). Indeed, amebae secreted products can degrade colonic mucin oligosaccharides independent of proteolytic activity (29). These observations confirm that virulent ameba have evolved mechanisms to overcome host defense mechanisms. However, as invasion is a dead-end for the parasite life cycle, it is not clear why trophozoites would actively degrade mucins and invade. It could be that CPs in principle serve a different function and under an aberrant host response, facilitate inadvertent parasite invasion.

After breaching the mucous protection, trophozoites get in contact with intestinal epithelial cells. As for intestinal mucins, adherence of ameba to host cells including epithelial cells is mediated by Gal-lectin molecule. This is followed by cytolysis, the mechanism of which is poorly understood. Gal-lectin mediated cell contact is essential for cell cytolysis. This was evident in studies using Chinese Hamster Ovary (CHO) cells deficient in terminal galactose/N-acetyl galactosamine residues and these mutant cells are resistant to amebic adherence and cytolytic activity (30). Rapid death of host target cells was observed within 5-15 minutes after contact with the parasite (31) and the cells were found to undergo both apoptotic and necrotic death (32, 33). Amebapores are thought to play an important role in this (34-36). These are pore-forming proteins which are inserted by the parasite into the target cell membranes upon contact (37). They have been shown to cause depolarization of epithelial cells and are directly involved in cell lysis (38). CPs also play key role in target cell killing (39). Overexpression of EhCP2 enhanced the parasites ability to destroy CHO monolayer *in vitro* and this is blocked by cysteine protease inhibitor, E-64 (40). However, overexpression of EhCP2 did not augment the

parasites ability to cause ALA in animal models (40) while general inhibition of CP activity by E-64 abrogated or reduced the virulence to cause ALA (41). This suggests that CPs are important for pathogenicity but it is unclear which CPs are specifically involved. A recent study (26) has shown that CP5 plays a major role in amebic virulence. In addition to the killing of epithelial cells, ameba induced degradation of the extracellular matrix (ECM) (42) and epithelial tight junctions (43) also might contribute to mucosal damage. This stems from the observation that proteinases play a major role in cytopathic effects such as detachment and rounding of cells seen *in vitro* (44). CPs also have been demonstrated to degrade laminin (45). Trophozoites have electron dense granules containing collagenase, which could degrade ECM (46). Ameba modulation of epithelial permeability changes is being studied. The epithelial barrier has several components of which tight junctions (TJ) play critical role. TJ link the adjacent cells and also maintains the polarity of cells by separating apical and basolateral portions of cell membrane. They selectively permit the passage of solutes through them and thus help in the homoestasis of gut physiology. *E. histolytica* has been shown to disturb the epithelial barrier function *in vitro* by causing a steep decrease in the Trans Epithelial Resistance (TER) of intestinal epithelial cell (IEC) monolayer grown in transwells (43). The parasite has also been specifically shown to increase paracellular permeability as measured by the abnormal passage of small molecules such as mannitol. Understanding the mechanism and the parasite factors involved in TJ alterations need further studies.

The role of the host immune response in the pathogenesis of amebic colitis is increasingly being appreciated. The extensive damage to colonic tissue is also a result of infiltration and lysis of host immune and inflammatory cells. Once the epithelial layer is destroyed, a massive infiltration of immune cells is seen at the area of invasion (47). Neutrophils are the first cells to be seen in the lesion. Based on *in vitro* studies, two potential mechanisms have been proposed. One study demonstrated that contact dependent killing of epithelial cells leads to the release of preformed IL-1α which acts in a paracrine fashion and induce the secretion of IL-8 (48), a potent chemoattractant for neutrophils. Co-culturing of amebic trophozoites with IEC resulted in the production of several proinflammatory cytokines and chemokines such as GRO- α, GM-CSF, IL-1α and IL-6. Another study

(49) showed that ameba killing of IEC releases pro IL-1β which is cleaved and activated to mature IL-1β by the ameba cysteine proteases. These cytokines can act on neighboring live cells and induce chemokine secretion. While all these reports suggest contact dependent killing of IEC is required for chemokine release, in our laboratory it has been elegantly demonstrated that soluble ameba components can induce, in the absence of cell contact, IL-8 secretion from IEC (50). Another mechanism of IL-8 induction is via prostaglandin E_2. COX-2 expression and PGE_2 production are increased in both IEC and macrophages following amebic infection (51-54). PGE_2 is a potent inducer of IL-8 (55) and as mentioned previously, ameba itself expresses COX-2 like enzyme and release PGE_2 that can act on host cells to induce IL-8. It should be noted that apart from the host cells, amebic trophozoites themselves have been found to produce a chemoattractant to neutrophils (56). All these mechanisms could be operating *in vivo* leading to the enhanced release of chemoattractants and consequent homing of immune cells to the ulcerated lesion. In the late invasive stages, plasma cells, macrophages and lymphocytes are seen in the lesion (57). The source of chemoattractants specific for each of these cells is not known. Cellular infiltration not only fails to kill trophozoites but even contributes to tissue destruction. Amebae have been shown to be very efficient in killing naive neutrophils and macrophages (58). The lysed immune cells release their toxic contents such as proteases, cathepsins, lysozymes, and reactive oxygen and nitrogen intermediates, which are lethal to host tissue and worsen the intestinal damage (59, 60). Indeed, studies in SCID-HU-INT mice in which human intestinal tissue is grafted on their back, have observed decreased tissue damage and colonic ulceration following depletion of neutrophils (61). The mechanisms by which ameba are resistant to neutrophil attack are not known. Ameba possess superoxide dismutase (62), NADPH:flavin oxidoreductase (63) and a cysteine-rich 29 kDa (64) protein that could detoxify reactive oxygen. Indeed, *in vitro,* highly virulent strains of ameba could resist neutrophils at a high ratio of 3000 neutrophils per ameba (65).

Aided by the infiltration and lysis of host inflammatory cells as described above, the trophozoites extend deep into the submucosa giving rise to the typical flask-shaped ulcer. At this stage, few macrophages are present and this paucity might result from parasite

secretion of monocyte locomotion-inhibitory factor (66). Invasion is also facilitated by locomotory activity of the trophozoite and signaling events in the parasite that regulate this process are intensively being studied (67). If the parasites reach blood vessels of intestine, they can get into the circulation and spread to soft tissues such as liver, lung or even brain. Clearly cysteine proteases play a key role in tissue degradation and invasion and the Gal-lectin is essential for target cell adherence and killing. CP-deficient or E-64 pretreated trophozoites have reduced virulence to cause ALA in animal models. Similar observations were made with parasites in which the expression of Gal-lectin and amebapores has been inhibited by the antisense technique (68, 69).

Another area of investigation is the mechanism of target cell death by *E. histolytica*. Most of the *in vitro* studies have been done in CHO and Jurkat T-cells. Following contact with the parasite, there is a rapid 20-fold increase of intracellular calcium (Ca^{2+}) level in the target cell (31). This is followed by membrane blebbing and cell death in 5-15 minutes. It is suspected that Ca^{2+} could act as a second messenger activating signaling events that eventually lead to cell death. While the mechanism of cell death caused by *E. histolytica* is controversial, recent evidence points to apoptotic death in immune cells. Cell death characteristic of both necrosis and apoptosis have been reported following attachment of host cells by ameba. Necrosis is characterized by cell swelling, rupture of cell membrane and release of cell contents while apoptosis has all the features opposite to the ones observed during necrosis. Initial reports suggested either necrotic or a non-classical apoptotic mechanism of cell death by *E. histolytica*. Ragland *et al.* (1994) observed DNA fragmentation (a marker for apoptotic death) in murine myeloid cell line FDC-P1 exposed to the parasite and this is not inhibited by overexpression of Bcl-2 (70). ALA studies in knock-out mice also indicated that ameba mediated apoptosis in hepatocytes is independent of Fas ligand or TNF receptor I (71). Recently the mechanisms of cell death of Jurkat T lymphocytes by *E. histolytica* have been elucidated. These studies suggest ameba induced cell death is apoptotic and is caspase-3 dependent but caspase 8 and 9 independent (72). Further, Jurkat cell and erythrocyte contact with ameba resulted in the host cell surface exposure of phosphatidyl serine (PS), a marker for apoptosis, and ameba preferentially phagocytosed apoptotic cells compared to necrotic cells (32, 73). Evidence

for apoptotic killing also came from another group of researchers who observed ROS mediated ERK1/2 activation leads to apoptotic killing of neutrophils by live trophozoites (74). The interaction between amebic trophozoites and intestinal epithelial cells needs to be explored. **To summarize, amebic pathogenesis involves distinct phases of colonization, degradation of mucins and killing of host cells. Multiple factors are involved and host inflammatory response contributes to the lesions seen in amebic colitis.**

1.3 Gal/GalNAc-lectin of *E. histolytica*

Gal/GalNAc lectin is the most well studied ameba virulence factor. This is the surface adherence protein that is essential for binding to mucins and varied target cells such as bacteria and host cells like colonic epithelial cells, red blood cells and immune cells (13, 58, 75-78). It has high affinity for complex, branched carbohydrate chains with Galactose or N-acetyl galactosamine terminal residues (79). Later it has been found that Gal-lectin has very high affinity to glycoproteins containing GalNAc-terminal oligosaccharides chains, as found in colonic mucin (80). Indeed, rat and human colonic mucins bind amebic trophozoites with 10000-fold affinity than galactose and N-acetyl galactosamine monomers (13).

Gal-lectin is a heterodimeric, 260 kDa glycoprotein consisting of 170 kDa heavy subunit and 31/35 kDa light subunit which are linked by disulfide bonds (Fig. 1.1) (81). There is no antigenic cross reactivity between heavy and light subunits (82, 83) and the molecule as a whole is quite distinct from any of the known types of lectins found in eukaryotes. The heavy chain is made up of 1276 amino acid (aa) residues which can be classified into three domains; extracellular, transmembrane and carboxy terminal cytoplasmic tail (82-84). The intracellular cytoplsmic tail has several potential phosphorylation sites and also has aa sequence similarity with that of β2 and β7 integrin cytoplasmic tails suggesting that it could play a role in intracellular signaling (85). The extracellular portion of heavy subunit is richly glycosylated and is divided into three distinct regions: a cysteine-tryptophan rich region (CT; aa 1-187), a cysteine poor region (CP; aa 188-377) and a cysteine-rich region (CR; aa 378-1208) that includes a pseudorepeat portion (PR; aa 378-

653) (Fig.1.1). Monoclonal antibodies (MAbs) against defined regions of Gal-lectin molecule are available and were shown to block the adherence of lectin to mucins and target cells (86, 87). It should be noted that different MAbs to Gal-lectin affect adherence and cytotoxicity functions differently suggesting the involvement of separate conformations of the molecule in its biological activity. For example, some monoclonal antibodies have been shown to enhance the trophozoite binding to target cells (88). Both adherence inhibiting and enhancing MAbs of Gal-lectin map to the cysteine rich region, indicating that carbohydrate recognition domain is present in the CR region of heavy chain.

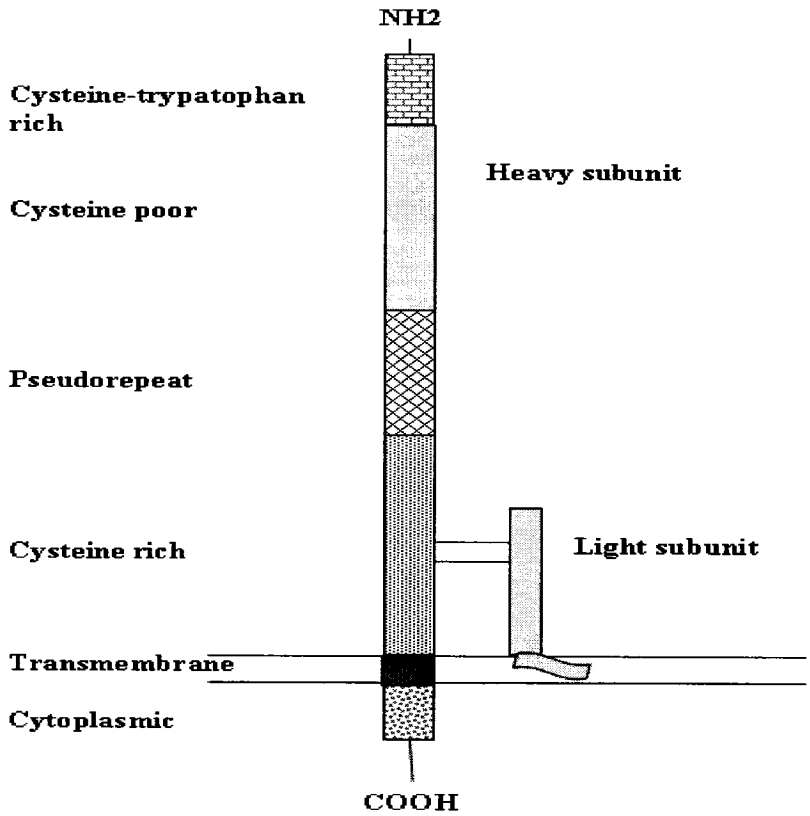

Fig. 1.1: Structure of E. histolytica Gal-lectin. Amebic Gal-lectin is composed of 2 subunits; Heavy subunit which is divided into extracellular, transmembrane and cytoplasmic resions and light subunit which is GPI-anchored and lacks cytoplasmic tail.

The light subunit has 2 isoforms of 31 kDa and 35 kDa and associate with the heavy chain to form 2 heterodimers: a 170/31 or 170/35 kDa Gal-lectin molecule. It is a glycoprotein with no transmembrane region, but contains a hydrophobic carboxy terminal domain which is a feature of glycosylphosphatidyl inositol (GPI)-anchored proteins. Its

function is poorly understood. MAbs against light chain do not inhibit adherence of trophozoites to target cells (89) and trophozoites deficient in light chain have reduced virulence features without any apparent defect in adherence (90). This suggests that light chain too plays a yet unidentified role in pathogenesis.

In addition to adherence, Gal-lectin was found to contain several immunological properties. It is highly antigenic and serum from amebiasis patients collected from different parts of the world consistently reacts with this molecule (91-93). The immunogenicity of Gal-lectin is also attributed to the CR region of heavy chain while light chain is poorly immunogenic as demonstrated by lack of reactivity with serum raised against native Gal-lectin. CR which has a sequence similarity to human CD59 that binds to purified C8 and C9 components of the complement prevents the formation of membrane attack complex and thus is thought to confer complement resistance to the parasite (94). High cysteine content of this region might also confer protection against both host and ameba-derived proteases (83). In short, the CR region of the heavy subunit is the most important region that is critical for all biological activities of this molecule.

Recently a 150 kDa intermediate subunit of Gal-lectin was also identified. A MAb against this protein significantly inhibited adherence to target cells, erythrophagocytosis and cytotoxicity *in vitro* (95, 96). It has similar binding properties as 260 kDa Gal-lectin discussed above and is found to be physically associated with the latter. In addition, several other adherence proteins that bind to host surface and extracellular matrix substances are also present. Examples for these minor adhesins are a 220 kDa protein and 80 kDa membrane proteins that bind to hyaluronic acid (97, 98), 140 kDa protein that has affinity to fibronectin (99) and a 96 kDa EhADH2 protein which is an alcohol dehydrogenase enzyme that also binds to fibronectin and laminin (100).

Because of the importance of Gal-lectin in colonization and cell killing, this molecule has been a potential vaccine candidate against amebiasis. Moreover, the lack of antigenic variation in this molecule makes it attractive to develop as vaccine. Beck *et al.* (101) demonstrated that the Gal-lectin heavy subunits of ameba isolates from three distinct

areas of the world (Bangladesh, Georgia, and Mexico) retain remarkable sequence conservation. Several studies reported success with Gal-lectin vaccination against hepatic abscess in gerbil and hamsters. Protection was observed with both native and recombinant portions of the Gal-lectin with varying efficacies (102). Initial vaccination studies using whole native lectin given parenterally, though showed good protection, reported larger liver abscesses in unprotected animals (103). Similar protection was observed using recombinant portions of the cysteine rich region (CR: 378-1208 aa) of heavy subunit of Gal-lectin. However, vaccination with cysteine poor (CP: 1-373 aa) region of Gal-lectin was associated with exacerbation of abscess size (104). Surprisingly, oral vaccination with whole lectin did not result in exacerbation and it is ascribed to the poor stimulation of systemic humoral response by this route of vaccine delivery (105). It is presumed that antibodies against CP region enhance amebic adherence. Vaccination in mice has been used to study the immune response elicited by Gal-lectin and recently Houpt *et al.* (106) showed that purified native and recombinant portion of Gal-lectin (that contains amino acids 578–1154 of the 170-kDa heavy chain of the lectin) protect against intestinal amebiasis in a mouse model. Subunit vaccines made from different portions of heavy chain are extensively investigated for their potential to stimulate immune response in mouse, hamster and gerbil models. The cysteine rich (CR) region that contains the carbohydrate recognition domain (CRD) was found to be the most protective portion of the Gal-lectin (102). This molecule contains both B and T cell epitopes as seen by the stimulation of humoral immune response and spleen cell proliferation in immunized animals (103, 107, 108). Indeed, *in vitro*, animal and field studies showed that mucosal IgA (against CRD of CR region) and activated immune cells confer protection against the parasite. Gaucher and Chadee (109) reported that a codon-optimized DNA vaccine targeting a portion of the Gal-lectin heavy subunit (including the CRD), stimulated a Th1-type Gal-lectin specific cellular immune response as well as inducing development of serum antibodies that recognized a recombinant portion of the heavy subunit. While vaccine studies confirmed that both humoral and cell mediated immune (CMI) responses mediate protection, the mechanism of how Gal-lectin stimulates CMI is not clear. Recent studies on Gal-lectin-macrophage interactions *in vitro* are shedding some light in this direction. Purified Gal-lectin by itself was shown to activate mouse bone marrow derived

macrophages to stimulate TNF-α production (110). It also stimulates IL-12 production from IFN-γ primed human macrophage cell line THP-1 (111). As IL-12 is the principal cytokine with the ability to drive a Th1 type immune response, it is thought that Gal-lectin could also act as an adjuvant by stimulating CMI. Interestingly, MAb inhibition studies point to the CRD region as mediating these effects. Recently (C. Ivory and K. Chadee, submitted for publication) the Gal-lectin was also found to activate dendritic cells (DC) by up-regulating co-stimulatory molecule expression and inflammatory cytokine induction. A full elucidation of potential mechanisms that could explain Gal-lectin's ability to activate immune system is awaited. **To summarize, Gal-lectin is a well characterized parasite virulence factor and is an excellent vaccine candidate. Studying its interaction with host cells would greatly help in understanding the pathogenesis and mechanism of protection against the disease.**

1.4 Modulation of host response by *E. histolytica*

All successful pathogens developed unique mechanisms to subvert or circumvent host defense mechanisms in order to survive and perpetuate their life cycle. Studies have shown that *E. histolytica* also developed several mechanisms to suppress deleterious host responses; both innate and immune mediated. As mentioned previously, the structural resemblance of Gal-lectin molecules to a host cell CD59 molecule confers protection against complement attack (94). Two more potential mechanisms by which the parasite overcomes complement mediated lysis is by synthesizing a CD59 like molecule (112) that can sequester membrane attack complex, and also by means of a complex of lipophosphoglycans (LPG) and proteophoshpglycans (PPG) on its surface that form a protective surface coat avoiding lysis by the alternative and membrane lysis pathways of complement during the invasion of tissue (113, 114). Moreover, cysteine proteases (CP) elaborated by the parasite cleave the complement components C3a and C5a to prevent immune cell homing and suppress the inflammatory response (115). CPs also cleave immunoglobulins and mucins and overcome these barriers (26, 116, 117). A 29 kDa surface protein of amebic trophozoite was found to have an anti-oxidant property by removing H_2O_2 thus making the parasite resistant to reactive oxygen-mediated killing by leukocytes (64).

E. histolytica has also been shown to have a down regulatory effect on host immune cells, particularly, macrophages and lymphocytes. Macrophages isolated from amebic liver abscess during the acute phase of infection in gerbils are deficient in their ability to respond to IFN-γ, develop respiratory burst and to kill trophozoites *ex vivo* (118). Two potential mediators were found in the suppressive phenomenon: macrophage migration inhibitory factor (MIF) and prostaglandin E2 (PGE_2). MIF is an oligopeptide secreted into the medium by ameba and is found to inhibit the locomotion of monocytes and also the respiratory burst of macrophages and neutrophils (119-120). This factor also inhibited delayed hypersensitivity reaction to the allergen 1-chloro-2-4 dinitrobenzene in guinea pigs, suggesting suppression of T-lymphocytes (121). The second suppressive mediator is PGE_2 which down regulates several macrophage functions. It was found that *E. histolytica* stimulates PGE_2 production in the liver during abscess development in hamsters (122). Inhibiting PGE_2 with indomethacin resulted in the reduction of abscess size. The ameba mediated suppression is a local event as evidenced by the fact that macrophages obtained from distal organs like spleen and peritoneum have normal responses to stimuli. *E. histolytica* suppresses IFN-γ-induced major histocompatibility complex (MHC) class IIa molecule, I-Aβ mRNA expression and TNF-α production in murine and gerbil macrophages in a PGE_2 dependent fashion (123, 124). The decreased ability of ALA derived macrophages to kill trophozoites *ex vivo* could be due to the modulation of iNOS expression and consequent nitric oxide production in a PGE_2 independent manner (125).

In addition to macrophages, T-lymphocyte functions are also down regulated by ameba trophozoites. *In vivo* or *in vitro* treatment of murine splenocytes with amebic antigens resulted in a decreased capacity of splenocytes to respond to mitogen-induced blastogenesis following a challenge with concanavalin A (Con A), phytohemagglutinin (PHA), and lipopolysaccharide (LPS) (126). Also serum from human ALA patients suppresses T cell proliferation in response to soluble amebic antigen. A similar observation was made with gerbils in which serum collected during the convalescent phase of ALA (20 days after intrahepatic injection) suppressed the T cell response to Con

A. This suppression is independent of NO and PGE_2 and is associated with decreased IL-2 production (127). T cells from mice immunized with 220 kDa lectin also fail to proliferate *ex vivo* in response to purified antigen. Interestingly, these cells were able to produce Th2 cytokines IL-4 and IL-10 that could potentially inhibit T cell responses (128). Ventura-Juarez *et al.* (129) observed *in situ* interactions between trophozoites and immune cells in human ALA biopsies and observed larger number of $CD8^+$ T cells than $CD4^+$ cells. As $CD8^+$ cells could be cytotoxic or suppressive type, it is assumed that presence of high $CD8^+$ cells leads to local suppression of the immune response, partly mediated by IL-10. The mechanisms for this T cell recruitment are not clear. **To summarize, *E. histolytica* has evolved multiple mechanisms to overcome host defense mechanisms and this modulation could be important in the pathogenesis of amebiasis.**

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*CHAPTER 2: TOLL LIKE RECEPTORS IN HOST DEFENSE

2.1 Introduction

Vertebrates have evolved an elaborate system of immune defense against constant threat by pathogens. In mammals there are two distinct arms of immune system; innate and acquired or adaptive. Innate immune system is activated rapidly, within minutes of a pathogen attack and serves two basic purposes; first, to kill/contain the pathogen and secondly to signal the acquired immune system which comes into the battle field a little later on. The major hurdle for innate immune function is pathogen recognition and self-non self discrimination. While adaptive branch evolved a mechanism of gene rearrangement to identify molecules unique to each pathogen, innate immune system developed germ line encoded Pattern Recognition Receptors (PRRs) which recognize Pathogen Associated Molecular Patterns (PAMPs) (note: recent literature suggests an alternate and more apt term, MAMP which stands for Microorganism Associated Molecular Pattern, keeping in view the fact that TLRs also recognize molecular patterns of non-pathogens) (1). PAMPs are molecules that are common to a group of organisms and are essential to the survival and/or virulence of the pathogens. Because of their importance to the parasite biology, PAMPs are also least subjected to mutations and structural alterations. For these reasons, recognition of PAMPs by PRR does not require complex receptor diversity as required for antibodies and T cell receptors (2).

There are two categories of PRRs, non-signaling and signaling (3). Non-signaling PRRs could be soluble factors or transmembrane proteins. The former includes C-reactive proteins or lectins which bind the microbes and make them susceptible to phagocytosis or complement mediated lysis. Example for non-signaling transmembrane proteins is scavenger receptors which recognize pathogens and help in internalizing and directing them to lysosomal killing. Signaling PRR could be either transmenbrane or cytosolic; Toll like receptors (TLRs) and NODs fall into these categories respectively.

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Our knowledge of TLRs started with the discovery of Toll in drosophila and then the human TLR-4, which recognizes LPS, in the context of septic shock (4, 5). The last decade witnessed an exploding research into TLRs that revolutionized our understanding of innate immune system. TLRs are evolutionarily conserved host defense molecules which have structural and functional homologues in plants, insects and mammals. They are type I transmembrane proteins with three distinct domains; N-terminal extracellular Leucine Rich Repeats (LRR) for ligand binding, a transmembrane domain and C-terminal intracellular signaling tail (6). The tail has a region that is common to TLR and IL-1 receptor family, designated as Toll/IL-1 Receptor (TIR) domain and is essential for the signaling function. A total of 13 mammalian TLRs (TLR 1-10 in human and TLR 1-9, 11-13 in mice) have been discovered to date (7). Table 2.1 shows the ligands and the microbes that are recognized by each TLR (7-9). As shown, TLR-2 is the most promiscuous receptor capable of recognizing a number of diverse PAMPs. In addition, it is apparent that a group of organisms can be recognized by different TLRs (Gram negative bacteria are recognized by both TLR-2 and 4) and the same TLR can sense different groups of organisms (TLR-2 recognizes both Gram positive bacteria, Gram negative bacteria and fungi).

Table 2.1: Ligands and microbes recognized by Toll like receptors

TLR	Ligand	Microbe	Comment
TLR-1	Triacyl lipopeptide	Bacteria, Mycobacteria	with TLR-2
TLR-2	**Bacterial**		
	Triacyl lipopeptide	Bacteria, Mycobacteria	with TLR-1
	Lipoteichoic acid	Group B Streptococcus	with TLR-6
	Peptidoglycan	Gram positive bacteria	
	Atypical LPS	Legionella, Leptospira, Porphyromonas, Bordetella	
	Lipoarabinomannan	Mycobacteria	with TLR-6
	Porins	Neisseria	
	Diacyl lipopeptide	Mycoplasma	with TLR-6

	Fungal		
	Zymosan	*Saccharomyces cerevisiae*	with TLR-6
	Phospholipomannan	*Candida albicans*	
	Glucuronoxymannan	*Cryptococcus neoformans*	
	Protozoan		
	Phospholipids (GPI) and GIPL	Trypanosoma	
	Lipophosphoglycan (?)	*E. histolytica*	
	Helminth		
	Phosphorylcholine-glycoprotein (ES-62)	Filarial nematode-*Acanthocheilonema viteae*	
	Viral		
	Haemagglutinin protein (?)	Measles virus	Also by TLR-4
	Host-derived	HCMV, HSV-1	
	Hsp60, 70 and 90	Host	
TLR-3	Double stranded RNA	Viruses, *S. mansoni*	
	Synthetic Poly(I:C)		
	Host cell mRNA		
TLR-4	**Bacteria**		
	Lipopolysaccharide	Gram negative bacteria	
	Viral		
	Envelop glycoproteins	RSV, MMTV	
	Plant product		
	Taxol	*Taxus brevifolia*	
	Fungal		
	Mannan	*Candida albicans*	
	Glucuronoxylomannan	*Cryptococcus neoformans*	Also by TLR-2
	Hsp60	*Chlamydia*	
	Host-derived		

	Hsp60, Hsp70 Surfactant protein A, Fibrinogen, Fibronectin		Also by TLR-2
TLR-5	Flagellin	Flagellated bacteria	
TLR-6	Mycoplasmal diacyl lipopeptide Fungal Zymosan Lipoteichoic acid		All with TLR-2
TLR-7 TLR-8	Viral single stranded RNA Guanine analogues-R484, Imiquimod siRNA Immune complexes containing self RNA	RNA Viruses Synthetic Host-derived	TLR-8 is inactive in mice
TLR-9	Unmethylated double stranded CpG DNA CpG oligonucleotides Immune complexes containing self DNA	Bacteria and virus Synthetic Host	
TLR-10	Unknown		Absent in mice
TLR-11	? Profilin-like molecule	Uropathogenic bacteria *T. gondii*	Inactive in human
TLR-12 TLR-13	Unknown Unknown		Absent in human

Heterodimerization of TLR-2 with TLR-1 or TLR-6 is required to recognize some ligands such as lipopeptides, lipoteichoic acid (LTA) and zymosan. Although much is known about TLRs 1–9 and 11, the ligands, modes of signaling and biological roles of TLRs 10, 12 and 13 have yet to be defined. It is evident that 9 TLRs can recognize the

whole range of microbes utilizing relatively conserved leucine rich repeat motifs and currently research is focused on studying TLR-ligand interactions to understand this diversity of recognition. Apart from few differences, in principle, all TLRs activate Th1 type proinflammatory immune response. This serves the two main functions of innate immune system; containing the pathogen and signaling the adaptive arm. TLRs are increasingly being shown to play crucial roles in host defense and inflammatory conditions during various infections, cancers and autoimmune states.

2.2 Distribution and regulation of Toll like receptor expression

TLRs are the primary sensors of invading pathogens and induce potent anti microbial defenses. Consequently they are expressed in diverse cell types, both in immune and non-immune cells. Antigen presenting cells such as macrophages, dendritic cells and B cells are the major immune cells while endothelial cells, fibroblasts and epithelial cells are the principal non-immune cells that express majority of TLRs (10). In addition, TLRs have been found in NK cells, monocytes, neutrophils, basophils, mast cells and regulatory T cells. The localization of TLRs varies with some (TLR-1, 2, 4, 5, 6) expressed extracellularly and others (TLR-3, 7, 8, 9) are present in intracellular components such as endosomes (9, 11). In the latter case, the corresponding ligands have to be internalized before activating the TLR signaling. However even in the case of TLR-2, the receptor is found to recruit to the phagosome following stimulation with its ligand, Zymosan (12). TLR-5 is found in the baso-lateral membrane of intestinal epithelial cells and its stimulation by flagellin requires the transepithelial transport of flagellin to the basolateral membrane or presence of flagellin in the lamina propria (13). These mechanisms are thought to represent a regulatory event in the functioning of TLRs to avoid an unintended activation by non-pathogens and host TLR ligands.

TLR expression in cells is not static but has been shown to be modulated in response to various stimuli such as vitamins, cytokines and pathogens. It is thought that increased disease susceptibility and poor adaptive immune responses in the elderly could be due to decreased TLR expression and function (14). Vitamin D3 suppresses TLR-2 and TLR-4 mRNA and protein expression in human monocytes leading to hypo-responsiveness of

monocytes to LPS and LTA in terms of reduced NF-κB activation and TNF-α production (15). Increased expression of TLRs has been reported during inflammatory conditions such as inflammatory bowel disease (IBD). Consistently, proinflammatory Th1 cytokines such as IFN-γ and IL-1β have been shown to up regulate the expression of TLRs in intestinal epithelial cells (IEC). Mueller *et al.* (16) recently demonstrated that Th2 cytokines, IL-4 and IL-13 suppress TLR-3 and TLR-4 expression in IEC and also dampened TLR activation induced by Th1 cytokines and LPS. This impaired TLR signaling and innate defense mechanisms are thought to make the host susceptible to chronic inflammatory conditions.

Recently several reports came out with regard to alteration of TLR expression during infectious conditions and by pathogens. Dolganiuc *et al.* (17) observed an increased expression of TLR- 2, 6. 7, 8, 9 and 10 in monocytes and T cells in Hepatitis C Virus-infected patients compared to controls. TLR4 was only upregulated in T lymphocytes, while TLR5 was selectively increased in monocytes of HCV-infected patients. In addition to TLRs, co-receptors such as MD-2 and CD14 were also increased in immune cells. Mycobacterial infection has been found to result in differential expression of TLRs (18). Whole blood from mycobacteria infected patients showed increased mRNA for TLR-1, TLR 2, TLR-4 and TLR-6. *In vitro*, mycobacterial components increased TLR-1 expression in THP-1 cells and PBMC from healthy donors when exposed to live Mycobacteria for prolonged period also exhibited increased expression of TLR-2 but not TLR-4. Cabral *et al.* (19) showed that treatment of human monocytes with *Borrelia burgdorferi* lysate, lipidated outer surface protein A, and triacylated lipopeptide Pam3CysSerLys4 (all TLR-2 agonists) results in the up-regulation of both TLR2 and TLR1 but down-regulation of TLR5. Consequently, TLR2 stimulation rendered cells hyporesponsive to a TLR5 agonist. On the contrary, monocytes stimulated with TLR5 ligands (including p37 or flaA, the minor protein from *B. burgdorferi* flagella) up-regulated TLR5. LTA, a ligand for TLR-2 was also found to induce the expression of its own receptor (TLR-2) in odontoblasts resulting in increased chemokine secretion and augmented dendrite cell maturation. (20)

Certain nematode and protozoan parasites also modulate TLR expression. Baseline expression of TLR1, TLR2, and TLR4, but not TLR9 was significantly lower in T cells of the filarial-infected individuals compared with the uninfected individuals in both endemic and non-endemic areas (21). Consequently, TLR function was significantly diminished in T cells of filarial-infected individuals as seen by decreased T cell activation/cytokine production in response to TLR ligands. This is thought to be a novel mechanism underlying T cell immune tolerance in lymphatic filariasis. However, no specific parasitic molecule has been implicated. *Trichomonas vaginalis* increased TLR-2, 4 and 9 expressions in HeLa cells in a p38 MAP kinase dependent manner (22). Surprisingly, inhibiting NF-κB did not abrogate this protozoan mediated increased TLR-2 or 4 expression. In certain inflammatory conditions, it is beneficial to suppress TLR expression. Indeed, vasoactive intestinal peptide, a neuron-immuno peptide has been found to reduce the severity of TNBS-colitis by decreasing TLR-2 and TLR-4 expression (23, 24). In addition, several substances such as carbon monoxide (25), nitric oxide (26), and macrophage migration inhibition factor (27) have been shown to alter TLR expression in different cells.

Apart from these observations, the signaling events involved in the differential regulation of TLRs, except in the case of TLR-2 and 4, have not been studied well. LPS, a TLR-4 ligand has been shown to increase TLR-2 expression via NF-κB but independent of MAP kinases (28, 29). This is not surprising given the fact that promoter region of TLR-2 contains several NF-κB binding sequences (30). On the contrary, An *et al.* (31) observed that LPS induced TLR-2, 4 and 9 expression is dependent on ERK MAP kinase and inhibiting p38 MAP kinase also suppressed TLR-2 and 4 expressions but enhanced TLR-9 expression in mouse immature dendritic cells. Expression of all the three TLRs is dependent on NF-κB. Also TLR-4 up regulation by CO was found to be p38 MAP kinase dependent in macrophages (25). The promoter of TLR-2 also has STAT-5 binding regions and this plays a role in IL-15 induced TLR-2 expression in T cells (30). Physiologically, a novel protein molecule associated with TLR-4 (PRAT4B) has been identified which is involved in the cell surface expression of TLR-4 (32). Down regulation of PRAT4B mRNA mediated by small interfering RNA decreased cell surface

TLR-4 on human embryonic kidney cell line HEK293 cells. The role of this molecule in microbial regulation of TLR expression needs to be explored. **To summarize, diverse stimuli including cytokines, microbes or microbial components can cause differential TLR expression, and these changes may be useful for either the pathogen and/or the host.**

2.3 TLR signaling and role in host defense

TLRs utilize the same molecules that are involved in IL-1R signaling. Following binding to the ligand, TLRs either homo or heterodimerize with other TLRs. The consequent conformational changes lead to the recruitment of TIR containing adaptor proteins. TLR signaling pathways can be primarily categorized to two classes; MyD88-dependent and MyD88-independent/TRIF-dependent (33). Two other adaptor molecules involved are TIRAP and TRAM. TIRAP/MAL bridges MyD88 with TLR-2 and 4 (34) while TRAM/TIRP/TICAM-2 is the adaptor between TRIF and TLR-4 (35). MyD88 is an essential signaling component for all TLRs except TLR-3. Its association with the TIR domain of cytoplasmic tail of TLRs results in sequential recruitment of IRAK-4, IRAK-1 and TRAF-6. Then TGF-β activated kinase 1 (TAK1) and TAK1 binding proteins TAB1 and TAB2 are recruited to TRAF-6 and TAK1 phosphorylates IKK-β and MAP kinase kinase 6 (MKK6) thus activating the transcription factor NF-κB and MAP kinases (36-38). This finally results in the induction of several genes involved in inflammatory response such as TNF-α, IL-1, IL-6, IL-8, and anti microbial peptides like defensins. This pathway is the classical TLR signaling and is typical for all TLRs (Fig. 2). TLR-4 requires accessory molecules such as CD14 and MD-2 for activation. In majority of cases, CD14 is essential for activation by LPS (39). It is also found to help in transferring the ligands to TLR-2 and TLR-3 (40, 41) and MD-2 is found to be required for the membrane localization of TLR-4 (42). Several co-receptors such as dectin-1, c-type lectin and CD36 also enhance TLR-2 signaling (43, 44).

In addition to this proinflammatory signaling pathway, activation of few TLRs such as TLR-3, TLR4, TLR-7/8 and TLR-9 also results in the type 1 IFN production. TLR-3 and 4 have been shown to induce IFN-α/β secretion, dendritic cell maturation and expression

of costimulatory molecules in MyD88$^{-/-}$ cells, utilizing other adaptor protein TRIF (45-51). TRIF mediated signaling has three branches. It can activate NF-κB in TRAF-6 dependent or independent pathways. The former overlaps with that of MyD88 dependent classical NF-κB pathway described above (52). The TRAF-6 independent mechanism involves receptor interacting protein (RIP)-1. RIP-1 can directly interact with TAK1 to activate NF-κB (53-55). In the third unique pathway, TRIF interacts with TBK-1 and IKK-ε (or IKK-i) that phosphorylates IRF-3 leading to type I interferon (IFN-β) production (35, 56-58). Activation of TLR-7/8 and 9 also leads to type I interferon (IFN-α) production in MyD88-dependent manner (51). As shown in Fig. 2.1, activation of interferon factors involves the adaptor protein TRAF-3 (50, 51) as opposed to TRAF-6 that is essential for NF-κB. In summary, TLR activation involves multiple signaling components, some of which are unique to a particular TLR. As seen from Fig. 2.1, NF-κB, the principal transcription factor that regulates a number of key genes involved in immune and inflammatory response is always activated by TLR signaling. Other important transcription factors activated by TLRs are IRF-3 and 7 that induce type I interferons. IRF-5 is activated by all TLRs except TLR-3 and acts synergistically with NF-κB by binding to the interferon responsive elements in the cytokine genes promoters to induce inflammatory cytokines (59, 60).

The consequences of TLR activation are many fold. TLRs recognize various components of bacteria, viruses, fungi and protozoa. In addition they also respond to host derived molecules like heat shock proteins and fibrinogen. Molecules induced by TLR activation include cytokines such as TNF-α, IL-1β, IL-6, IL-12, IFN-α/β; chemokines like IL-8; surface molecules such as MHC II, CD 80, CD86; antimicrobial peptides such as defensins. Chemokines and cytokines released as a sequel to TLR activation recruit and activate the immune cells such as macrophages and neutrophils which ingest the invading pathogens and limit the spread of infection. These cells also produce nitric oxide and reactive oxygen species. and the ensuing inflammation plays a crucial role both in resolving the infection and also in contributing to host tissue damage. TLRs have also been linked with phagosome maturation, defensin production, actin remodelling and angiogenesis (61-64). Increased co-stimulatory molecule expression by TLR activation

aids in efficient antigen presentation by APCs and also influences the type of ensuing adaptive response. CD4 T cells that differentiate to Th1 cells produce proinflammatory cytokines IL-6, IL-12, IL-18 and TNF-α. while Th2 cells secrete IL-4 and IL-13 (65). The Th1 response is protective against viral, bacterial and protozoan infections and Th2 responses are protective against helminthes. Antigen processing cells activated by TLR ligands secrete the Th1 instructive cytokines IL-12 and IL-18 thus skew the immune response towards Th1 type (46). Also it has been observed that Th1 and Th2 responses are mutually suppressive. For this reason, TLR activation can be beneficial to counter Th2 mediated allergic responses. For example, CpG DNA, a ligand for TLR-9 has been shown to inhibit eosinophilia and airway hypersensitivity by shifting the balance to Th1 type (66). Nonetheless, TLR-4 activation by low doses of LPS or when LPS was administered through inhalation, results in Th2 response (67). TLR-2 activation has also been shown to induce Th2 response under some circumstances (68). Recently, TLRs have been found to play a role in the regulation of immune response via direct or indirect influence on the function of CD4+ CD25+ T regulatory cells, which results in their induction and subsequent suppression of the immune response or a reversal of suppression (69, 70).

Several inflammatory conditions such as bacterial sepsis, autoimmune disorders and chronic inflammatory diseases such as IBD are the result of dysregulated activation of the innate or adaptive immune system. Apart from their beneficial effects as described above, TLRs have also been implicated in the immunopathology of many of these diseases (71). In order to avoid an uncontrolled TLR activation, cells have several molecules that function as negative regulators of TLR signaling. IRAK-M is expressed in monocytes and macrophages and its expression is increased following TLR agonist stimulation. This molecule lacks the kinase activity and inhibits the dissociation of IRAK-1 and IRAK-4 from TLRs by either blocking their phosphorylation or stabilizing the TLR/MyD88/IRAK-4 complex (72). This excludes TRAF6 from the signaling cascade and as such prevents NF-κB and MAP kinase activation by MyD88 dependent TLR signaling. Other examples for suppressors of TLR activation are Tollip, SOCS-1 and IRF-4 which are cytoplasmic, and SIGIRR and ST2L which are membrane bound (73-

76). **In summary, TLR activation involves a complex network of adaptor molecules and signaling intermediates, results in an inflammatory response and also signals the adaptive immune system.**

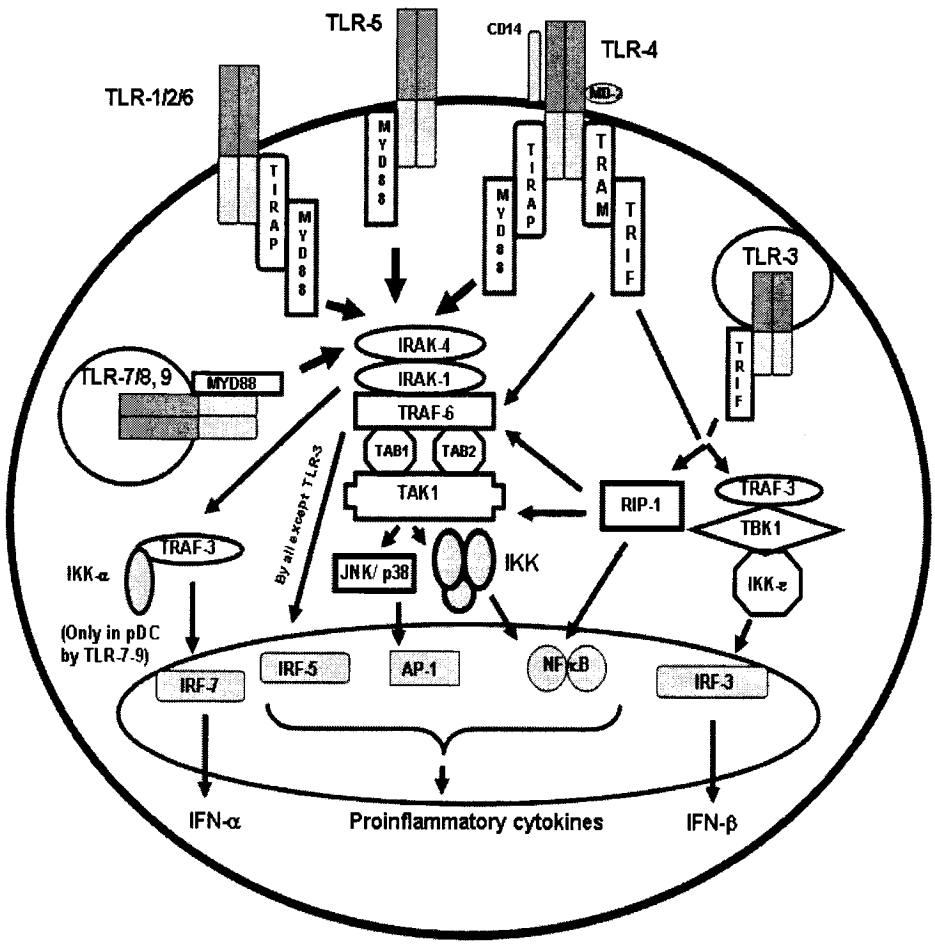

Fig. 2.1: TLR signaling: TLR-1, 2, 4, 5 and 6 are present extracellularly. TLR-7/8 and 9 are exclusively endosomal and TLR-3 is both extracellular and endosomal. TLRs 1, 2, 5, 6, 7/8 and 9 are exclusively MyD88 dependent; TLR-3 is exclusively TRIF dependent and TLR-4 utilizes both MyD88 and TRIF. Signaling via MyD88 involves IRAK-4/ IRAK-1/TRAF-6 complex that activate TAK1 which in turn activates JNK, p38 and IKK complex ultimately leading to early AP-1and NF-κB transcription factors. In addition, the MyD88/IRAK/TRAF-6 complex activates IRF-5 and IRF-7, which induces cytokine and IFN-α production respectively. TRIF signaling by TLR-3 and 4 involves 3 cascades. RIP-1 associates with TRIF and also with TRAF-6 and activates the classical pathway. RIP-1 also directly activates TAK1 and NF-κB in a TRAF-6 independent mechanism. Finally, recruitment of TRAF-3 to TRIF forms the complex TBK1/IKK-ε that activates IRF-3 and induces IFN-β. pDC-plasmacytoid dendritic cells.

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CHAPTER 3: INTESTINAL EPITHELIAL CELLS IN MUCOSAL INFECTIONS

3.1 Intestinal epithelial cells as sensors of microbes

A single layer of intestinal epithelial cells (IEC) that separate the host from environment is a complex dynamic tissue spanning a surface area of about 200 square meters with a villous architecture. It serves vital functions such as digestion and absorption of nutrients, electrolytes and water from lumen (1). Simultaneously, it forms a highly regulated, selective barrier for toxins and microbes present in the lumen. Recently, much knowledge has been gained on the role of IEC as first line of host defense against enteric pathogens. Defensins (2), nitric oxide (3), trefoil factor (4) and IgA (5) are some of the important host molecules secreted by IEC that ward off microbes away from gaining systemic access. In addition IEC also sense the microbes and signal the host immune system. The colonization of gut by pathogens results in altered epithelial gene expression leading to a coordinated immune and inflammatory response mediated by a spectrum of cytokines, chemokines and increased expression of surface receptors such as MHC II, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM) (Fig. 3.1) (6-14). Activation of the proinflammatory transcription factor NF-κB is the key event in this altered IEC gene expression and the majority of these observations (14-17) are based on interactions between human and mouse intestinal epithelial cell lines with bacterial pathogens and on studies in mouse colitis models. Jung *et al.* (6) reported that human colon epithelial cell lines (T84, HT29, Caco-2) produced a specific array of four proinflammatory cytokines, IL-8, monocyte chemotactic protein (MCP)-1, GM-CSF, and TNF-α in response to invasive strains of bacteria (*Salmonella dublin*, *Shigella dysenteriae*, *Yersinia enterocolitica*, *Listeria monocytogenes*, enteroinvasive *Escherichia coli*). In contrast, cytokine gene expression was not altered after infection of colon epithelial cells with noninvasive bacteria. Subsequently, Eckmann *et al.* (7) undertook global gene expression profiling in *S. dublin*-infected HT-29 and T84 cells and observed up-regulation of GM-CSF, IL-8, macrophage inflammatory protein (MIP)-2α, transcription factors (interferon regulatory factor (IRF)-1) and human leucocyte antigen (HLA) class I genes. Inhibition of NF-κB resulted in the repression of many of the bacterial-induced genes underscoring the pivotal role of this transcription factor.

Bacterial induced chemokine IL-8 by IEC was also found to be the key player orchestrating mucosal inflammation in shigellosis (18). Stokes *et al.* (19) studied T84 cell response to 8 different strains of *Vibrio cholerae* and observed 2-5 fold increase in the mRNA of several genes involved in innate mucosal immunity, intracellular signaling, and cellular proliferation. These include cytokines like IL-8, GRO-α, Gro-β, Gro-γ, MIP-3α, TNF-α, IL-5, transcription factors Jun-B, Early growth response (EGR)-1 and Activating transcription factor (ATF)-3. In addition to the pathogens, probiotics also alter IEC gene expression. Caco-2 cells treated with probiotic strain, *E. coli* Nissle 1917 (EcN) expressed 10-fold induction of MCP-1, MIP-2α and MIP-2β (20). MCP-1 production by EcN was also confirmed in mouse intestinal epithelial cells. Recently, IEC response to the gut protozoan parasite *Giardia lamblia* was studied (21). Differentiated Caco-2 cells infected with *G. lamblia* isolate WB-A11 expressed a novel chemokine profile of CCL2 (MCP-1), CCL20 (MIP3α), CXCL1 (MIP-2), CXCL2 (GRO-β), and CXCL3 (GRO-γ). *E. histolytica* trophozoites (22, 23) and soluble factors (24) can also induce IL-8 from IEC both *in vitro* and *in vivo*. Using high density gene expression arrays, Zhang and Stanley (25) studied the epithelial cell responses to *E. histolytica* and *Shigella flexneri* in human colonic xenografts in severe combined immunodeficient mice (SCID-HU-INT mice) and found up-regulation of a number of chemokines including IL-8, GRO, MCP-1, MCP-2, MCP-3, ENA-78 from IEC in both the infections. In addition to the pathogens, recently the purified toxin A of *Clostridium difficile* was also found to induce IL-8, GRO-α and MCP-1 from HT-29 and primary human colonic cells in NF-κB dependent fashion (26).

Chemokines are broadly divided into three families, C-X-C (α), C-C (β), and C (γ), based on the presence and position of the conserved cysteine residues (27) and have chemoattractant properties to immune cells. While some have effect on specific immune cells, many have seemingly overlapping functions. This is owing to the presence of restricted or shared chemokine receptor expression on different immune cells (28). For example, the receptor CXCR2 that recognizes the CXC chemokine IL-8 is exclusively present on neutrophils, making the IL-8 specifically chemotactic to these cells. However CCR2, the receptor for CC chemokine MCP-1 is present on monocytes, basophils and T

cells. In addition to their chemotactic property, chemokines can also activate the immune cell functions. Kim *et al.* (29) tested various chemokines for their ability to activate cytotoxic CD8+ cells and found MCP-1 to be the most potent. While the exact role of each chemokine in host defense and in pathological responses is not well known, Yung *et al.* (30) studied expression of 12 chemokines in response to *S. dublin* and proinflammatory cytokines and reported that their regulated secretion by IEC results in temporal and spatial mucosal chemokine gradients that are important for both early and late phases of inflammatory response.

As seen from above examples, chemokine secretion appears to be a signature response of IEC to signal both innate and adaptive immune systems. This results in the homing of immune cells and has dual effects on pathogenesis. The release of toxic substances such as nitric oxide (NO), hydrogen peroxide (H_2O_2) and lysozyme by immune cells could kill the invading pathogen and eliminate the infection. On the other hand, this could also damage the epithelial tissue leading to acute inflammation of the intestine. Thus, it seems the immune cell infiltration contains the infection at an early stage, even though it is at the cost of extensive mucosal destruction. This was illustrated in a rabbit model of *Shigella* infection (31). Neutralization of IL-8 resulted in reduced neutrophil infiltration and epithelial inflammation, however this is accompanied by bacterial overgrowth in lamina propria and increased passage into mesenteric blood.

It should be mentioned that despite these observations, IEC have been found to respond poorly to some purified bacterial products and this is attributed to TLR signaling defects. *In vitro* studies using colonic epithelial cell lines demonstrate that IEC are hypo-responsive to peptidoglycan and LPS owing to the low expression of TLR-2, TLR-6 (32), TLR-4 (33) together with high expression of Toll inhibitory protein (Tollip) (32). These mechanisms are thought to help in maintaining homeostasis in the face of constant and harmless presence of luminal bacteria. On the contrary, Rakoff-Nahoum *et al.* (34) propose that microbial recognition by TLR is essential for intestinal homeostasis. This study reported that activation of TLRs by commensal microflora is critical for the protection against gut injury and associated mortality. Nonetheless, increased expression

and defective functioning of these receptors was observed during inflammatory conditions such as IBD (35- 37) and indeed cytokines have been shown to modulate TLR expression in IEC (38, 39). It seems that intestinal epithelium has evolved dual abilities; not to elicit an aberrant response to commensals under physiological state and the potential to respond under pathological conditions. **To summarize, intestinal epithelial cells sense the microbes and respond primarily by secreting chemokines and cytokines that mediate mucosal inflammation.**

3.2 Modulation of IEC responses by immune cells

Classical studies on mucosal immunity focused primarily on immune cells. However, recent observations that epithelial cells express receptors and respond to cytokines has dramatically changed our understanding of gut immunology. In fact, this observation is not surprising given the microenvironment that surrounds the IEC which is replete with cytokine producing immune cells. IEC live in close proximity of intraepithelial lymphocytes, dendritic cells, macrophages and granulocytes. Consistent with the basal location of these cytokine sources, IEC possess various cytokine receptors on their baso-lateral surface (Fig. 3.1) and are activated by cytokines in an autocrine and paracrine fashion. In addition, cytokines can also modulate the basic physiological functions in IEC. Later studies revealed the epithelial presence of receptors for various cytokines such as IL-1, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-15, IL-17, IFN-γ, TNF-α and GM-CSF that take part in innate and adaptive immunity (40, 41). IEC also express MHC class I, class II receptors, non-classical MHC molecules (12) and chemokine receptors such as CXCR1, CXCR4, CCR5 and CCR6 (Fig. 3.1). While interactions between epithelial IEC-MHC molecules and T cells have been extensively studied (43), the role of IEC chemokine receptors is not well studied. The surface expression of these receptors is also dynamic and is modulated by various stimuli. LPS and IFN-γ modulates IL-1, IL-6 receptors (43) and IFN-γ also enhances TNF-α receptor II (44) and MHC class II antigens and thus endows them with the ability to present antigen (45, 46). These studies suggest that in general, the immune mediated signals induce a global phenotypic switch in IEC from cells expressing classical epithelial functions such as barrier and ion transport to those with significant immune functions such as chemokine/cytokine

induction, antigen presentation and leucocyte trafficking. Despite the observations that IEC do express several receptors for immune mediators and are activated by different cytokines, surprisingly, not many reports are available that looked at this phenomenon in the context of infectious agents.

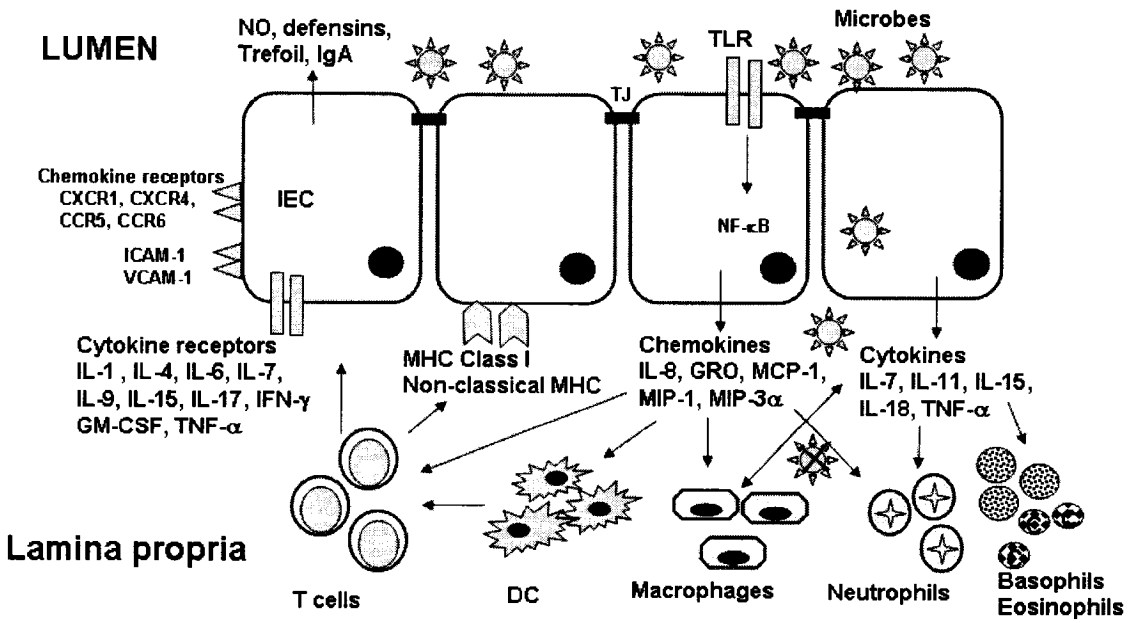

Fig. 3.1: Intestinal epithelial cell (IEC) - immune cell interactions. Epithelial cells secrete innate defense molecules (NO, defensins, IgA), chemokines and cytokines. Chemokines recruit immune cells. Cytokines from IEC activate immune cells and influence the immune response. Cytokines produced by immune cells in turn modulate IEC responses. MHC molecules on IEC present antigens to recruited T cells. Microbes also translocate through IEC and encounter immune cells directly. NF-κB regulates IEC responses. IEC also express chemokine receptors. Tight junctions (TJ) form a physical barrier to the passage of microbes.

To simulate the *in vivo* situation, researchers have adopted different modifications of *in vitro* co-culture systems to critically analyze how immune cells alter epithelial response to a given microbe. Majority of co-culture studies were done with no contact using semi-permeable transwell plates (Fig. 3.2) and all these studies show that epithelial response to pathogens can be modified by immune cell secretions without cell-cell contact. Haller *et al.* (47) reported that nonpathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. Non-pathogenic *E. coli* and *L. sakei* induced IL-8, MCP-1, IL-1β and TNF-α from Caco-2 cells in the presence of underlying peripheral blood mononuclear cells (PBMC) while naive Caco-2 cells remained

hyporesponsive, suggesting that sensitization of IEC by neighboring immunocompetent cells constitutes a crucial step for recognition of non-pathogenic microbes. Interestingly,

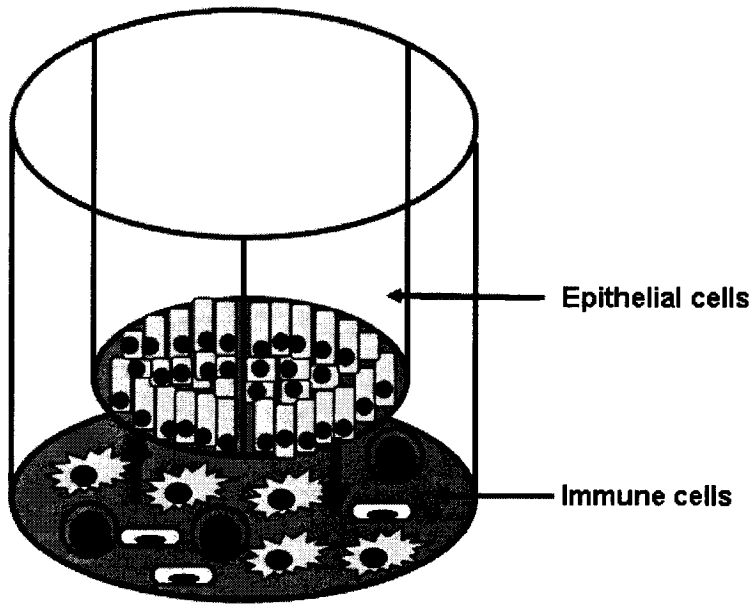

Fig. 3.2: *In vitro* model for IEC-immune cell co-culture. Intestinal epithelial cells are grown on semi-permeable transwell membrane with immune cells in the bottom well without direct contact. This facilitates bi-directional cross talk via soluble factors.

another intestinal commensal *Lactibacillus johnsonii* induced less inflammatory response but more TGF-β in immune cell sensitized IEC. IL-1β and TNF-α were found to mediate the priming of IEC by leucocytes. Another gram-negative commensal bacteria *Bacteroides vulgatus* also induced IκB degradation, NF-κB transcriptional activity in rat IEC (IEC-6 cell line and primary) and these effects are inhibited when IEC were grown in transwell cultures in the presence of lymphocytes (48). This inhibition was observed only in the presence of PBMC or peripheral blood lymphocytes (PBL) but not monocytes. The same authors in a later study (49) investigated more critically, the molecular mechanism of immune-epithelial cell cross-talk on Gram-negative, non-pathogenic, enteric bacteria-induced NF-κB signalling and pro-inflammatory gene expression in IEC using HT-29/MTX as well as Caco-2 transwell cultures. Naive HT-29 cells remained hyporesponsive to both *E. coli* and *B. vulgatus* but in the presence of PBMC or lamina propria mononuclear cells (LPMC), produced IL-8 in response to *E.*

coli (and its LPS) but not *B. vulgatus*. Interestingly, both the bacteria induced IκB phosphorylation in PBMC sensitized IEC but only *B. vulgatus* failed to trigger IκB-α degradation and NF-κB transcriptional activity. This suggests that immune cell mediated tolerance of IEC is stimulus-specific. Another potential mechanism for this differential regulation is that immune cell signals increase the expression of TLR-4 and its co-receptor MD-2 in IEC which makes the cells more responsive to LPS; and *B. vulgatus* is shown to down regulate TLR-4 expression and also block PBMC-induced up regulation of MD-2 making IEC hyporesponsive to this particular microbe. Moreover, differential LPS induced-IL-8 expression was observed when PBMC from Crohn's disease (CD) and ulcerative colitis (UC) patients were used. While no significant affect was observed with the former, UC derived PBMC dramatically decreased he LPS-induced IL-8 mRNA. Finally, when co-culturing was done with purified cell populations from healthy volunteers, hyporesponsiveness to *B. vulgatus* was found to be mediated by lymphocytes but not monocytes suggesting that different immune cells confer differential IEC responsiveness to microbes. However, the same authors (50) previously reported that co-culture of HT-29 or Caco-2 with $CD14^{low}$ monocytes led to reduced inflammatory response by IEC against commensal bacteria. Secretions from IEC have been shown to induce a phenotypic switch in peripheral blood monocytes from a $CD14^{high}$ to a $CD14^{low}$ /$CD16^{low}$ with immunosuppressive functions. These cells secreted immunoregulatory cytokine IL-10 and IL-1 receptor antagonist, and antagonized lymphocyte-mediated activation of the intestinal epithelium in response to intestinal and food derived bacteria. Two recent studies (51, 52) reported induction of chaperone stress proteins, Hsp25 and Hsp72 in mouse intestinal epithelial cells co-cultured with lymphocytes and IL-2 was found to be the mediator. It should be noted that in all these studies, bacteria were added apical to the IEC with immune cells below and it was possible that bacterial products could have translocated through IEC to gain access to immune cells whose response might have altered IEC cytokine production. Also, bi-directional cross talk is involved in this differential IEC response wherein epithelial secretions could also have modified immune cells. Indeed it has long been known that epithelial cells contribute to mucosal immune homeostasis in many ways. However, reports of specific modification of immune cell functions by IEC secretions in the absence of co-culture have been rare.

Kanzato *et al.* (53) showed that paracrine factors from Caco-2 cells increased the phagocytic capacity of differentiated THP-1 macrophages. While the mediators from IEC are thought to be TGF-β or MCP-1, the exact identity of these effective substances is not known. Stockmann *et al.* (54) reported that supernatants of HIV-infected immune cells decreased transepithelial resistance (TER) in HT-29/B6 cells and this effect could be mimicked by recombinant TNF-α. It was also demonstrated that colonic epithelial cell lines (HT-29, Caco-2, HCT-116 and Colo-320) down-regulate IL-8 expression in human intestinal microvascular endothelial cells in a TGF-β1 dependent manner (55). **To summarize, immune cells modify intestinal epithelial cell responses and in many instances, these responses are anti-inflammatory and cyto-protective.**

3.3 Stress response and IEC

Heat shock proteins (Hsp) are a family of endogenous proteins that are highly conserved across all species and are induced by a variety of stress stimuli such as thermal (56, 57), chemical (58), oxidative stress (59, 60), depletion of ATP (61), free radicals (62), and during different patho-physiological conditions such as ischemia (62) and hypoxia (63). Recently, several microbial agents have also been shown to induce stress response in intestinal epithelial cells (51, 52, 64-66). It should be noted that even though the words heat shock proteins and stress proteins are used synonymously, the latter include in addition to Hsp; gp96, glucose-regulated proteins (GRP) and ubiquitin (67). Several classes of Hsp are present based on molecular weight of which the major six families are; Hsp110, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps which includes Hsp27 and Hsp8.5 (68). Hsps can also be grouped into two categories; constitutive and inducible. Constitutive Hsp are normally present in the cell and function as molecular chaperones by folding proteins into their proper configuration and help in transferring proteins across cell membranes. Inducible Hsps are produced under conditions of stress and serve to stabilize proteins, preventing their denaturation. Overall, the major functional properties of Hsp are; a) to maintain normal protein homeostasis by assisting in protein folding, b) facilitating assembly and disassembly of protein complexes, c) inhibition of improper protein aggregation (as occurs during thermal/chemical denaturation), d) direction of newly formed proteins to target organelles for final packaging, degradation or repair (69-

76). Induction of Hsps by mild stress conditions confers protection from subsequent lethal stress and this phenomenon is referred to as stress tolerance. Hence, it is thought that Hsps are an endogenous defense mechanism for cells to overcome deleterious effects of toxic agents.

Hsp expression is transcriptionally regulated by heat shock factors (HSF). Four HSFs have been identified in vertebrates; HSF-1, 2 and 4 are present in mammals and HSF-3 is specific to avians (58, 77-79). It is well established that HSF-1 is the key transcription factor that is necessary for Hsp induction in all conditions in different cell types while HSF-2 is more a developmental regulator (80, 81). HSF1 is constitutively present in cells but under this condition its transcription initiating activity is limited by binding with Hsp90 multichaperone complex (82). Under stress, several denatured proteins compete with the HSF1 for Hsp90 multichaperone complexes, resulting in unbinding of HSF1 that facilitates formation of its homotrimers which acquire transcriptional activity following nuclear translocation. This is an oversimplification of HSF-1 activation but it is suffice to mention that it is regulated at multiple levels; the oligomeric status, its DNA-binding ability, posttranslational modification, transcriptional competence, nuclear/ subnuclear localization, as well as its interactions with regulatory cofactors or other transcription factors (83). Nonetheless, transcription is initiated by the binding of HSF to the heat shock response element (HSE) in the promoters of Hsp genes. HSE are characterized by the presence of specific nucleotide motif which is an inverted repeats of nGAAn (81, 84).

Functionally, Hsp27 (murine Hsp25) and Hsp70 (murine Hsp72) have been extensively studied with regard to their abilities to suppress apoptosis induced by various factors. Both have been found to possess powerful anti apoptotic properties through different mechanisms such as chaperone activity, ability to inhibit several key components of apoptotic machinery and induction of proteasomal mediated degradation of apoptosis-regulatory proteins. Overexpressed Hsp27 or Hsp70 prevented cell death induced by hyperthermia, oxidative stress, cytotoxic drugs, staurosporin and ligation of death receptors Fas/Apo-1/CD95 (85). However, Hsp70 might also enhance apoptosis in a cell

specific manner as overexpression of Hsp70 was found to enhance TCR/CD3- and Fas/Apo-1/CD95-mediated apoptosis in Jurkat T cells (86).

In vivo IECs express all Hsps though with varying degrees at different locations in the gut. Several factors such as inflammation, bacterial load, acidity, nutrients like glutamine and short chain fatty acids, immune cell mediators etc. regulate Hsp expression in IEC (87). While much information is available on Hsp induction by physical, chemical and nutritional stressors, microbial regulation of Hsp in IEC has not been well studied. High Hsp expression in IEC induced by the intestinal flora is thought to maintain the barrier function and protect the host from the damaging effects of toxic agents. Gut inflammation as a result of changes in the intestinal flora seen during antibiotic treatment and *C. difficile* colitis might result from reduced protective factors from commensal bacteria. It has been shown that metronidazole treatment decreases colonic mucosal Hsp25 and Hsp72 expression and decreases barrier function to *C. difficile* toxin A (51). Kojima *et al.* (88) showed that *E. coli* LPS induces Hsp25 but not Hsp72 in young adult mouse colon (YAMC) cells in a p38 and ERK MAP kinase dependent manner. This increased Hsp25 protected the cells against actin depolymerization induced by the oxidant monochloramine. Bacterial superantigen was the only second purified microbial product that has been shown to induce a similar heat shock protein expression from IEC. *Staphylococcal aureus* enterotoxin B (SEB) induces Hsp25 and Hsp72 but not Hsc73 in mouse small intestinal epithelial cells (52). Inhibition of ERK1/2 but not p38 significantly inhibited SEB induced Hsps. Probiotics have excellent beneficial effects in different intestinal inflammatory conditions such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), pouchitis, and antibiotic associated diarrhea. However, the mechanism of this protection is poorly understood. Petrof *et al.* (66) demonstrated that treatment of mouse colonic epithelial cells with conditioned medium of probiotic bacterial mix VSL#3 induces the expression of Hsp25 and Hsp72. Again this is accompanied by protection against oxidative damage through stabilization of actin. Similar observations were also reported by Tao *et al.* (64) who found up-regulation of Hsp25 and Hsp72 by soluble factors of probiotic bacteria *Lactobacillus* GG from young adult mouse colon cells. An acid and heat stable low molecular weight peptide from the

conditioned medium of bacteria induced these Hsps in p38 and JNK MAP kinase dependent manner. Recently cytokines and immune cell secretions have been shown to induce Hsp expression in IEC. Musch *et al.* (52) showed that co-culture of mouse IEC with lamina propria lymphocytes also induced Hsp25 and Hsp72 and this induction is inhibited when neutralizating antibody against IL-2 are added during the co-culture. Role of lymphocytes in IEC Hsp expression was also confirmed in RAG-$1^{-/-}$ mice (51). RAG-$1^{-/-}$ mice are devoid of B and T cells and colonic mucosa from these mice express greatly reduced levels of Hsp72 with not much change in Hsp25 and Hsc73. Sasaki *et al.* (89) reported that TGF-β and IL-1β activate HSF-1 and induces Hsp47 expression in human fibroblasts. **To summarize, microbes and immune mediators can induce heat shock protein expression in intestinal epithelial cells.**

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CHAPTER 4: NF-κB SIGNALING

4.1 Introduction

Signaling is an essential event by which a living cell communicates with environmental cues by sensing and responding to them. Signaling pathways modulate plethora of cell functions under both physiological and pathological states such as growth, proliferation, apoptosis, defense against pathogens etc. While an appropriate regulation of cell signaling is beneficial to the host, the consequences of an aberrant activation/suppression could be disastrous. Signaling cascades involve several components which are intricately connected via a myriad of networks and broadly consist of five steps; receptor-ligand interaction, intracellular signal transduction, nuclear events such as transcriptional/post-transcriptional modulation, cellular responses and phenotypic responses. A thorough deciphering of signaling events contributes immensely to our understanding the pathogenesis of diseases and also to discovering drugs against myriad of health problems such as infectious diseases, diabetes and cancer. Some examples of well studied pathways include, G-protein coupled receptor, protease activated receptor, cytokine receptor signaling, NF-κB signaling, Toll receptor signaling, Wnt signaling, JAK/STAT signaling, MAP kinase, phosphoinositide 3 kinase signaling, calcium and calmodulin signaling (1).

NF-κB (nuclear factor κB), the best-studied and characterized signaling mediator, was first identified as the transcription factor required for κ chain in B cells. Today it is recognized as a ubiquitous molecule that is activated by plethora of stimuli including pathogens, cytokines, growth factors, stress etc. It regulates a number of genes involved in inflammation and immune response and thus assumes a central role in host defense (2). Its significance is evident from the fact that all most all effects of TLR signaling described in chapter 2, are mediated by this transcription factor. The diverse genes that are regulated by NF-κB include those encoding cytokines (IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, TNF-α, IL-1β, LT-α, LT-β, GM-CSF), chemokines such as (IL-8, MIP-1α, MCP, RANTES, eotoxin), adhesion molecules (ICAM-1, VCAM, E-selectin), acute phase proteins (SAA), inducible enzymes of inflammatory response (iNOS, COX-2), anti-microbial peptides (defensins), molecules for adaptive immune signaling (MHC I and II,

B7.1, B7.2) and apoptosis regulators (c-IAP-1, cIAP-2, BCL, Fas ligand, *c-myc*, cyclin D1). Given this long list of genes, it is not surprising that NF-κB has become the target molecule of choice to modulate various disease processes. This lead to an intense research aimed at understanding the various aspects of this transcription factor under different conditions. Its activation was found to be rapid and is regulated very tightly. It is a family of five transcription factors and exists as either homo or hetero dimers which differ in their ability to bind the promoter regions of target genes and induce the gene transcription (3). There are 5 genes of NF-κB family in mammalian cells: *RelA*, *RelB*, *c-Rel*, *NF-κB1* and *NF-κB2*. These genes gives rise to 7 NF-κB proteins- Rel A (p65), Rel B, c-Rel, p50 and its precursor protein p105, and p52 and its precursor protein p100. P50 and p52 correspond to the amino terminal halves of the precursor proteins p105 and p100 respectively. All 5 members share a 300 aminoacid Rel Homology Domain (RHD) at N-terminal, which is essential for DNA binding, dimerization and binding to the IκB family of NF-κB inhibitory proteins. However, the transactivation domains (TAD) are present in the c-terminal of only Rel A, Rel B and c-Rel (Fig. 4.1). TADs interact with the basal transcriptional activators such as TBP, TFIIB, CBP etc (4-6). The glycine rich regions (GRR) are important for co-translational processing of p105 to p50 and post-translational processing of p100 to p52. The prototypical NF-κB is composed of p50 and p65 which has the greatest transcriptional ability where as p50/p50 and p52/p52 homodimers repress the gene transcription owing to the absence of TADs (7). Various other combinations of dimers are known to exist and mice lacking individual subunits display distinct phenotypes, suggesting specific role of dimers in inducing distinct sets of NF-κB target genes (3). The dimers of NF-κB exist in a latent form in the cytoplasm, bound to the Inhibitory κB (IκB) proteins. The major members of the IκB family include IκB-α, IκB-β, IκB-γ, IκB-ε, and BCL-3. The hallmark of IκB proteins is the presence of ankyrin-repeating domain that mediates interaction with NF-κB proteins (Fig. 4.1). Also p105 and p100, the precursor proteins respectively of NF-κB p50 and p52, contain ankyrin repeating domains and act as inhibitors of NF-κB. BCL-3 is unique because it contains a TAD in addition to ankyrin repeats. Consequently p50 and p52 units of NF-κB when form a complex with BCL-3, acquire transcriptional activation properties. Various stimuli

that activate NF-κB cause degradation of inhibitory proteins and nuclear translocation of NF-κB subunits (8). Once in the nucleus, NF-κB transcription factors recognize a specific consensus sequence in the promoter regions of target genes which contain one or more κB-enhancer consensus sequences 5'-GGGRNYYYCC-3', where R is a purine, Y is a pyrimidine and N is any nucleic acid. Binding of NF-κB together with recruitment of co-activators such as CBP, TBP and TFIIB then stimulates gene transcription.

It should be mentioned that NF-κB-IκB complexes can also shuttle between the cytoplasm and the nucleus in unstimulated cells, but the nuclear export is more efficient and, therefore constitutively, the NF-κB-IκB complexes remain mainly cytoplasmic. This is facilitated by the existence of nuclear export signals (NES) on IκB-α and also the incomplete masking of nuclear localization signals (NLS) on NF-κB subunits. Majority of the studies on NF-κB activation have focused on IκB-α but NF-κB is also sequestered by another isoform IκB-β. However, complexes of IκB-β can not enter nucleus because of the absence of nuclear export signals (NES) on IκB-β and also the complete masking of NLS on NF-κB molecules by this isoform (2).

4.2. Activation of NF-κB

Fundamentally, there are two mechanisms by which the transcription factor NF-κB is activated; one depends on the phosphorylation and degradation of inhibitory proteins with subsequent release of NF-κB dimers, while the second pathway depends on the post-translational modifications of NF-κB molecules. These two pathways are complementary and are found to be required for optimal NF-κB activation. Four pathways exist that involve degradation of IκBs: classical, alternative, atypical and p105 pathways (Fig. 4.2) (9).

Classical pathway:

This is triggered by proinflammatory cytokines TNF-α, IL-1β and TLRs and involves a sequential recruitment of various adaptors to the cytoplasmic membrane and activation of kinases that converge at the level of Inhibitory Kappa B Kinase (IKK) complex. IKK

complex consists of regulatory unit, a scaffolding protein NF-κB essential modulator (NEMO) or IKK-γ and two catalytic subunits, IKK-α (or IKK-1) and IKK-β (or IKK-2). IKK phosphorylates IκB-α at serine 32 and 36 positions which is then tagged by

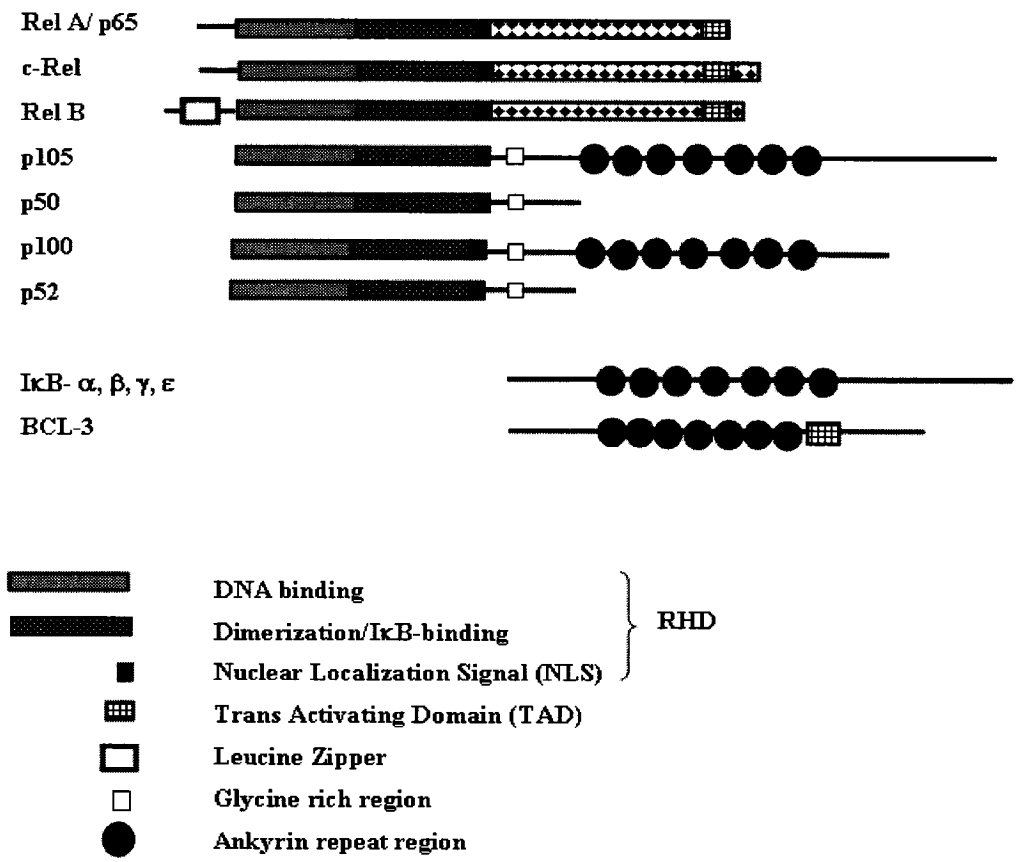

Fig. 4.1: Structure of NF-κB and IκB molecules: All NF-κB/Rel molecules have a common NH_2-terminal Rel Homology Domain (RHD). C-terminal TAD is present only in Rel A(p65), Rel B and c-Rel. LZ is essential for transcativation by Rel B. IκB molecules (includes p105 and p100) possess ankyrin repeat regions which mediate interaction with NF-κB proteins. GRR is essential for processing of p105 and p100 into their respective NF-κB molecules; BCL-3 is unique in that it possesses both ankyrin repeats and TAD.

ubiquitin. Polyubiquitination of IκB-α is mediated by ubiquitin ligase complex designated β-TrCP-SCF (10, 11). Several other components make up this complex, including the ubiquitin-conjugating enzyme and a subunit, Cu11. Ubiquitinated IκB-α is recognized by 26S regulatory subunit of proteasome followed by proteolytic digestion of inhibitory protein. Following degradation of the inhibitory IκB, the NF-κB subunits are

released with their Nuclear Localization Signals (NLS) exposed. Thus, they get translocated to the nucleus and induce transcription of a number of genes. A simplistic mechanism of this pathway is depicted in Fig. 4.2. In addition, diverse stimuli activate initiator caspases 1, 2, 8 and 10 that again converge on and activate IKK complex and thus, the classical p50/p65 nuclear translocation (12).

Alternative pathway:

A second pathway that depends on inhibitory protein degradation involves the activation of IKK-α homodimer via NF-κB inducing kinase (NIK). The inhibitory protein p100 is the downstream target for IKK-α. Following phosphorylation on specific serines at both C- and N-terminals, p100 is cleaved by proteasome, generating p52 subunit of NF-κB. P52 then forms heterodimer with Rel B, moves to the nucleus and activate a set of target genes. This is found to be triggered by cytokine lymphotoxin β, B-cell activating factor (BAFF), CD40 ligand and by certain viruses like Epstein-Barr virus and human T cell leukemia virus (13-17).

Atypical pathway:

A third mechanism by which IκB is degraded and activates NF-κB is IKK-independent. DNA damaging agents such as UV and doxorubicin induces IκB-α degradation via p38 MAP kinase activated casein kinase 2 (CK2) (18, 19). CK2 phosphorylates IκB-α on N-terminal serine residues thereby induces proteasomal degradation of the inhibitory protein. Another pathway wherein tyrosine but not serine residues of IκB-α are phosphorylated is activated by oxidative stress. N-terminal Tyr42 play a role in this and Syk protein tyrosine kinase is required for hydrogen peroxide mediated NF-κB activation (20, 21). As evidenced above, all these mechanisms require phosphorylation of IκB and indeed, cells expressing phosphorylation deficient- IκBα mutant fail to activate NF-κB (22). In addition to IκB-α and p100, IκB-β and IκB-ε are also targeted for phosphorylation on serine 19 and 23 by IKK complex that triggers the inhibitor protein degradation and release of NF-κB dimers (23, 24).

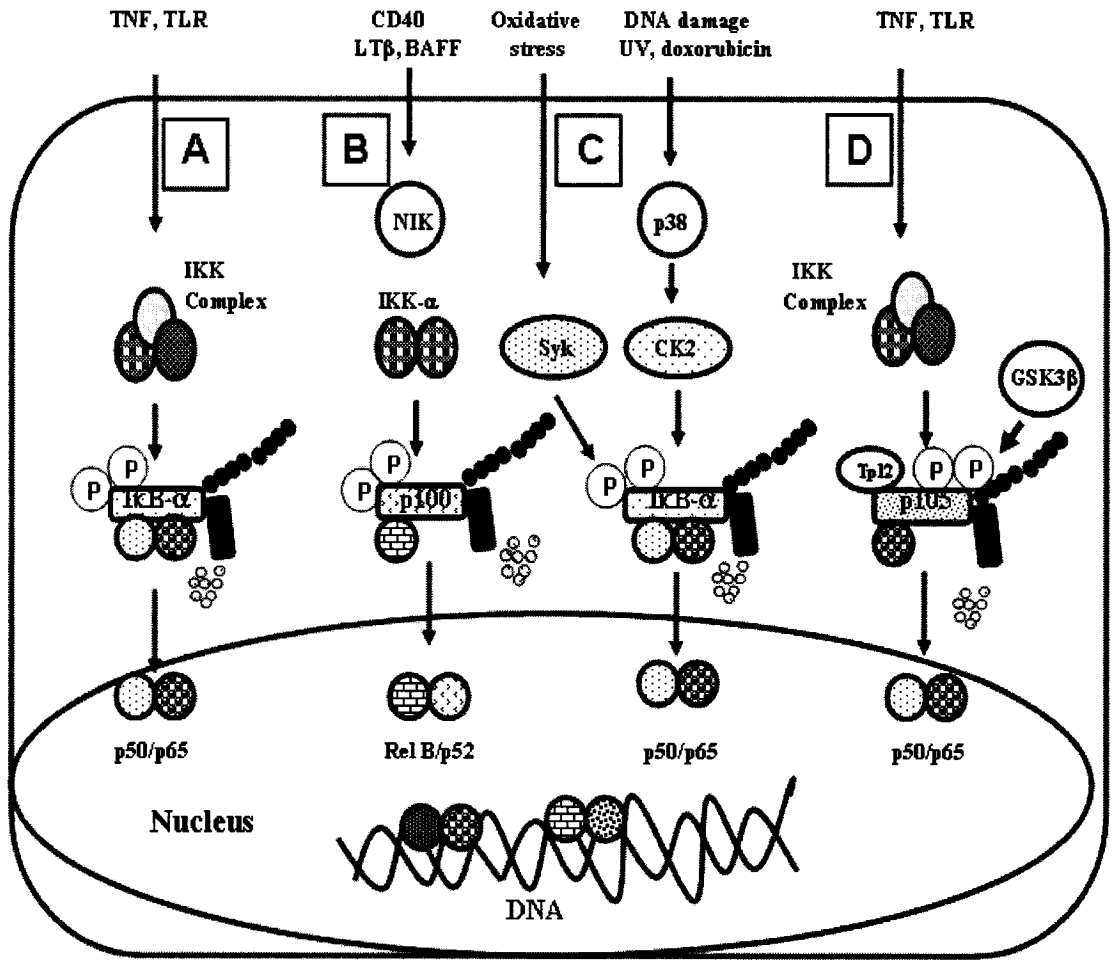

Fig. 4.2: Pathways for NF-κB activation. A) Classical activation involves phosphorylation of IκB-α by IKK complex, ubiquitination, degradation by proteasome and activates p50/p65 dimers. These dimers bind to κB enhancer elements in the promoters of target genes and induce transcription. This is the most common pathway initiated by majority of stimuli. B) Alternative pathway, activated by few stimuli involves phosphorylation of p100 by IKK-α homo dimer, followed by cleavage of p100 to p52 that moves into nucleus with Rel B. C) Atypical pathway is similar to classical pathway except that IκB-α is phosphorylated by CK2 or Syk and is IKK-independent. D) p105 pathway involves phosphorylation of p105 by IKK complex, GSK3β and Tpl2, processing into p50 that forms dimer with p65 and translocate to nucleus.

P105 pathway:

Another ankyrin containing inhibitory molecule p105 is subjected to phosphorylation-dependent degradation by several kinases. Similar to that observed with p100, degradation of p105 generates another NF-κB protein p50. IKK-mediated phosphorylation of p105 on ser927 and ser932 is induced by TNF-α (25) and glycogen

synthase kinase 3β (GSK-3β) plays a dual role in p105 phosphorylation and degradation. Phosphorylation of ser903 and ser907 by GSK-3β prevents the degradation and stabilizes the inhibitory protein. However, phosphorylation at these sites is essential for IKK mediated phosphorylation of ser927 and ser932 that induces degradation of p105 (26). Mitogen activated kinase kinase kinase (MAP3K), Tpl2/Cot, is also found to regulate p105 phosphorylation and this phenomenon plays an important role in lipopolysaccharide (LPS)-activated extracellular signal-related (ERK) signaling (27-29).

Two more inhibitory proteins, IκB-ζ (30) and BCL-3 (31) exist in nucleus. The potential kinases that phosphorylate these inhibitors and how this modulates NF-κB activation are poorly understood. IκB-ζ is required for the expression of a set of genes such as IL-6 during TLR and IL-1 signaling (32). Recently GSK-3β is shown to constitutively phosphorylate BCL-3 and target the latter for proteasomal degradation (33). **To summarize, NF-κB activation in principle, requires the degradation of inhibitory proteins (IκBs) that bind the NF-κB molecules and this degradation requires phosphorylation by upstream kinases. There are stimulus-dependent signaling events, which involve activation of specific kinases that have substrate specificity to a particular inhibitory protein.**

4.3 Post-translational modifications of NF-κB

In addition to the pathways described above that depend on degradation of inhibitory proteins, it was found that posttranslational modifications of NF-κB subunits themselves could increase the transactivating potential of this transcription factor. In fact, degradation of IκB and subsequent nuclear translocation of NF-κB are not sufficient to elicit maximal transcriptional response. A second phase of events which occurs primarily, but not exclusively, in nucleus involve phosphorylation and acetylation of NF-κB or acetylation of histones that surround the promoter regions of NF-κB target genes (3, 9). These events define the strength and duration of NF-κB induced gene activation.

4.3.1 Phosphorylation of NF-κB

It has been observed that cells lacking kinases such as T2K, GSK3β, TRAF-associated NF-κB activator (TANK)-binding kinase 1 (TBK1 or NAK) or that have been treated with PI3K inhibitors, have defective NF-κB activation despite efficient IκB degradation in response (34-37). Several kinases that function both in the cytoplasm and nucleus were found to target the RelA or p65 subunit of NF-κB (Fig. 4.3). Serines (Ser) at four positions are phosphorylated by specific kinases; Ser276 and Ser311 in the RHD, and Ser529 and Ser536 in the TAD regions of p65 are phosphorylated in a stimulus-dependent manner. Catalytic domain of Protein kinase A (PKA_c) mediated Ser276 phosphorylation in the cytoplasm, induced by LPS modulates DNA binding and oligomerization properties of NF-κB (38). PKA_c phosphorylation of Ser276 also enhances the ability of p65 to recruit histone acetyltransferases such as CREB-binding protein (CBP) and p300 (39) and to displace p50-histone deacetylase (HDAC)-1 complex from DNA (40). With this dual effect, PKA_c potentiates the NF-κB activity. On the other hand, TNF-α stimulates mitogen and stress activated kinase-1 (MSK1) which phosphorylates the same Ser276 in the nucleus resulting in an increased NF-κB transcriptional activity (41). TNF-α also phosphorylates Ser311 through the action of another kinase, PKCζ leading to a similar enhancement of transcriptional response (42). As with PKA_c, PKCζ phosphorylation of p65 enhances its interaction with CBP and RNA polymerase II on IL-6 promoter. Ser529 and Ser536 in the TAD of p65 are phosphorylated by casein kinase II (CKII) and IKK respectively. IL-1β (43) and TNF-α (44) activate CKII and phosphorylation of p65 on Ser529 by this kinase requires prior IκBα degradation. Ser536 phosphorylation of p65 was extensively studied and found to occur by several kinases via various signaling pathways. IKKs are the major kinases involved in ser536 phosphorylation. Lymphotoxin B (45) and oncoprotein Tax (46) phosphorylate p65 on Ser536 via IKKα. In case of

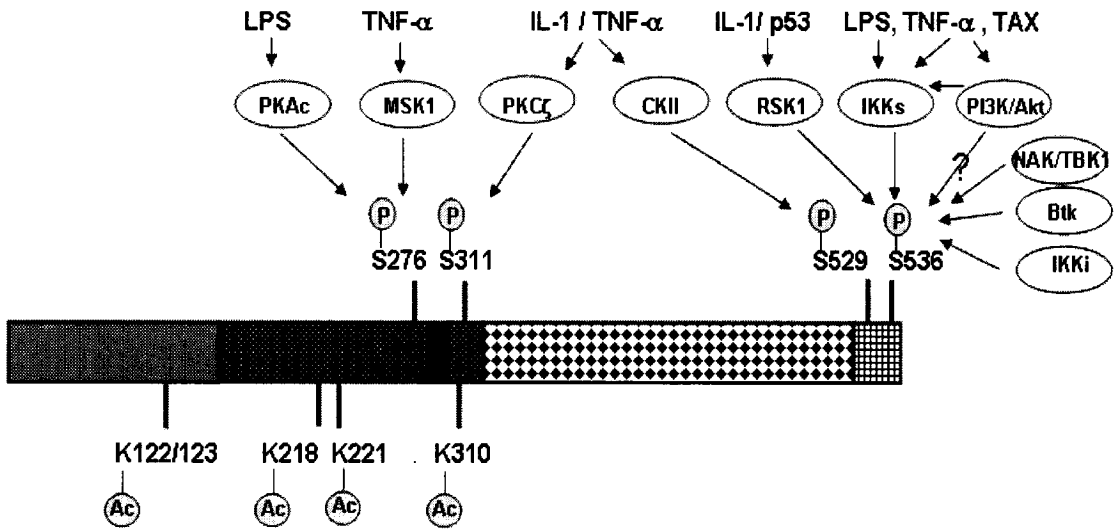

Fig. 4.3: Post-translational modifications of p65. Sites for serine (S) phosphorylation (P) and the kinases involved are shown on the top. PI3K/ Akt phosphorylates S536 via IKK and also through unidentified kinases. Sites for lysine (K) acetylation (Ac) are depicted in the bottom. ▒ : DNA binding domain, ▒ : Dimerizarion / IκB-binding domain, ■ : Nuclear localization signal, ▦ : Transactivation domain.

lymphotoxin B, NIK is required for IKKα activation (45). Conversely, IKKβ mediated Ser536 phosphorylation was observed in LPS treated monocytes/macrophages (47) and CD40 triggered hepatic stellate cells (48). LPS induced S536 phosphorylation is also mediated by a novel kinase, Bruton's tyrosine kinase (Btk) (49). Recently, the novel IKK, IKK-i/IKK-ε was found to play a role in the constitutive but not cytokine-induced phosphorylation of S536 of p65 (50). DNA damaging agents, similar to their ability to induce IκB degradation independent of IKKs, also induce S536 phosphorylation of p65, which does not involve IKKs. Drugs such as doxorubicin and etoposide have been found to activate NF-κB via p53 pathway that depends on ribosomal S6 kinase-1 (RSK1) induced phosphorylation of Ser536 (51). Ser536 is phosphorylated by several other kinases such as GSK3β (52), PI3 kinase/Akt (53, 54) and NF-κB activating kinase (NAK; also called TBK1) (55). While some kinases directly phosphorylate p65, others such as PI3 kinase and Akt act indirectly via other kinases. The downstream effectors that mediate PI3K/Akt pathway have not been identified and remain a point of controversy. IKKα, IKKβ and p38 have been implicated in carrying out the Ser536 phosphorylation by PI3 kinase/Akt (53, 56). It seems there are still unidentified kinases such as the one

that phosphorylates Ser468 upon T cell co-stimulation (57). Majority of these observations were made in cell lines and the precise role of p65 phosphorylation in an *in vivo* situation remains to be clarified. Despite the presence of several phospho acceptor sites that are phosphorylated by specific kinases in a stimulus-dependent fashion, it is not clear if it confers unique functional advantage in a given situation or is a redundant phenomenon.

The other transactivating domain (TAD) containing NF-κB proteins, Rel B and c-Rel also undergo phosphorylation (58, 59). However several issues such as which genes are specifically targeted by Rel B and c-Rel proteins, the effect of these phosphorylations on p65 transactivating potential etc. merit further investigation. Not much is known about the phophorylation of other NF-κB proteins that do not have the TAD, p50 and p52. Though evidence exists that p50 is phosphorylated (60) upon stimulation, the significance is not clear. PKA, calmodulin II, CKII and PKG are potential kinases that target Ser337 on p50 (61). As these NF-κB proteins lack the transactivating region, phosphorylation might affect only the DNA binding ability and could indirectly influence the transcriptional responses induced by TAD containing NF-κB subunits. **To summarize, NF-κB p65 is phosphorylated principally on four serines leading to recruitment of transcriptional co-activators or displacement of repressors resulting in enhanced transcriptional response. Various kinases are involved in phosphorylating p65 at specific positions following activation by different stimuli.**

4.3.2 Acetylation of NF-κB

Similar to phosphorylation, acetylation was also found to be an important regulator of NF-κB function. As opposed to phosphorylation, acetylation occurs exclusively in the nucleus where acetyltransferases are localized. Acetylation by histone acetyltransferases (HATs) regulate the subcellular localization, DNA binding, interaction with transcriptional cofactors and transactivating potential of various transcription factors (62, 63). Lysine (Ly) 218, 221 and 310 are the three major acetylation sites on NF-κB p65 molecule that modulate distinct biological responses (Fig. 4.3) (64). Acetylation at Ly221 enhances DNA binding by p65 and reduces association with IκBα. On the other hand,

acetylation of Ly310 is required for full transcriptional activity of p65 and does not affect DNA binding or IκBα interaction. Acetylation at two other sites, Ly122 and Ly123 has a negative effect by reducing p65 binding to DNA and consequent reduction in the NF-κB transactivating potential (65). P50 subunit is acetylated at Ly431, 440 and 441 leading to increased transcriptional response by p50 heterodimers (66, 67). (p50 homodimers are repressive because of the absence of TAD). It is possible that phosphorylation is required for subsequent acetylation. In case of p65, phosphorylation at ser276 and 311 enhances association with p300/CBP and this leads to increased acetylation and transcriptional activation (40, 42). Several puzzles remain unanswered such as the stoichiometry of RelA acetylation, the relationship between signal intensity, acetylation and ensuing transcriptional response. It is suffice here to mention that in addition to the post-translational modification of NF-κB proteins themselves, similar modifications of histones that surround the NF-κB target genes also equally regulate NF-κB transcriptional activity (3).

4.4 Suppression of NF-κB activation by pathogens

All pathogens, viral, bacterial or eukaryotic, developed various strategies to exploit the hosts in order to continue their life cycle. On the other hand, hosts too evolved several defense mechanisms to ward-off the pathogens. A successful parasite must be able to overcome these defense mechanisms. This constant interaction and evolution might eventually terminate in commensalism or symbiotic relationship. In the former, both host and pathogen remain harmless to each other while in the latter both get benefited by each other. Because of the importance of NF-κB as a regulator of apoptosis, inflammatory and immune responses, it became a potential target for pathogens to modulate host response for their replication and survival. Studying how pathogens modulate the NF-κB system thus provides an exciting paradigm for host-pathogen interactions.

Invasion of the host by pathogen, in majority of cases, is associated with activation of NF-κB which regulates various immune/inflammatory processes that are required for host defense against the infection. It is increasingly being recognized that various steps of NF-κB activation are targets for microbes to interfere with immune and inflammatory

responses (Table 4.1). TLR signaling which primarily results in NF-κB activation is the target for vaccinia virus. This virus produces the protein A52R, a homologue of MyD88 that is required for TLR and IL-1 signaling induced NF-κB activation. A52R acts as a dominant negative regulator of MyD88 and suppresses the NF-κB pathways requiring this scaffolding protein (68). IL-1 is required for host defense against this virus and by this mechanism the pathogen effectively overcomes the host responses, which are deleterious to the virus.

A large number of bacteria target a further downstream molecule, IκB whose degradation is essential for NF-κB activation. Different species of gram negative extracellular bacteria *Yersinia* secrete type III secretory molecule, Yersinia Outer proteins (Yop) that inhibit NF-κB by different mechanisms. YopJ of *Y. enterocilitica* associates with IKK-β, inhibits the kinase activity and consequently, the phosphorylation and degradation of IκB-α (69). This would suppress the NF-κB nuclear translocation and inflammatory cytokine induction. While the precise mechanism of how YopJ blocks IKK activity is not understood, Mukherjee *et al.* (70) recently showed that YopJ acetylates the critical serine/threonine residues required for IKK-β phosphorylation and thus competitively inhibits the phosphorylation.

Table 4.1: Mechanism of NF-κB inhibition by microbes

Mechanism of NF-κB inhibition	Pathogen	Molecule responsible	Comments
I. At the receptor level Mimics MyD88 and blocks initiation of signaling	Vaccinia virus	A46R A52R	Signaling pathways that require MyD88 as adaptor (IL-1 and TLR) are blocked
II. Blocks regulatory protein function upstream of IKK a. interfering with ubiquitination	*Y.pseudotuberculosis* *Y.pestis*	YopJ	Cleaves ubiquitin by cysteine protease activity
b. blocks tyrosine phosphorylation	*Y.pseudotuberculosis*	YopH	Blocks T and B cell activation
III. Direct inhibition of IKK activity (indirectly blocks IκB phosphorylation)	*Yersinia*	YopJ	Acetylates IKK-β and blocks its phosphorylation
IV. At IκB level a. blocks phosphorylation	Pox viruses Measles virus		By dephosphorylation
b. blocks ubiquitination	HIV Avirulent Salmonella UPEC	Vpu protein Soluble factors	Blocks β-TrCP Blocks β-TrCP or deubiquination
c. blocks degradation	Probiotics	Soluble factors	Blocks proteasome activity
d. mimics IκB (binds NF-κB)	African swine fever virus	A238L	

V. Prevents nuclear transport of p65 subunit	Epstein-Barr virus *Bordetella bronchiseptica* *Toxoplasma gonodii*	ZEBRA	
VI. Prevents DNA binding and transcriptional activity of NF-κB	*Mycobacterium ulcerans* *Bacteroides vulgatus* *Schistosoma mansoni* *Salmonella enterica* serovar typhimurium	Soluble toxin Excretory-secretory product SspH1	Prevents p65 phosphorylation Mediated by TGF-β via histone deacetylation Disrupts protein complex formation at κB site of E-selectin Via interaction with PKN1 in nucleus

Several signaling intermediates require ubiquitin or ubiquitin-like molecules such as SUMO-1 for activation. Such modifications are involved at several regulatory events in NF-κB pathway: a) TRAF6 ubiquitination mediated by Ubc13 and Uev1A, b) ubiquitination of SCF complex mediated by unknown ligase and c) addition of ubiquitin onto phospho- IκB mediated by the β-TrCP-SCF. YopJ of *Y. pseudotuberculosis* and *Y. pestis* acts as cysteine protease and targets these ubiquitins, preventing the functions of many regulatory proteins. This virulence factor was found to inhibit MAP3K via direct association or indirectly by inhibiting ubiquitin conjugation (71). These effects are primarily observed in macrophages but can also occur in epithelial cells, fibroblasts and lymphocytes. In both T and B cells, YopH of *Y. pseudotuberculosis* acts as tyrosine phosphatase that prevents phosphorylation of some early proteins that are activated following antigen-specific receptor signaling (72). Though no specific inhibition of NF-κB pathway was studied, decreased costimulatory molecule and cytokine expression was observed. As the phosphorylation of earliest signaling molecules such as PKC and CD3ζ

were suppressed, it is hypothesized that YopH could also interfere with NF-κB activation. Apart from the inhibition of events proximal to IκB, measles virus was found to directly inhibit IκB phosphorylation in neuronal cells through an unknown mechanism (73).

A further downstream mechanism is inhibition of degradation of IκB even after its phosphorylation. Many pox viruses including vaccinia virus inhibit IκB degradation by dephosphorylation of IκB-α or interfering with its degradation by an unknown phenomenon (74). Vpu protein of HIV-1 interferes with ubiqutination of IκB in a unique way. It targets β-TrCP, a component of ubiquitin ligase complex that is required for IκB ubiquitination and subsequent proteasomal degradation (75). The non-pathogenic species of intracellular bacteria, salmonella were also found to shut down NF-κB activation by a similar mechanism of interfering with IκB ubiquitination. This is thought to help colonization of these species without inducing mucosal inflammation (76).

Uropathogenic *E. coli* (UPEC) prevents the degradation of IκB thereby stabilizes this inhibitory molecule and inhibits NF-κB activation. However, the precise mechanism is not known (77). Nonetheless, this results in a decrease of anti-apoptotic proteins and increases apoptosis of urothelial cells. UPEC also inhibits MAP kinase signaling in a contact dependent or independent mechanism. The latter is thought to be mediated by undefined soluble factors. Some pathogens like African swine fever virus possess a protein, A238L that mimics IκB and binds NF-κB dimers even after host IκB is degraded (78). Recently, commensal bacteria (a mix containing *Streptococcus thermophilus* and several species of *Lactobacillus* and *Bifidobacteria*) were shown to inhibit NF-κB activation by a novel mechanism of proteasome inhibition without affecting ubiquitination (79). Inhibition of proteasome activity also suppressed the cleavage of inhibitory p105 protein. Together this blocked the release of NF-κB molecules from the inhibitory proteins.

Some pathogens inhibit the nuclear translocation of NF-κB molecules after they are released from IκB. A viral protein ZEBRA in Epstein-BarrVirus (EBV) binds p65 molecule and prevents nuclear localization of NF-κB (80). A soluble toxin of *Mycobacterium ulcerans* blocks phosphorylation of p65 subunit and prevents its DNA binding activity leading to suppression of immune cell functions (81). *Bordetella bronchiseptica* also inhibits nuclear translocation of NF-κB p65 subunit in response to TNF-α in respiratory epithelial cells through an unknown mechanism involving type III secretory system (82).

Further downstream, pathogens modulate the nuclear events of NF-κB activation. SspH1, a *Salmonella enterica* serovar Typhimurium TTSS effector, localizes to the mammalian nucleus and down-modulates production of proinflammatory cytokines by inhibiting NF-κB-dependent gene expression. Though the precise mechanism is not known, it could be mediated via the interaction with PKN1, a serine/threonine protein kinase that is found to regulate the NF-κB induced gene expression (83). Haller *et al.* (84) proposed a novel mechanism of NF-κB inhibition that involves histone acetylation. Non-pathogenic, enteric bacteria *Bacteroides vulgatus* induces an initial NF-κB and then a persistent TGF-β1 signaling. It was found that TGF-β1 inhibits *B. vulgatus* induced NF-κB activation by deacetylating the histones surrounding the NF-κB recruitment site in the promoter of IL-6 gene and thus suppresses the latter's expression.

Evidence for parasite mediated NF-κB suppression came from studies in *Toxoplasma gondii* and *Schistosoma mansoni*. *T. gondii* blocks inflammatory stimuli-induced nuclear translocation of NF-κB and inhibits IL-12 and TNF-α production that are required for host resistance of this infection (85). Similarly, excretory-secretory products of *S. mansoni* inhibits a protein complex formation at κB binding sites in the promoter of E-selectin, thereby reduces the expression of this adhesion molecule in lung endothelial cells. This prevents recruitment of inflammatory cells that could effectively eliminate the schistosomula (86).

It should be noted that pathogens also exploit NF-κB activation to promote inflammation and spread infection to other organs (87). From these observations it is apparent that microbes usurp or mimic a eukaryotic activity and refine this activity to produce an extremely efficient mechanism to combat NF-κB signaling. **To summarize, various pathogens developed different means to suppress NF-κB as a potential mechanism to circumvent host defense.**

4.5 Modulation of NF-κB activation by stress response

It has been found that activation of cellular stress response inhibits subsequent proinflammatory responses in various cell culture models. This has been unequivocally linked to the inhibitory effects of both heat shock proteins (Hsp) and heat shock transcription factor (HSF) on NF-κB activation. Similar effects are also observed in animal models of inflammation wherein inhibitory effects of stress response are associated with protection against organ injury and death (88-92).

In vitro, stress response was found to inhibit IL-1β (93), TNF-α, IL-6 (94), iNOS (95, 96), IL-8 (97), RANTES (98), complement components C3 & C5 (99), ICAM-1(100) and Cox-2 (101) expression in diverse cells types including monocytes, epithelial, endothelial and neuronal cells. While original observations reported inhibition of NF-κB mediated gene expression following heat shock protein induction by different stimuli, the exact mechanism and involvement of specific Hsps in this inhibition were not described. Recent studies looked into the identity of Hsps and mechanism of Hsp-mediated NF-κB inhibition, which has been shown to be operating by different mechanisms; inhibiting IκB degradation (by regulating IKK activity and increasing phosphatase activity), inducing IκB-α expression, preventing NF-κB nuclear translocation and finally by inhibiting gene transcription by repressing the promoter activity of inflammatory genes (Table 4.2). Hsps have been shown to associate with IKK complex and modify its ability to phosphorylate IκB-α. Hsp90 positively regulates IKK activity by its association with IKK complex and various stimuli that dissociate Hsp90 from IKK leads to suppressed NF-κB activation (102, 103). Heat shock or exposure to sodium arsenite dissociates

Hsp90 and insolubilizes IKK complex reducing the kinase activity in alveolar epithelial cell line A549 (104). Sustained PKC activation by PMA treatment in colonic epithelial cells also leads to dissociation of Hsp90 and makes the IKK complex vulnerable to proteasome degradation and consequently suppresses NF-κB activation by TNF-α (105). Interestingly, the gastric pathogen *H. pylori* induced IL-8 production is dependent on Hsp90. *H. pylori* phosphorylates Hsp90 and treatment with geldanamycin, an inhibitor of Hsp90 blocked *H. pylori* induced NF-κB activation and IL-8 production in gastric epithelial cells (106).

Hsp70 has been shown to have contrasting effects on IKK activity. Ran *et al.* (107) reported a negative regulation of IKK activity by Hsp70. Hsp70 directly interacts with IKK-γ and prevents the formation of functional IKK complex thereby inhibits IKK activity. Over expressing Hsp70 inhibited phosphorylation and degradation of IκB-α, induction of anti-apoptotic gene expression and increased apoptosis in response to TNF-α. However, in a recent study, Lee *et al.* (108) reported a positive role for Hsp70 in restoring IKK activity following heat shock. It was shown that over expression of Hsp70 did not inhibit TNF induced NF-κB activation but helped in the resolubilization of IKK complex following heat shock in BEAS-2B, a bronchial epithelial cell line. Thermal stress in cells with reduced Hsp70 expression resulted in delayed recovery of IKK

Table 4.2: Modulation of NF-κB activation by stress response

Target event	**Molecules and mechanisms**	**Positive/Negative**
Receptor activation	Hsp60 via TLR-4	Positive
Modulation of regulatory protein function	Hsp70 blocks ubiquitination of TRAF-6	Negative
	Sequestration of TRAF2 into stress granules	Negative
IKK activity	Hsp90 acts as chaperone for IKK	Positive
	Hsp70 acts as chaperone for IKK	Positive
	Hsp70 interacts with IKK-γ prevents	Negative

	IKK complex formation Hsp27 interacts with and blocks IKK-α	Negative
NF-κB/IκB complex	Hsp27 acts as ubiquitin binding protein and promotes IκB degradation Hsp70 binds to the complex and stabilizes	Positive Negative
IκB synthesis	HSF-1 binds to HSE in IκB-α promoter and induces transcription	Negative
Phosphatase activity	Decreases phosphorylation and degradation of IκB	Negative
Nuclear transport of NF-κB	Hsp70 binds to p65, c-Rel and p50 Hsp60 (?)	Negative Negative
Transcriptional repression	HSF-1 binds to cytokine gene promoters and represses transcription and/or interfere with NF-κB binding	Negative

activity and cellular inflammatory response to TNF-α. Here Hsp70 played a chaperone function for IKK by renaturating and restoring the latter's kinase activity in response to TNF-α. It is thought that the role of Hsp70 in NF-κB activation could be cell and stimuli specific. As mentioned earlier in this chapter, several regulatory proteins require ubiquitination for their activation and a recent report by Chen *et al.* (109) suggests that Hsp70 interacts with TRAF-6, a signal transducer in TLR signaling and prevents its ubiquitination. This mechanism was found to inhibit LPS induced iNOS and COX-2 expression in murine macrophage cell line RAW264.3. Though, no specific Hsp was identified to be responsible, TRAF2 was found to sequester into stress granules following thermal shock and thus inhibit TNF induced NF-κB activation (110). Another Hsp that associates with and regulate IKK activity is Hsp27. Park *et al.* (111) for the first time showed that phosphorylated Hsp27 interacts with both IKK-α and IKK-β but its

association is increased only with IKK-α following TNF-α treatment in HeLa cells, resulting in suppression of IKK activity. However, acting downstream of IKK, Hsp27 enhances IκB-α ubiquitination and promotes proteasomal degradation of IκB-α leading to increased NF-κB activity (112). Though no specific Hsps have so far been implicated, stress response in general is found to augment intracellular phosphatase activity and inhibition of phosphatases at least partially reversed the inhibitory effect of stress response on LPS-induced IκB-α degradation (113, 114)

IκB-NF-κB complex exists via an interaction between ankyrin repeats of IκB with nuclear localization sequences (NLS) in NF-κB. As mentioned earlier, phosphorylation of IκB is essential for the dissociation of this complex. Hsp70 has been shown to contain the NLS (115) and hence can directly interact with IκB-α ankyrin region and interferes with IκB-α phosphorylation, probably by steric hindrance. This hypothesis was recently confirmed in an *in vivo* study. Chen *et al.* (116) reported that heat shock treatment of rats suppresses subsequent sepsis induced NF-κB activation and found that Hsp70 forms a complex with NF-κB/IκB and prevents the degradation of IκB and decreased translocation of NF-κB.

It is also evident that Hsps induce IκB expression. A 20 bp heat shock response element has been identified in the promoter region of IκB-α. Increased IκB-α expression following stress could stabilize the NF-κB-IκB complex and hinder NF-κB activation (117). In IEC, heat shock was associated with constant levels of IκB-α that hampered LPS induced NF-κB DNA binding activity and increased IκB-α expression was found to be partially responsible for this effect (118).

In contrast to the Hsp70- IκB-α interaction as reported above, association of Hsp70 with NF-κB subunits was proposed. Feinstien *et al.* (119) suggested that Hsp70 could interfere with p65 nuclear translocation in glial cells, though no specific interaction was demonstrated. One of the mechanisms hypothesized was that because of the presence of NLS on Hsp70 molecule, it may compete with p65 for nuclear pore complexes and

decrease latter's accumulation in nucleus. The interaction between Hsp70 and NF-κB molecules was demonstrated by Guzhova *et al.* (120) who reported that in human lymphoma cells Hsp70 is specifically associated with p65, c-Rel and p50 but not with IκB-α. By binding to NF-κB molecules and preventing their nuclear translocation, Hsp70 could suppress NF-κB activation by PMA and LPS.

Hsp60 has both pro and anti-inflammatory properties. It was found to have opposite effects on innate and adaptive immune systems, probably via activating different Toll like receptors. In innate cells such as dendritic cells, it induces maturation and a Th1 cytokine response which is TLR-4 dependent (121) while in T cells, signaling through TLR-2, it suppresses nuclear translocation of NF-κB and inflammatory cytokines and induced IL-10 secretion (122). The exact mechanism of NF-κB inhibition is not known. *In vivo*, it was found to exert a protective effect against Th1 mediated inflammations such as experimental immune arthritis and type1 diabetes (123, 124).

Several studies reported decreased inflammatory responses following HSF inhibition. However, it is not clear if this is mediated directly by HSF-1 or indirectly by reducing Hsp expression. Wirth *et al.* (125) observed increased IκB degradation in the absence of HSF-1. This has two opposing effects in a mouse model of toxin inhalation. $HSF^{-/-}$ mice had greater activation of NF-κB, particularly p50. The consequent increase in the activity of p50/p50 homo dimers suppressed TNF-α expression while increased p50/p65 hetero dimers increased GM-CSF expression. This could be the result of differential involvement of NF-κB complexes in the transcription of these two cytokine genes. Nonetheless, this study demonstrated that HSF-1 mediated lung protection against cadmium instillation involves specific cytokine repression via inhibition of NF-κB activation *in vivo*. Recently, Chen and Curie (126) reported exacerbated pro-inflammatory activation of angiotensin II-induced NF-κB and AP-1 in vascular smooth muscle cells following siRNA knockdown of HSF-1 expression. Accumulating evidence from recent studies shows that heat shock transcription factor (HSF)-1 by itself inhibits NF-κB activation, independent of Hsp expression. This occurs primarily at the level of

gene transcription but no direct interaction between HSF-1 and NF-κB signaling molecules has been established. It seems that HSF-1 acts, as a transcriptional repressor for NF-κB induced genes by poorly understood mechanism. Promoter region of TNF-α also has HSF-1 binding site that represses transcription and lack of HSF-1 thus leads to sustained TNF-α expression (127). A similar transcriptional inhibition occurs with regard to IL-1β via binding of HSF-1 with the transcription factor, NF-IL6 (C/EBPbeta) (128). **To summarize, heat shock response antagonizes NF-κB mediated inflammatory cytokine response by various means such as inhibiting IKK activity, inducing IκB expression and by transcriptional repression of cytokine genes.**

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SECTION II: MANUSCRIPTS

Chapter 5: Manuscript I

Regulation of Toll-Like Receptor-2 Expression by the Gal-lectin of *Entamoeba histolytica*

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Abstract

The Gal/GalNAc lectin (Gal-lectin) of *Entamoeba histolytica* is a surface molecule involved in parasite adherence to host cells and is the most promising subunit vaccine candidate against amoebiasis. As macrophages are the major effector cells in host defense against amoebae, we studied the molecular mechanisms by which Gal-lectin activates macrophages. Microarray analysis showed that Gal-lectin up-regulated mRNAs of several cytokines and receptor genes involved in pro-inflammatory responses. The mechanism whereby the Gal-lectin regulates Toll-like receptor 2 (TLR-2) expression in macrophages was studied. Native Gal-lectin increased TLR-2 mRNA expression in a dose- and time-dependent fashion; peak response occurred with 1.0 μg/ml after 2 h stimulation. By immunofluorescence, enhanced surface expression of TLR-2 was observed after 12 h. Using non-overlapping anti-Gal-lectin monoclonal antibodies that map to the carbohydrate recognition domain, amino acid 596-1082 was identified as the TLR-2 stimulating region. The Gal-lectin increased TLR-2 gene transcription and the half-life of the mRNA transcripts was 1.4 h. Inhibition of NF-κB suppressed TLR-2 mRNA induction by the Gal-lectin. Moreover, cells pre-treated with an inhibitor of p38 kinase (SB 208530) inhibited Gal-lectin-induced TLR-2 mRNA expression by 40%. We conclude that the Gal-lectin activates NF-κB and MAP kinase-signaling pathways in macrophages culminating in the induction of several genes including TLR-2 and hypothesize that this could have a significant impact on macrophage activation and contribute to amoebic pathogenesis.

Introduction

Entamoeba histolytica, an intestinal protozoan parasite, is the causative agent of human amoebiasis. Invasive amoebiasis claims around 70,000 lives annually making it the fourth leading cause of death and the third cause of morbidity, due to a protozoan infection (1). The Gal-lectin of amoebae is the major surface glycoprotein involved in parasite adherence to colonic mucin and mucosal epithelial cells. This adherence is an absolute prerequisite for colonization and invasion. The Gal-lectin is shown to be highly immunogenic and is the most promising subunit vaccine candidate. It is a 260 kDa, heterodimeric protein consisting of 170 kDa and 35 kDa subunits, linked by disulphide bonds. The cysteine rich region (CRR) of the heavy subunit encompasses the carbohydrate recognition domain (2). A protective efficacy ranging from 67% to 83% was reported using recombinant CRR of the heavy subunit (3). Th1-type of immune response characterized by cell-mediated immunity (CMI) has been shown to confer resistance against amoebiasis. We have previously shown that the Gal-lectin induces the expression of several pro-inflammatory molecules including nitric oxide, TNF-α and IL-12 from macrophages (4-6). However, the signal transduction pathways initiated by the Gal-lectin leading to priming or activation of macrophages is not known.

Toll-like receptors (TLRs) are a recently discovered Pattern Recognition Receptors (PRRs) that recognize conserved molecular patterns on pathogens. Activation of TLRs on the surface of antigen presenting cells (APCs) induces the expression of co-stimulatory molecules and secretion of pro-inflammatory cytokines via activation of the transcription factor NF-κB (7). TLRs thus play a crucial role in skewing the immune response to Th1 type and are aptly called adjuvant receptors. Recently, a number of reports have suggested a potential role for TLRs in intestinal inflammation (8-10).

With the notable exception of LPS, very few studies have examined the intracellular mechanisms initiated by pathogens or their products that activate host immune cells. Of the multitude of signaling cascades, NF-κB and MAP kinase pathways play a central role in cellular response to diverse stimuli. The transcription factor NF-κB regulates several

pro-inflammatory molecules such as IL-12 and TNF-α (11). Recently it has also been shown to regulate TLR-2 gene expression (12). MAP kinases play a critical role in regulating the expression of several cytokines through activation of transcription factors (13-15). Extending our molecular characterization studies to decipher the mechanisms of macrophage activation by the Gal-lectin, we studied the regulation of TLRs by the Gal-lectin. Using specific inhibitors, we determined the role of downstream signaling mediator, NF-κB and upstream signaling intermediates such as MAP kinases in Gal-lectin induced TLR-2 mRNA expression.

Herein, we report that TLR-2 expression in response to the Gal-lectin is dependent on NF-κB and p38 MAP kinase in murine macrophages. By monoclonal antibody inhibition studies, we identified that the region between aa 596-1082 of the CRR mediates this response. This is the first report of a parasite molecule up-regulating Toll expression and also implicating MAP kinase in TLR-2 gene regulation. We hypothesize that enhanced expression of TLR-2 could have a profound impact on macrophage activation, skewing the immune response to protective Th1 type. Our study supports the current contention that in the design of an ideal subunit Gal-lectin based-vaccine, the CRD regions should be included to optimize Th1 responses. Furthermore, Gal-lectin induced up-regulation of TLR expression in macrophages or other immune cells could play a role in amoebic pathogenesis.

Materials and Methods

Cultivation of *E. histolytica* and preparation of secretory components, soluble amebic proteins and purified Gal-lectin

E. histolytica (strain HM1-IMSS) was cultivated in our laboratory as previously described (16). *E. histolytica* secretory components (EhSc) were prepared by collecting secretory/excretory components of live amoebae incubated in Hanks' balanced salt solution (2 x 10^7/ml) in the absence of serum at 37°C for 2 h. Viability of amoebae after 2 h was 95% as determined by trypan blue exclusion assay. Soluble amoebic proteins (SAP) were prepared from trophozoites in mid log growth phase (3 days) by freeze-thaw

lysis. Lysed trophozoites were centrifuged at 15,000x g at 4°C for 10 min and supernatants collected. Protein concentration in the EhSc and SAP was quantified by the method of Bradford (17) using bovine serum albumin as standard. The Gal-lectin was purified by mAb affinity chomatography as described (18). Endotoxin contamination was undetectable by the E-Toxate assay (sensitive to 0.05-0.5 endotoxin unit/ml: Sigma) in EhSc at 10 μg/ml, SAP at 50 μg/ml and Gal-lectin at 1 μg/ml.

Preparation of polyclonal Ab and MAb against the Gal-lectin

Pre-immune serum and rabbit polyclonal anti Gal-lectin serum was prepared as described [3]. The production of murine anti-Gal-lectin MAbs has been previously described (19). The epitopes recognized by MAb 1G7 (IgG2b), 7F4 (IgG2b), H85 (IgG2b), 3F4 (IgG1) and 8C12 (IgG1) were mapped to the CRR of the Gal-lectin (Fig. 5.6) using a series of successive deletion peptides of the 170kDa Gal-lectin subunit (20). Monoclonal antibodies 3F4 and 7F4 act as internal isotype-matched controls.

Cell culture and reagents

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI (Life Technologies Inc., ON, Canada) medium with 10% FCS. The cells were grown in tissue culture flasks at 37°C in 5% CO_2 and passaged regularly to maintain logarithmic growth. PD98059, a specific inhibitor of ERK kinase and SB208530, a specific inhibitor of p38 kinase were purchased from Calbiochem (San Diego, CA). LPS from *Escherichia coli* serotype 0111:B4, cycloheximide, PDTC, and anti Goat IgG FITC conjugate (F7367) are from Sigma (St. Louis, MO). Wortmannin and Herbimycin-A are from Alexis Biochemicals (Qbiogene, Carlsbad, CA). All other antibodies, NF-κB consensus and mutant oligonucleotides are from Santa Cruz Biotechnology (Santa Cruz, CA). NE-PER nuclear and cytoplasmic extraction kit was from Pierce (Rockford, IL). Poly (dI-dC) was from Amesham Pharmacia biotech. All primers and Actinomycin-D are from Invitrogen.

Northern blot analysis

Total cellular RNAs were extracted using TRIZOL reagent (Life Technologies, Rockville, MD) according to manufacturer's instructions. Aliquots (10 μg) of the total RNAs were fractionated in a 1% agarose gel containing 10mM sodium phosphate buffer and transferred to a nylon membrane. After UV cross-linking, the membranes were prehybridized for 3h at 42°C in hybridization solution (5X SSPE, 50X Denhardt's solution, 50% formamide, 0.25mg/ml of salmon sperm DNA and 0.1% SDS) followed by incubation with ^{32}P labeled-probe in the same solution for 14 h. The membranes were washed in 2X SSC for 15 min twice at room temperature and in 0.1%SSC +0.1% SDS for 15 min twice at 50°C (0.5% SSC+0.1% SDS at 60°C twice for actin) and were exposed to Kodak XAR-5 film with intensifying screen for 12-18 h at -70°C. Blots were scanned and densitometric results analyzed with the UN-SCAN IT Gel program. The mTLR-2 DNA probe was prepared by amplifying 2300 bp coding region by RT-PCR and cloning into TA vector. Actin probe consisted of a 1.25 kb *Pst*I fragment of pBA-1.

Western blot analysis

Nuclear or cytoplasmic extracts were collected from 2 X 10^7 cells using the Pierce NE-PER kit as per manufacturer's instructions. Protein concentrations were estimated by micro BCA method (Pierce). Equal amounts of the samples were then separated in 12% SDS-Polyacrylamide gels and transferred onto nitrocellulose membrane (Bio-Rad, CA). The membranes were blocked in 3.5% skim milk-TBS-T (20nm Tris-HCl (pH 7.5), 500mM NaCl. 0.1% Tween-20) at 4°C overnight, incubated with primary antibodies in skim milk-TBS-T at 4°C overnight, washed three times with TBS-T and incubated with HP-conjugated anti mouse or goat Ig in skim milk-TBST overnight at 4°C. After three washes each in TBS-T and TBS-3T, the blot was developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ)) according to the manufacturer's instructions.

Nuclear run-on assay

TLR-2 gene transcription was detected in a PCR-based nuclear run-on assay (5). Following 2h stimulation, macrophages (2 x 10^7/sample) were washed in PBS and

resuspended in lysis buffer (320 mM sucrose, 5mM $MgCl_2$, 10mM Tris-HCl, 1% Triton X-100, pH 7.5), incubated on ice for 10 min, and centrifuged at 1300 x g for 15 min at 4°C. The nuclear pellets were washed once in lysis buffer and the nuclei divided into two aliquots, each resuspended in 100 µl of storage buffer (50mM Tris-HCl, pH8.0, 40% glycerol, 0.1mM EDTA, 5mM $MgCl_2$, and 1mM DTT) and frozen in liquid nitrogen until use. Elongation of the nascent RNA *in vitro* was done by adding 100 µl of transcription buffer (20mM Tris-HCl, pH 8.0, 300 mM KCl, 10mM $MgCl_2$, 200mM sucrose, 48µM EDTA and 1mM DTT) with or without ribonucleotide phosphates (1mM each of rATP, rCTP, rGTP and rUTP) to 100 µl of nuclei and incubating at 30°C for 30 min. The reactions were stopped by adding 1ml Trizol and RNA extracted for RT-PCR analysis. A difference between samples incubated with and without rNTP is indicative of active transcription.

Electrophoretic mobility shift assay

All buffers, including those in the NE-PER kit were added with a mixture of protease inhibitors (1.0 µg/ml each of aprotinin, leupeptin, pepstatin, 0.5 mM PMSF, and 1 mM EDTA). Double stranded NF-κB consensus oligonucleotide was labeled with [γ^{32}P]-ATP, using T4 polynucleotide kinase. Unlabeled nucleotides were removed using Sephadex G-25 columns. The binding reaction consists of 20 µl total volume of 0.5 ng of DNA probe, 5 µg of nuclear extract, 1 µg of poly (dI-dC) in the binding buffer (12 mM HEPES, 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM of DTT and 12% glycerol) (pH 8.0), and incubation for 30 min at room temperature. For competition assay, nuclear extracts were incubated with 50-100-fold excess of unlabeled probe before the addition of labeled probe. For supershift assay, 2 µl of anti p65 NF-κB was incubated with nuclear extract for 2 h before binding reaction. DNA-protein complexes were resolved by electrophoresis on 6% poly-acrylamide gels at 4°C in TBE buffer (90 mM Tris-borate, 2mM EDTA). Gels were subsequently dried and autoradiographed with intensifying screens at -70°C.

Immunofluorescent assay

Macrophages were grown on cover slips and stimulated with Gal-lectin or LPS for 12 h. Cells were washed with 1x PBS, fixed in 4% paraformaldehyde at room temperature for 1 h, washed again and permeabilized with cold absolute methanol for 20 min at -20°C. The cells were incubated with primary antibody or control goat IgG (in PBS) for 1 h at room temperature, washed and FITC-conjugated secondary antibody (in PBS-Evans's blue) was added and incubated at room temperature for 1 h in dark. Finally the cover slips were mounted onto microscopic slides using Vectashield as a quenching solution and examined under fluorescent microscope.

Microarray study

GE Array Q series Mouse NF-κB signaling pathway specific-gene array membrane (MM-016; SuperArray Biosciences Corporation, Frederick, MD) was used to study the differential expression of genes involved in NF-κB mediated signal transduction. RNA was extracted using TriZol reagent (Invitrogen) from RAW 264.7 cells stimulated with lectin or with medium alone. Probe labeling and hybridization to the membranes were done as per the manufacturer's protocol. Membranes were exposed to X-ray film and data acquisition done using the ScanAlyze software. The numerical data generated by ScanAlyze was analyzed using another software, GEArray Analyzer. Both these softwares are free and can be downloaded from the website, www.superarray.com. Initially, all raw signal intensities were corrected for background by subtracting the signal intensity of negative control, PUC18. This was followed by normalization of all signal intensities to that of the housekeeping gene GAPDH. These corrected, normalized signals were then used to estimate the relative abundance of particular transcripts.

Results

Gal-lectin induces pro-inflammatory response from macrophages

From our previous observations on Gal-lectin induction of TNF-α and IL-12 from macrophages, we hypothesized that this molecule might be stimulating an NF-κB-mediated pro-inflammatory pathway. Accordingly, microarray studies were done using

NF-κB specific gene array with control and Gal-lectin-stimulated macrophages (Fig. 5.1). As shown in Table 5.1, the Gal-lectin up-regulated several cytokines and molecules involved in inflammation and down-regulated others. Of particular interest to the present objective, is the up-regulation of TLR-2. Thus, we further probed the mechanism of regulation of TLR-2 by Gal-lectin with a view to decipher the signaling pathways activated by the Gal-lectin.

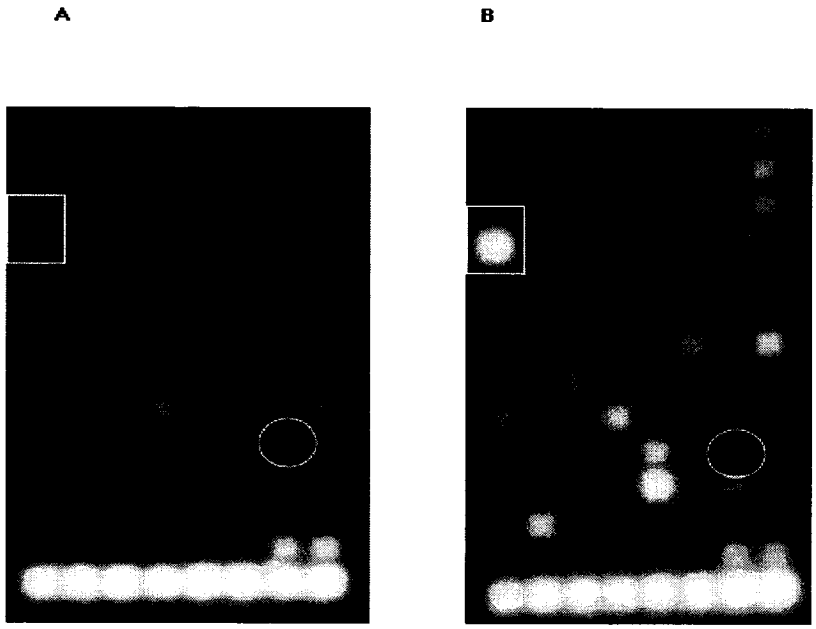

Fig. 5.1: Gal-lectin induces pro-inflammatory response from macrophages. Macrophages were treated with either medium alone or with 500 ng/ml of Gal-lectin for 2 h, RNA extracted and microarray experiment was done as described in experimental procedures. One up-regulated (IL-1β) (□) and one down-regulated (TLR-4) (O) gene is shown.

Table 5.1: Differential gene expression induced by *E. histolytica* Gal-lectin in macrophages

Functional Gene Grouping	Up-regulated	Down-regulated
Adhesion molecules	Intercellular Adhesion Molecule-1	-
Cytokines	Interleukin -1α	
	Interleukin -1β	-
	Tumor necrosis factor-α	
Transmembrane receptors	Toll-Like Receptor (TLR)-2	TLR-4
		Cell death protein
Adaptor proteins	TNF-receptor associated factor 1	-
Signal transduction kinases	NF-κB-Inducing Kinase	-
	P38 MAPK	
Transcription factors	Nuclear Factor-κB-1	
	Nuclear Factor-κB-2	-
	Interferon Regulatory Factor	
Acute phase proteins	Serum Amyloid A 1	-
	Serum Response Factor	

Table 5.1: Macrophages were treated with either medium alone or 500 ng/ml of Gal-lectin for 2 h and differential gene expression was studied as described. The experiment was done twice and genes increased, at least by 3-fold (up-regulated) or decreased by less than half (down-regulated) in Gal-lectin treated cells compared to that of control cells in both the experiments are represented.

Gal-lectin stimulates TLR-2 mRNA and protein expression in macrophages

To determine if TLR-2 mRNA expression is altered in response to amoebic components in general, cells were stimulated with *E. histolytica* secretory components (EhSC), soluble amoebic proteins (SAP) and purified Gal-lectin for 2 h and relative mRNA levels were detected by Northern blot analysis. As shown in Fig. 5.2, EhSC and SAP which contain Gal-lectin and other proteins stimulated TLR-2 mRNA expression moderately while purified Gal-lectin induced significant expression (3-4 fold) of TLR-2 mRNA indicating the relative potential of this molecule. Based on this and microarray data, subsequent studies were done on TLR-2 regulation by the purified Gal-lectin. As shown in Fig. 5.3A, Gal-lectin stimulated TLR-2 mRNA expression in a dose-dependent fashion. Significant expression was induced with as little as 250 ng/ml of Gal-lectin and peak response observed with 1.0 μg/ml. In a detailed time course experiment with a sub-optimum dose of 500 ng/ml of Gal-lectin, TLR-2 mRNA expression was induced by 1h, peaked at 2h, and returned to basal levels by 12 h (Fig. 5.3B). The surface expression of TLR-2 protein was significantly increased when stimulated with 1.0 μg/ml of lectin for 12 h (Fig. 5.4) and was comparable to that stimulated with LPS (data not shown), demonstrating the potency of the Gal-lectin in stimulating TLR-2 mRNA as well as protein expression in macrophages.

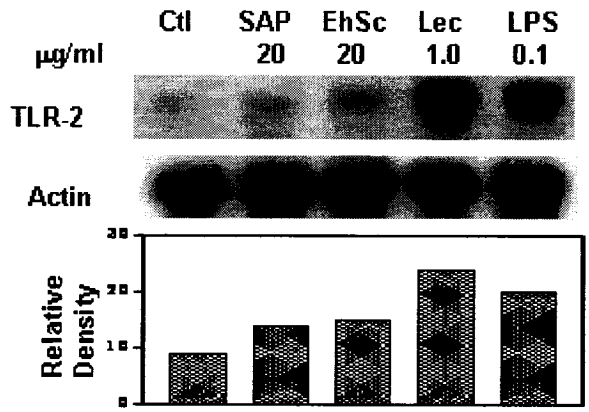

Fig. 5.2: Regulation of TLR-2 mRNA by *E. histolytica* components. Macrophages were stimulated with soluble amoebic proteins (SAP), secretory components from amoebae (EhSc), Gal-lectin (Lec) or LPS for 2 h and analyzed by Northern blot using TLR-2 probe. The membrane was stripped and re-probed with actin for standardization. Quantity of TLR-2 mRNA was normalized to actin levels and expressed as densitometric units (TLR-2/Actin) in the histogram with identical lane designations for Northern blot and histogram. Blot is representative of three experiments.

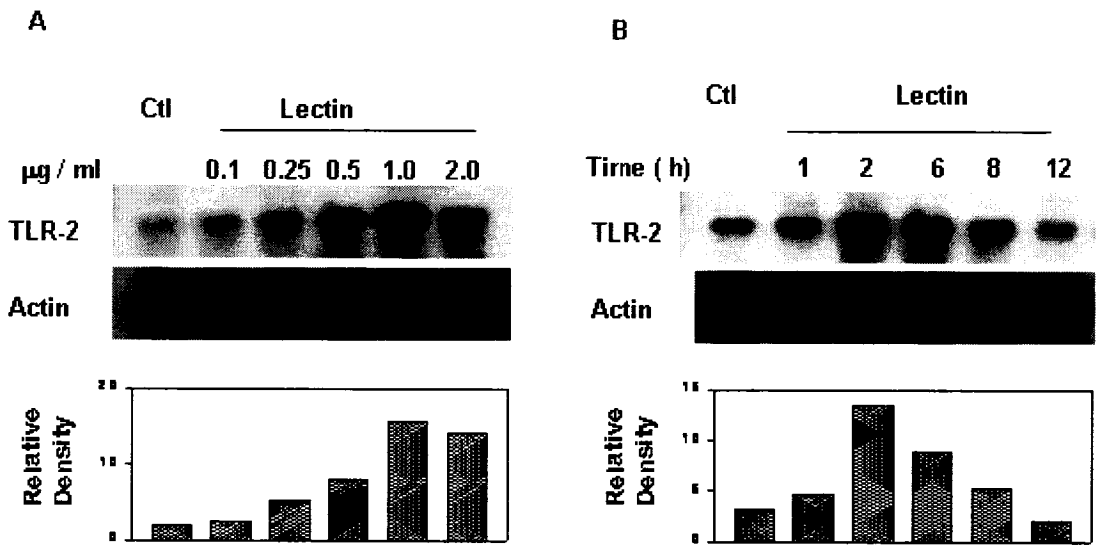

Fig. 5.3: Dose- and time-dependent expression of TLR-2 mRNA by the Gal-lectin. A). Macrophages were stimulated with increasing amounts of Gal-lectin for 2 h prior to extraction of total RNA and Northern analysis. **B)** Macrophages were stimulated with 500ng/ml of Gal-lectin and RNA collected at different time points for Northern analysis. TLR-2 mRNA levels were normalized to actin and histograms are representative of three separate stimulations.

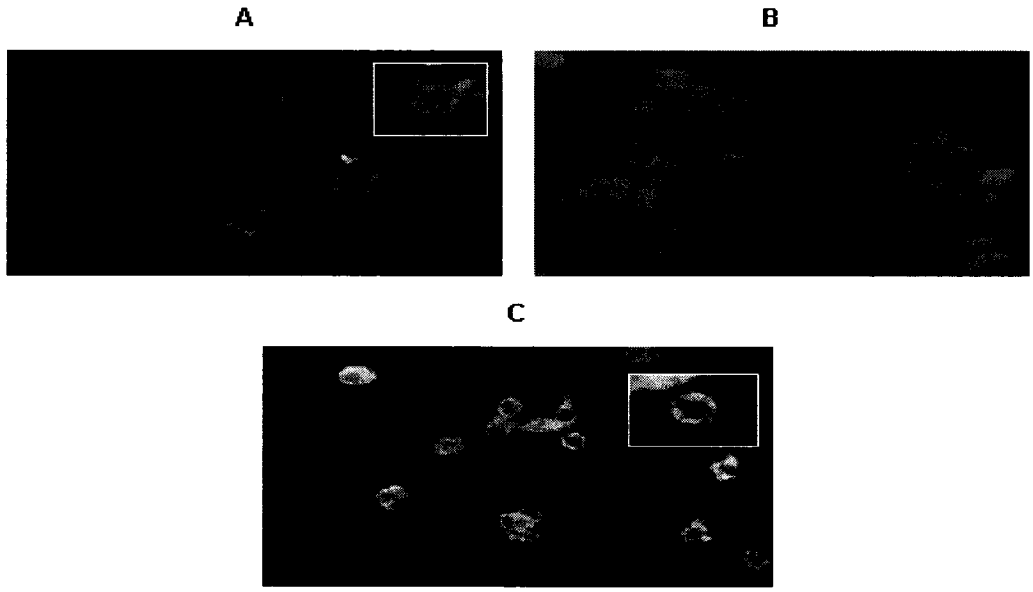

Fig. 5.4: Increased surface expression of TLR-2 protein by the Gal-lectin. Macrophages were stimulated with medium or 1.0 μg/ml of Gal-lectin for 12 h and protein expression assessed by immunofluorescent assay using FITC-tagged secondary antibody as described in experimental procedures. A) Control cells were unstimulated and probed with anti TLR-2 antibody; B) isotype controls were stimulated with Gal-lectin and probed with normal goat IgG: C) Gal-lectin stimulated cells probed with anti TLR-2 antibody.

Polyclonal antiserum and MAbs against the CRR of the Gal-lectin inhibit TLR-2 mRNA expression

To address the specificity for the Gal-lectin, polyclonal rabbit immune serum (1:1000) raised against the native Gal-lectin was used to inhibit TLR-2 induction. As shown in Fig. 5.5, anti Gal-lectin serum inhibited Gal-lectin-induced TLR-2 expression by 90% while LPS-mediated response remains unaffected. Pre-immune serum at 1:1000 did not affect TLR-2 expression. To identify the specific region of the Gal-lectin that stimulates TLR-2 mRNA expression, we examined the inhibitory effects of a panel of monoclonal antibodies which map to non-overlapping epitopes on the CRR of the Gal-lectin (Fig. 5.6). Three MAbs, 1G7 (aa 596-818), 8C12 (aa 895-998) and H85 (aa 1033-1082) markedly inhibited TLR-2 mRNA expression in response to Gal-lectin, whereas, MAb 3F4 (aa 895-998) and 7F4 (aa 1082-1138) did not (Fig. 5.7). The latter MAbs also acted as internal isotype controls. Thus, aa 596-1082 of the CRR is responsible for stimulating TLR-2 mRNA expression in macrophages.

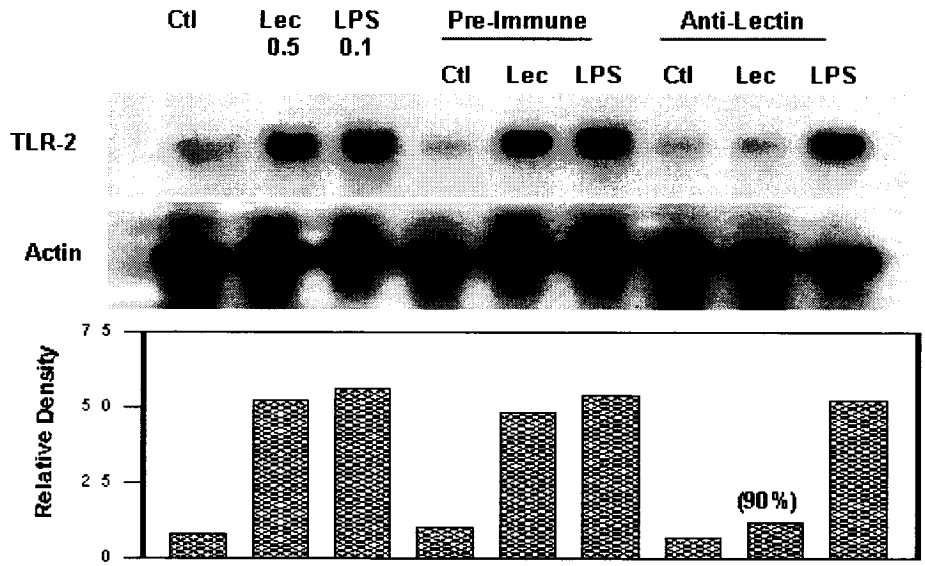

Fig. 5.5: Polyclonal anti-Gal-lectin serum specifically inhibits Gal-lectin-induced TLR-2 mRNA expression. Purified Gal-lectin (500 ng/ml) or LPS (100ng/ml) were treated with either medium, pre-immune rabbit serum (1:1000) or polyclonal anti-lectin serum (1:1000) for 2 h at 4° C prior to incubation with macrophages for 2 h at 37° C. Results of scanning densitometric analysis of Northern blot are presented as histogram with identical lanes for Northern blot and histograms. Value in parenthesis shows percentage inhibition relative to homologous controls.

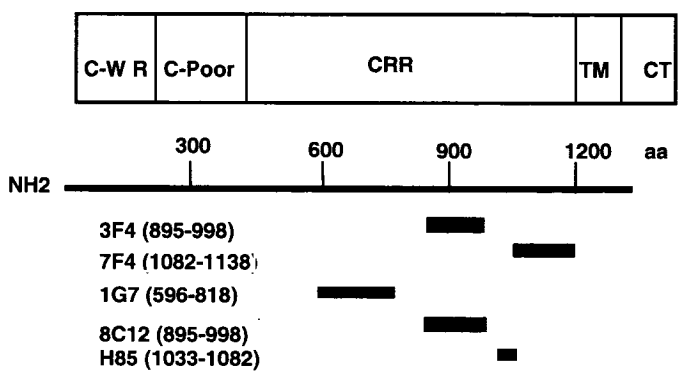

Fig. 5.6: Epitope mapping of the binding sites by monoclonal antibodies to the Gal-lectin. The location of the epitopes recognized by mAbs as determined by reactivity with deletion constructs of the170-kDa subunit (19) is shown. C-W R= Cysteine-Tryptophan Rich, C-poor= Cysteine poor, CRR= Cysteine Rich Region, TM= Transmembrane domain, CT= Cytoplasmic Tail

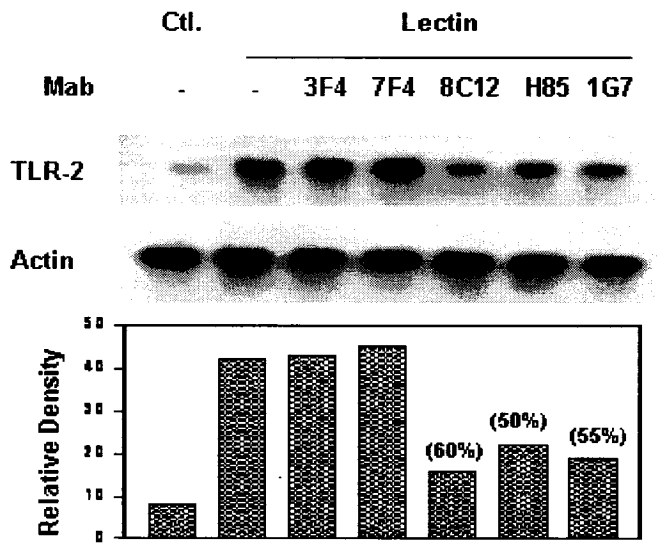

Fig. 5.7: Monoclonal antibody inhibition study to identify the region of the Gal-lectin responsible for TLR-2 mRNA induction. The Gal-lectin was treated with 1.0 μg/ml each of affinity purified mAbs against the Gal-lectin at 4°C prior to stimulating the cells for 2 h. Results of scanning densitometric analysis of Northern blots are presented as histogram and are expressed as densitometric units with identical lane designations for blots and histograms. Values in parenthesis show percentage inhibition of the response relative to that induced in the presence of 7F4 or 3F4 (isotype matched controls). Similar results were obtained from three independent experiments.

Regulation of TLR-2 mRNA expression in response to Gal-lectin

Previous studies have shown that the pro-inflammatory cytokines, TNF-α and IL-1β can up-regulate TLR-2 mRNA expression in murine macrophages (21). As Gal-lectin can also stimulate pro-inflammatory cytokines from macrophages, we determined if TLR-2 induction by Gal-lectin occurs directly or via autocrine stimulation with these cytokines. As shown in Fig. 5.8, no change in TLR-2 mRNA expression was observed when macrophages are stimulated with Gal-lectin in the presence of neutralizing antibodies against TNF-α and IL-1β, confirming that TLR-2 mRNA expression is independent of these cytokines. Moreover, TNF-α and IL-1β stimulated TLR-2 mRNA expression similar to the Gal-lectin and this induction was completely inhibited by their respective neutralizing antibodies. To determine if *de novo* protein synthesis is required for TLR-2 mRNA expression, cells were pre-treated with different concentrations of cycloheximide for 30 min and then stimulated with Gal-lectin for 2h. Cycloheximide treatment markedly increased TLR-2 mRNA expression in both control and stimulated cells (Fig. 5.9)

indicating that newly synthesized proteins negatively regulate TLR-2 mRNA expression. Actinomycin-D treatment followed by RNA chase experiment was done to examine the stability of the TLR-2 mRNA transcript induced by Gal-lectin in comparison to that of LPS. $T_{1/2}$ for TLR-2 mRNA transcripts induced by the Gal-lectin was calculated to be 1.4 h while that induced by LPS was 2.5 h (Fig. 5.10). These results show that TLR-2 mRNA induced by lectin is stable and undergoes slow post-transcriptional degradation similar to that of LPS. The molecular regulation of TLR-2 gene expression was determined by a PCR based nuclear run-on assay. As shown in Fig. 5.11, a three fold-increase of TLR-2 gene transcription was observed in cells stimulated with Gal-lectin, similar to that of the positive control LPS.

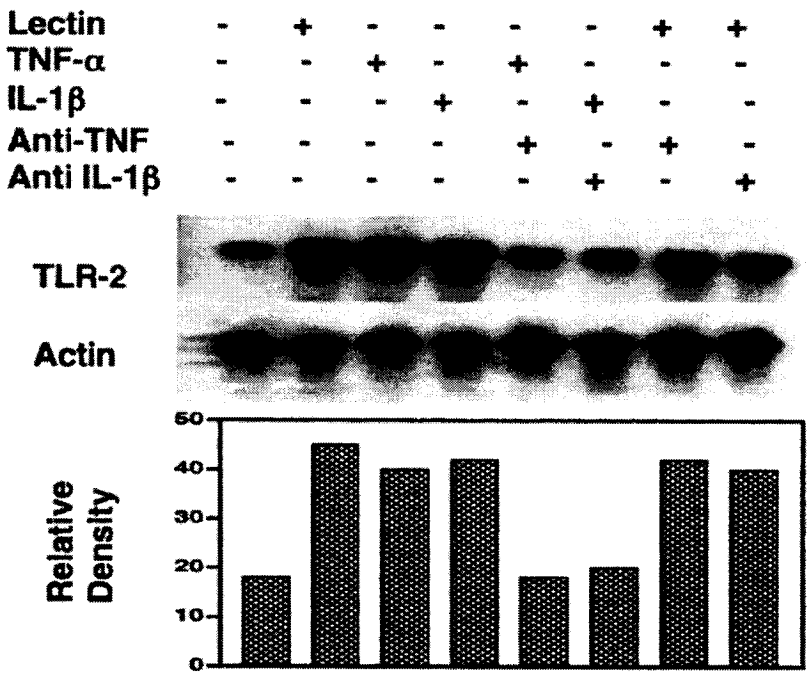

Fig. 5.8: Gal-lectin induced TLR-2 mRNA is independent of TNF-α and IL-1β. Macrophages were stimulated with Gal-lectin (500 ng/ml) or medium only in the presence or absence of neutralizing antibodies against mouse TNF-α (2 μg/ml) or IL-1β (10 μg/ml) for 2 h and total RNA collected for subsequent Northern blot analysis. Under the same conditions recombinant mouse TNF-α (10 ng/ml) and IL-1β (10 ng/ml) were used as specific controls. Results are from three separate experiments.

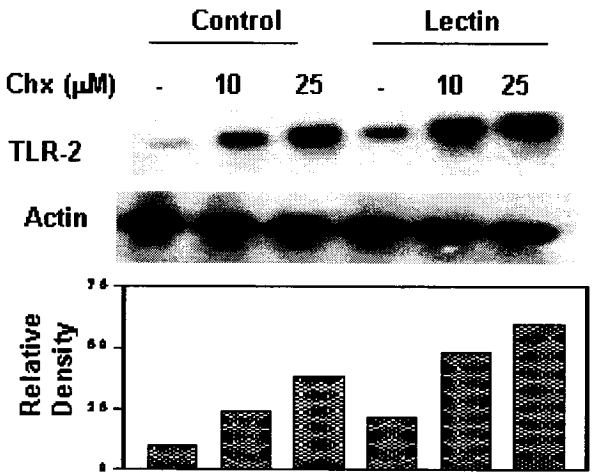

Fig. 5.9: Effect of cycloheximide treatment on TLR-2 mRNA induction by the Gal-lectin. Cells were treated with the protein synthesis inhibitor cycloheximide or medium for 30 min before stimulation with medium or Gal-lectin for 2 h. Results of scanning densitometric analysis of Northern blots are presented as histogram and are expressed as densitometric units with identical lane designations for blots and histograms. Results are from three independent stimulations.

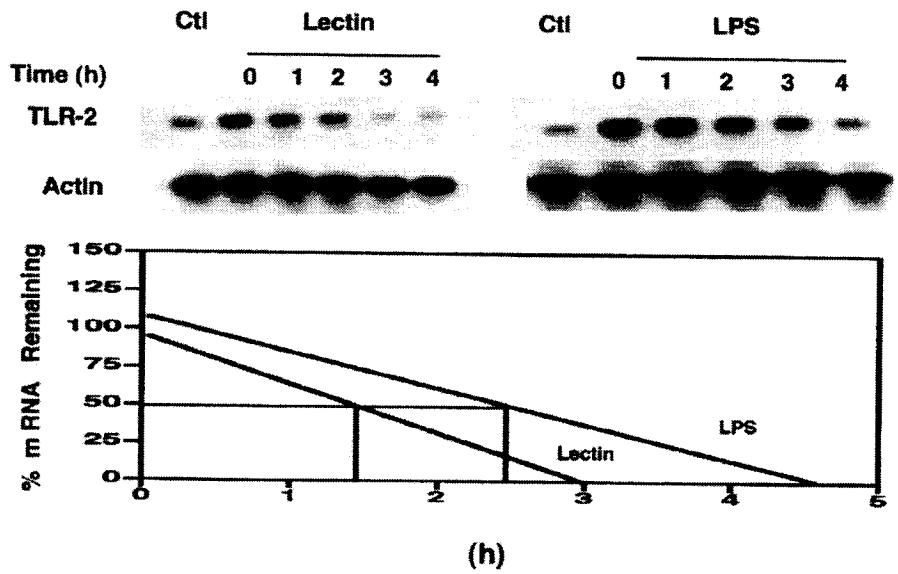

Fig. 5.10: Stability of TLR-2 mRNA in response to Gal-lectin and LPS. Cells were stimulated for 2 h with 500 ng/ml of Gal-lectin or 100 ng/ml of LPS. The transcriptional inhibitor actinomycin-D (2.0 μg/ml) was added and total RNA collected at 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h for Northern blot analysis. TLR-2 mRNA levels are expressed as percentage of mRNA remaining where 100% represents mRNA expression (normalized to actin) at the time of actinomycin treatment (0 h). The $t_{1/2}$ (50% stability) of TLR-2 mRNA transcripts as calculated from the graph are 1.4 h (r=0.974) and 2.5 h (r=0.9677) for Gal-lectin and LPS, respectively. Results are from two experiments.

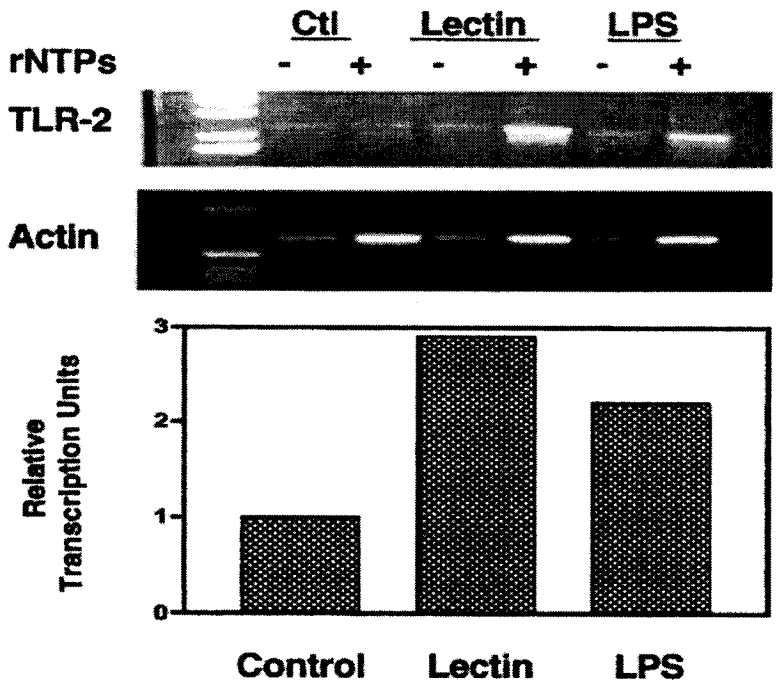

Fig. 5.11: Gal-lectin stimulates TLR-2 mRNA gene transcription in murine macrophages. Cells were stimulated for 2 h with medium alone, Gal-lectin (500 ng/ml) or LPS (100ng/ml). Nuclei were isolated and *in vitro* transcription done as described. After densitometric scanning, TLR-2 mRNA levels were normalized to those of actin. Basal rate of transcription (in control cells) was assigned an arbitrary value of 1 and fold-increase of transcription expressed as Relative Transcription Units. Similar results were obtained from two independent experiments.

NF-κB is involved in Gal-lectin-induced TLR-2 mRNA expression

NF-κB has been shown to regulate TLR-2 gene transcription in murine macrophages (12). Hence its role in Gal-lectin-induced TLR-2 gene transcription was determined using two NF-κB inhibitors pyrollidine dithiocarbamate (PDTC) and SN50 peptide. As shown in Fig. 5.12A, PDTC inhibited Gal-lectin-induced TLR-2 expression by 64% at 10 μM concentration and completely suppressed TLR-2 expression at 25 μM, Moreover, the specific NF-κB inhibitor SN50 inhibited TLR-2 mRNA induction by 80% (Fig. 5.12B) confirming a role for this transcription factor in Gal-lectin signaling. In support of this, we observed nuclear translocation of the two subunits of NF-κB, p65 and p50 as early as 30 min following Gal-lectin stimulation (Fig. 5.12C). Furthermore, EMSA was done to check the DNA binding activity of NF-κB that was activated by the Gal-lectin. As

shown in Fig. 5.12D, Gal-lectin treatment significantly increased the DNA binding activity of NF-κB. Specificity was determined by pretreatment of the nuclear extracts with 50 and 100-fold concentration of cold probe and by mutant NF-κB consensus sequence. As expected, pretreatment of nuclear extracts with cold probe inhibited DNA binding by the labeled probe in a dose-dependent fashion while pretreatment with mutant probe did not inhibit subsequent binding by labeled probe. The P65 subunit of NF-κB has been implicated in the transcativating function of this transcription factor (11). Thus, to determine if this subunit is activated by the Gal-lectin, supershift assay was done using anti p65 antibody. Fig. 5.12E shows a clear supershifted band, validating its role in Gal-lectin signaling.

Role of MAP kinases in Gal-lectin induced TLR-2 mRNA expression

MAP kinases play a key role in cellular responses to diverse stimuli by acting as signaling intermediates in the expression of cytokines (13-15). Accordingly, we tested if the Gal-lectin can activate the MAP kinase pathways. By Western blot studies employing antibodies against phosphorylated forms of the three important MAP kinases; ERK, JNK and p38, we observed that the Gal-lectin activates p38 kinase (Fig. 5.13A) but not ERK or JNK (data not shown). Specific inhibitors of the MAP kinase pathways were then used to check their role in Gal-lectin-induced TLR-2 mRNA expression. As shown in Fig. 5.13B, pretreatment of cells with SB203580, a specific inhibitor of the p38 kinase pathway, significantly inhibited (by 40%) TLR-2 mRNA induction by Gal-lectin while the ERK inhibitor had no effect. In order to understand further the upstream signaling events in Gal-lectin signaling, we studied the effects of two inhibitors Wortmannin, a PI3 kinase inhibitor and Herbimycin-A, a specific tyrosine kinase inhibitor on Gal-lectin-induced TLR-2 mRNA expression. Neither of these inhibitors had any significant effect on TLR-2 mRNA expression (Fig. 5.14), suggesting that PI3 kinase and tyrosine kinases do not play a role in Gal-lectin mediated TLR-2 expression.

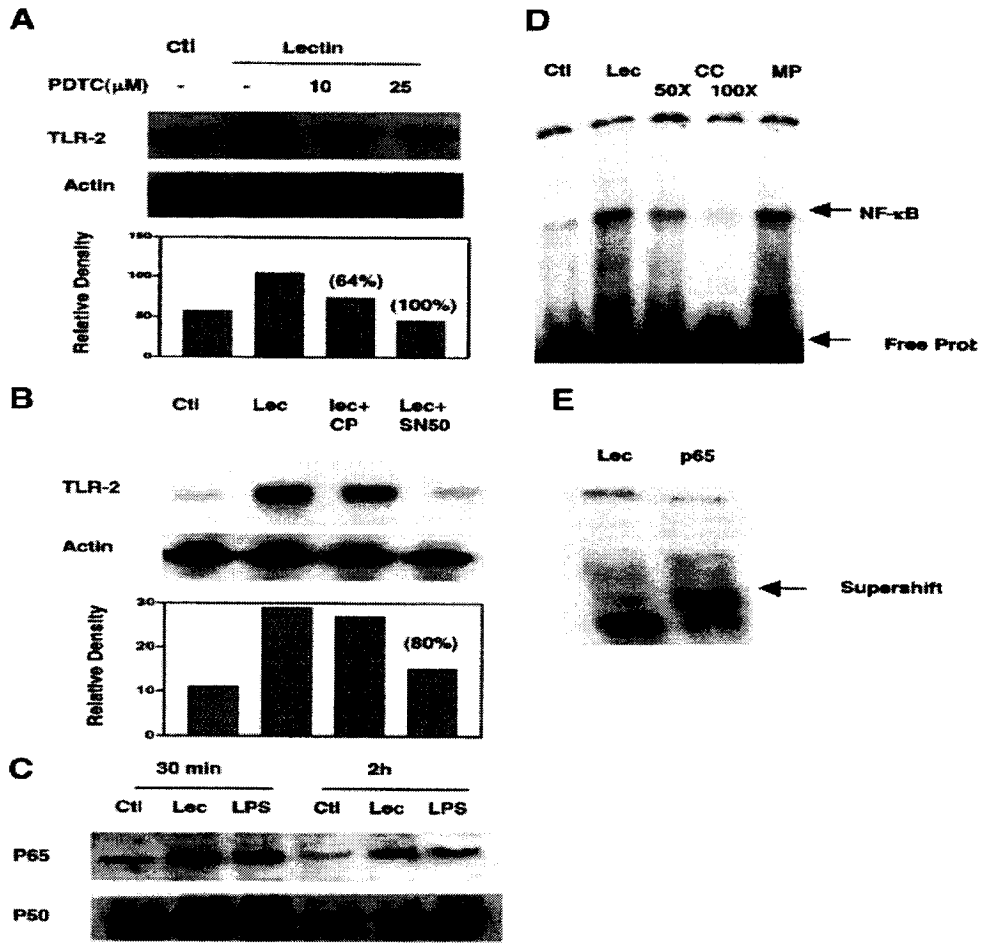

Fig. 5.12: NF-κB is involved in Gal-lectin signaling. A). Cells were pretreated with NF-κB inhibitor pyrollidine dithio carbomate (PDTC) at different concentrations or medium alone for 30 min before stimulating with lectin (500 ng/ml) for 2 h and Northern analysis.Values in parenthesis indicate percentage of inhibition relative to that observed with lectin. B) Cells were pretreated with 100 μg/ml of either control peptide (CP) or SN50 peptide (SN50) for 1h before stimulation with 500ng/ml of Gal-lectin for 2 h. Figure in parenthesis show percentage of inhibition relative to that observed with control peptide. C) Western blots showing the nuclear translocation of NF-κB subunits by the Gal-lectin. Cells were stimulated with 500 ng/ml each of lectin or LPS for 30 min and 2 h and nuclear extracts collected as described. 30 μg of protein was fractionated by SDS-PAGE and probed with antibodies against the two NF-κB subunits p65 and p50. D) NF-κB activation by the Gal-lectin. Macrophages were stimulated with Gal-lectin (500 ng/ml) for 30 min. Nuclear extracts from stimulated and unstimulated cells were prepared as described in experimental procedures and then assayed for DNA-binding activity by EMSA. Arrows indicate positions of DNA complexes. E) Supershift with anti p65 antibody. To check the specificity and identify the subunits induced by Gal-lectin, the nuclear extracts were pre-incubated anti p65 antibody for 2 h before EMSA. Supershifted band (arrow) with p65 is shown.

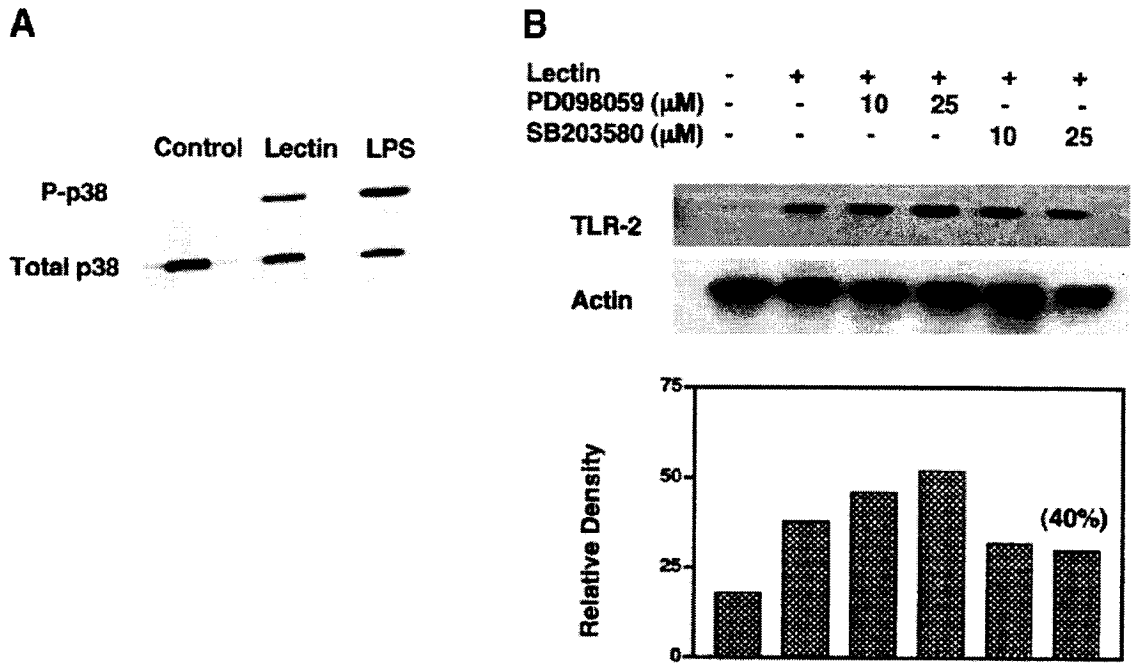

Fig. 5.13: P38 MAP kinase is activated by the Gal-lectin. A) Gal-lectin induces the phosphorylation of p38 kinase. Cells were stimulated with Gal-lectin (1.0 μg/ml) or LPS (1.0 μg/ml) for 30 min, cytoplasmic extracts prepared using NE-PER kit and 100 μg of protein per lane were fractionated by SDS-PAGE. The membranes were probed with antibodies that specifically recognize the phosphorylated form of P38. To check equal loading, membranes were subsequently stripped and re-probed with antibody that recognizes total p38. B) P38 kinase is involved in Gal-lectin-induced TLR-2 mRNA expression. Cells were treated with indicated concentrations of specific MAP kinase inhibitors for 30 min before stimulating with Gal-lectin for 2 h. Values in parenthesis show percentage of inhibition relative to that induced by the Gal-lectin. Results are representative of three separate experiments.

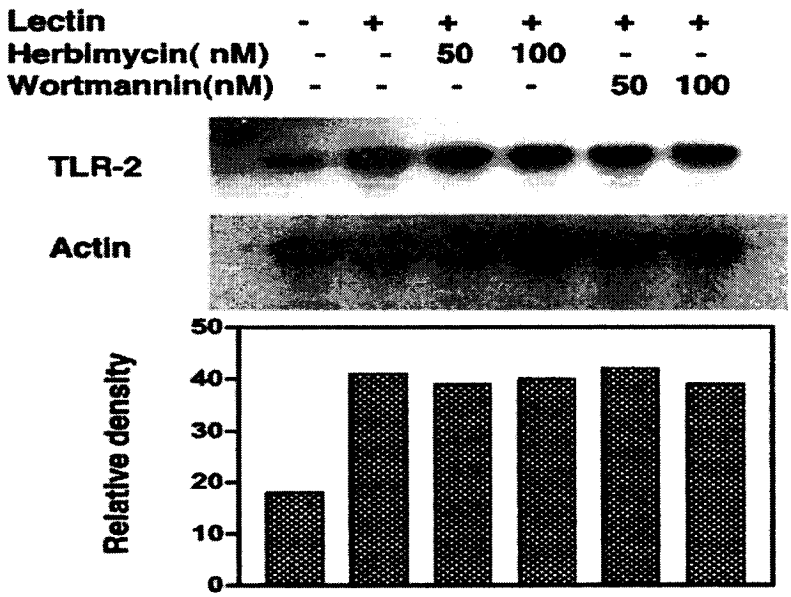

Fig. 5.14: Gal-lectin-induced TLR-2 mRNA expression is independent of tyrosine kinases and PI3 kinase. Cells were pretreated with herbimycin-A (tyrosine kinase inhibitor) and wortmannin (PI3 kinase inhibitor) at indicated concentrations for 30 min prior to stimulation with Gal-lectin for 2 h. Total RNA was extracted and Northern blot analysis done as described. TLR-2 mRNA levels were normalized to those of actin and expressed as densitometric units with identical lane designations for blots and histograms. Results are representative of two independent treatments.

Discussion

DNA microarray technology is increasingly becoming a powerful tool which allows a global view of gene profiling in a rapid and efficient fashion (22) and several researchers are reporting microarray analysis of mRNA levels from macrophages in response to variety of pathogens (23, 24). Hence we initially exploited this technique to have simultaneous analysis of 96 genes involved in NF-κB signaling pathway. The observed gene profile, over all, supports our previous observations of pro-inflammatory cytokine induction and suggests the involvement of the important transcription factor NF-κB in Gal-lectin signaling. As we are interested in the regulation of Toll receptors and deciphering the signaling events involved in this activation, we focused our study on regulation of TLR-2 induction by the Gal-lectin. Of the 10 TLRs identified to date in mammals, TLR-2 appears to be the most promiscuous one that can be activated by a variety of stimuli. In this study, we show that the Gal-lectin induces robust TLR-2

expression in murine macrophages. To our knowledge, this is the first purified molecule isolated from a parasite that induces Toll receptor expression in macrophages. The others include LPS from Gram-negative bacteria (21), *H. influenzae* (25), and *M. avium* (26). In addition, several parasitic components have been shown to signal via TLR-2 and activate the Toll pathway. These include GPI anchors and protein Tc52 from *T. cruzi* (27, 28) and schistosomal lyso-phosphatidyl serine (29). Although our study shows that Gal-lectin can stimulate TLR-2 expression, it is premature to hypothesize that it is a ligand for TLR-2.

Using a panel of monoclonal antibodies, we identified the region of Gal-lectin that stimulates TLR-2 mRNA expression and protein production. This is of paramount importance in the design of a subunit vaccine. The same region of the Gal-lectin stimulates TNF-α and IL-12 production by macrophages. It is interesting to note that IFN-γ priming was not necessary for the Gal-lectin-induced TLR-2 mRNA or protein expression by murine macrophages. Thus, even unprimed antigen presenting cells (APCs) exposed to Gal-lectin can express high levels of this Toll receptor, which can potentially contribute to skewing the immune response to protective Th1 type. In addition, other workers have shown that all the functional properties of Gal-lectin such as parasite adherence, cytotoxicity and complement resistance are mediated by the CRR, a part of the large extracellular domain (2, 30). In this context, it would be of interest to explore the structural properties that confer such a diverse functionalities to this molecule, and to the CRR in particular.

NF-κB encompasses a very important family of transcription factors and is critical for the inducible expression of several genes involved diverse cellular responses (11). The promoter region of murine *TLR-2* contains the following consensus binding sequences: two NF-κB, two CCAAT/enhancer binding protein, one cAMP response element–binding protein and one STAT (12). Hence, it is not surprising that NF-κB plays a central role in TLR-2 gene expression. In fact, LPS and *M. avium* have been shown to regulate TLR-2 mRNA via NF-κB (12, 26). We also observed that NF-κB plays a critical role in Gal-lectin-induced TLR-2 expression. This is consistent with the observation that

no new protein synthesis is required for TLR-2 induction by the Gal-lectin as initial NF-κB activation does not require *de novo* protein synthesis (31). Although both Gal-lectin and LPS regulate TLR-2 in an NF-κB-dependent manner, there are clear differences in the mechanisms used by these diverse molecules. While MAP kinases do not play a role in LPS-mediated TLR-2 regulation, p38 kinase was a significant intermediate in Gal-lectin mediated TLR-2 induction. Also, LPS induced a much robust DNA-binding by NF-κB than Gal-lectin at 30 min (data not shown). These observations together with a clear difference in the stability of TLR-2 mRNA, suggest that LPS induces a sustained expression of TLR-2 probably though activation of other transcription factors in addition to NF-κB, while Gal-lectin induces a less stable expression of TLR-2 which appears to be dependent mainly on NF-κB. This is supported by the fact that NF-κB was not absolutely necessary for LPS-induced TLR-2 gene expression (12) while inhibition of this single transcription factor by SN50 peptide resulted in 80% inhibition of TLR-2 mRNA expression induced by the Gal-lectin. However, promoter analysis studies are warranted to assess the full spectrum of transcription factors involved in Gal-lectin induced TLR-2 gene expression and to identify the putative responsive elements in the gene promoter region. These differences are also reflected in the kinetics of protein expression where a significant expression with Gal-lectin was observed only at 12 h while LPS stimulation resulted in a relatively rapid induction by 6 h (data not shown). Although we showed that both Gal-lectin and LPS induces TLR-2 gene transcription, differential post-transcriptional regulation of TLR-2 mRNA might be responsible for these quantitative and kinetic differences.

The family of MAP kinases includes the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase. P38 MAP kinase is an important mediator of stress-induced gene expression (32) and it appears to differentially regulate TLR-2 mRNA expression in different cell types in response to different stimuli. P38 kinase and ERK pathways have been shown to positively regulate TLR-2 mRNA expression in response to PMA in T-lymphocytes (33) while nontypeable *Hemophilus influenzae* and glucocorticoids were reported to enhance TLR-2 expression in human epithelial cells via a negative cross-talk with p38 kinase (25). We showed that the p38 kinase pathway is a

significant mediator of TLR-2 induction by the Gal-lectin. Several lines of experimental evidence support this contention. First, Gal-lectin induced a marked phophorylation of p38 kinase as early as 30 min. Second, pretreatment of cells with SB203580, specific inhibitor of p38 kinase blocked Gal-lectin-induced TLR-2 mRNA expression by 40%. In contrast, inhibiting the ERK pathway resulted in slight increase in TLR-2 mRNA expression, confirming an earlier report of negative regulation by ERK (21) in response to LPS stimulation. However, the clear discrepancy in signal transduction pathways activated by Gal-lectin and LPS is that Gal-lectin does not activate ERK or JNK while LPS potently induces both. As well, LPS mediated-TLR-2 mRNA induction was not significantly inhibited even at 50 μM concentration of SB208530 (21). Although the exact mechanism for this differential activation is not known, modulation of different membrane receptors may be involved.

MAP kinases are one of the downstream targets for protein tyrosine kinases (PTKs) and inhibition of PTK and PI3 kinase activities has been shown to modulate transcription factor activation and gene induction (11, 34-36). Both have been implicated in induction of proinflammatory molecules such as iNOS and TNF-α in response to LPS and IFN-γ (37, 38). However, specific inhibitors at a concentrations shown to inhibit respective target kinases, fail to influence the TLR-2 mRNA expression by Gal-lectin. PKC is one of the prominent downstream target of PI3-kinase (39) and we previously observed that *E. histolytica* activated and translocated PKC to the membrane fraction in murine macrophages (40). Also, previous reports have shown that these kinases are involved in transcription factor activation and gene induction (40, 41). However, we fail to associate these kinases with TLR-2 expression indicating that Gal-lectin induced NF-κB and p38 are independent of PI3 kinase and tyrsosine kinases.

While this study clearly helps in developing an ideal subunit vaccine against this important pathogen, its contribution to furthering our understanding of amoebic pathogenesis, and intestinal inflammation in general, cannot be ignored. Our observation of up-regulation of TLR-2, via NF-κB, by Gal-lectin is of paramount importance in this context. The contribution of NF-κB, particularly the p65 subunit, to intestinal

inflammation has been well documented (42-44). Up-regulation of TLR-2 could definitely be one of the diverse consequences of NF-κB activation. Indeed, several reports are revealing an increased expression of TLRs in both epithelial cells and lamina propria macrophages during inflammatory conditions (8-10, 45). Recently, TLR-2 was shown to contribute to liver injury by *Salmonella* infection via Fas ligand expression on NKT cells in mice (46). Based on this work and other studies, a working model for the role of TLR-2 in amebic pathogenesis can be hypothesized (Fig. 5.15) wherein *E. histolytica* through Gal-lectin enhances the surface expression of TLR-2 in lamina propria mononuclear cell (LPMNCs) which renders these cells more responsive to diverse ligands of gut pathogens or even commensals which gain access to these underlying cells though the leaky gut. During the course of *E. histolytica* infection, there is ample opportunity for epithelial cells and LPMNCs to be exposed to Gal-lectin either through direct contact with the trophozoites or through exposure to the secreted products and/or lysates of dead amebae. The consequent activation of TLRs in intestinal epithelium results in further influx of inflammatory cells and development of colitis, thus perpetuating an uncontrollable intestinal inflammation. Thus, this study further supports the current contention of regulating the TLR function as an attractive therapeutic target for controlling intestinal inflammation during infections with gut pathogens. It should be emphasized that this model proposes a role for TLR-2 in the exacerbation of the mucosal inflammation rather than in initiating the pathogenesis. Future proteomic studies might identify novel parasitic molecules such as lipoproteins or lipophosphoglycans as potential ligands for Toll receptors, which might directly implicate the innate pattern recognition receptors in amebic colitis.

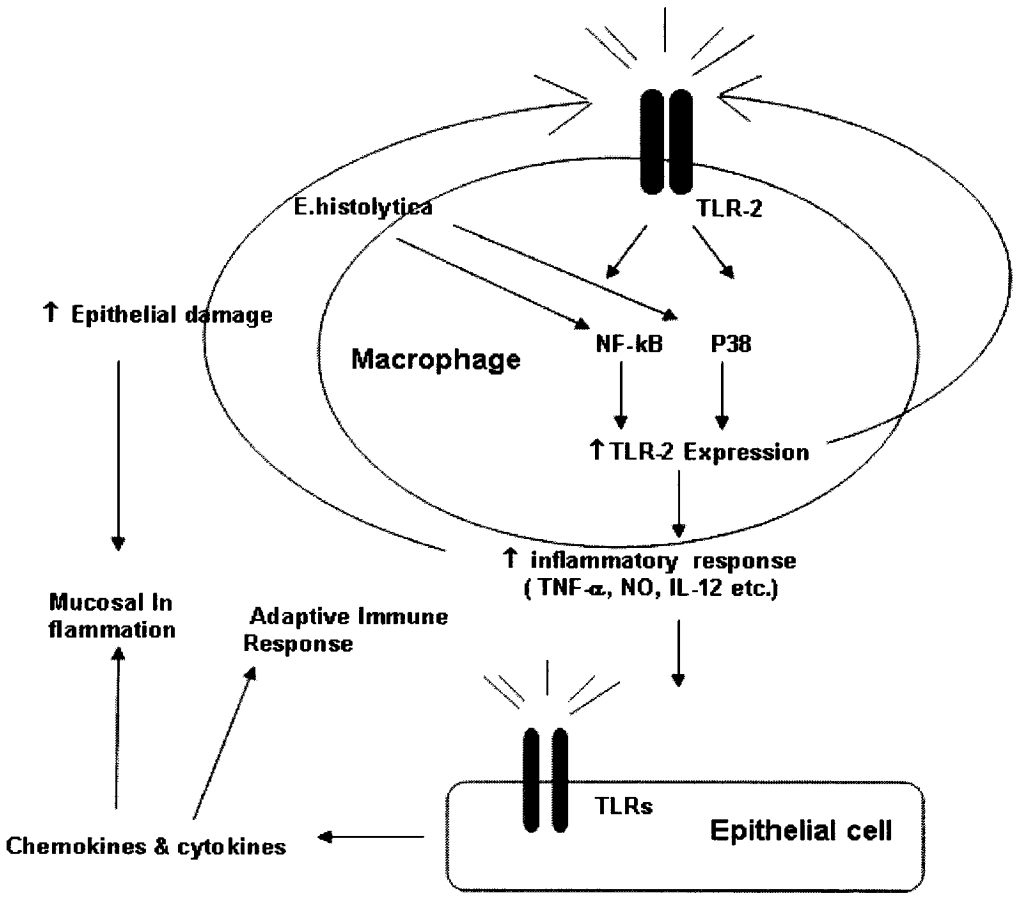

Fig. 5.15: Hypothetical model for the role of TLR-2 in amebic pathogenesis. Enhanced TLR-2 expression in lamina propria mononuclear cells (LPMNC) by Gal-lectin of *E,histolytica* via NF-κB and p38 pathways will amplify the Toll signaling activated by diverse ligands in gut. The resulting amplified proinflammatory cytokine response from leukocytes in turn can up-regulate Toll receptor expression in epithelial cells (47). Consequently, epithelial cells that are hitherto unresponsive/hyporesponsive to gut antigens begin responding to diverse Toll ligands. The resulting production of chemokines and cytokines while helping signaling the adaptive response, also contributes to inflammatory reaction by recruiting phagocytes to the site of tissue damage.

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Connecting Statement I

In the first manuscript we showed that the Gal-lectin induces Toll like receptor-2 expression in macrophages and hypothesized that amplified immune cell responses would contribute to pathogenesis of amebiasis. However, it is not clear how immune cells home into the lamina propria during amebic colitis. As intestinal epithelial cells sense the invading pathogens and signal the immune system and it has been previously shown that amebic components induce IL-8 secretion from IEC, we determined the mechanism of amebic induction of macrophage chemokine, MCP-1 from IEC. It should be mentioned that while Gal-lectin is a potent activator of immune cells, we failed to see any responses in IEC to this molecule in our studies and hence employed a more realistic substance, soluble amebic components.

Chapter 6: Manuscript II

Induction of Monocyte Chemotactic Protein-1 in intestinal epithelial cells by *Entamoeba histolytica* is mediated via PI3 kinase/P65 pathway

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Manuscript under revision for *Infection and Immunity*

Abstract

The role intestinal epithelial cells play in the pathogenesis of amebic colitis is poorly understood. Herein, we demonstrate that soluble *Entamoeba histolytica* proteins (SAP) induce the chemoattractant, Monocyte Chemotactic Protein (MCP)-1 mRNA and protein expression in colonic epithelial cells. SAP failed to activate any of the MAP kinase pathways and IKK activity. Inhibiting the classical pathway of NF-κB activation did not affect SAP induced MCP-1 expression. Instead we find that it is dependent on post-translational modification of NF-κB p65 subunit. SAP induced phosphorylation of p65 and enhanced NF-κB transcriptional activity, which are PI3 kinase dependent. Treatment with PI3 kinase inhibitor LY290004 significantly abrogated the activation of Akt, p65 and MCP-1 mRNA induction. We conclude that colonic epithelial cells play a role in the initiation of inflammation by secreting chemokines in response to soluble amebic components.

Introduction

Entamoeba histolytica is an enteric protozoan parasite that is responsible for the disease amebiasis in humans. The disease affects 50 million people globally and is the fourth leading parasitic cause of death (1). Host inflammatory responses are thought to play an important role in the pathogenesis of intestinal amebiasis. However, the role of intestinal epithelial cells and mediators of colonic inflammation have not been fully elucidated.

Invasive amebiasis is characterized by infiltration of immune cells such as leukocytes and lymphocytes (2). It has not been well established if this cellular infiltration is a cause or consequence of inflammation. The parasite has been previously shown to elicit IL-8 production from colonic epithelial cells (3, 4). IL-8 is a potent chemoattractant primarily for neutrophils and its secretion alone does not explain the homing of other immune cells such as monocytes and lymphocytes seen in the amebic lesions (2). Monocyte chemotactic protein, MCP-1 belongs to a group of C-C or β-chemokines and is produced by a variety of cells, including intestinal epithelial cells (5). MCP-1 is potently chemotactic for monocytes, lymphocytes and basophils (6). MCP-1 levels are increased during various inflammatory conditions that are characterized by cellular infiltration (7-9). Recently, amebic infection in a human intestinal xenograft model has been shown to increase a number of genes in the epithelial cells including MCP-1 (10). However, the mechanism of induction is not known and also it is not clear if ameba components themselves can induce this chemokine.

The transcription factor NF-κB regulates a number of genes involved in immune response and inflammation (11). It is composed of two subunits most commonly of P65 and P50. Only P65 has the transactivating domains and hence is critical for NF-κB activity. Various pathogens and pathogen molecules activate NF-κB and there by induce an inflammatory response. NF-κB is activated by different pathways including classical, alternate, atypical and p105 pathways (12). All these pathways involve phosphorylation and subsequent degradation of IκB, increased nuclear translocation of NF-κB subunits,

increased binding to DNA in the promoter regions of target genes and increased gene transcription. However, it is increasingly being recognized that post-translational modifications of NF-κB p65 subunit is equally important in the transactivating function of the transcription factor (13). The two important post-translational events that determine the strength, duration and efficacy of NF-κB function are phosphorylation and acetylation. Indeed, phosphorylation appeared to be a requirement for subsequent acetylation. Importantly, this p65 phosphorylation can lead to transcription of a set of NF-κB target genes independent of IκB degradation (14, 15). IKK, Akt, NIK, Casein kinase II have been implicated in the phosphorylation of cysteine residues at different positions of P65 subunit (12). In this study we show that soluble amebic proteins can induce MCP-1 from IEC by a unique pathway involving PI3 kinase and NF-κB p65.

Materials and Methods

Cells, reagents and amebic components

Human adenocarcinomal cell line Caco-2 cells from ATCC were grown to confluent monolayers in minimum essential medium with 5% serum and penicillin and streptomycin for 5-7 days. Antibodies against MCP-1, total p65, phospho-p65 are from Santa Cruz Biotech. Anti IκB, phospho-IκB, total and phospho Akt antibodies and LY 290004 are from Cell signaling. PD98059 and SB203580 are from Calbiochem. All other reagents are from Sigma. Soluble Amebic Proteins (SAP) were prepared by three cycles of freeze-thaw lysis of log phase *E. histolytica* virulent strain HM1: IMSS and quantified by the BCA assay (Pierce) as described previously (16). For transwell studies, amebic trophozoites were added to the Corning's transwell inserts with pore diameter 0.6 μM with Caco-2 cells in the bottom well.

Real-time PCR

Total RNA was extracted with TriZol reagent (Invitrogen), quantified and 5 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and oligo d(T) as per manufacturer's instructions. 2 μl of cDNA was used for real-time PCR. Real-time

primers used are forward: GATCTCAGTGCAGAGGCTCG, reverse: TGCTTGTCCAGGTGGTCCAT for MCP-1, forward: GAAGATGGTGATGGGATTTC, reverse: GAAGGTGAAGGTCGGAGT for GAPDH. Amplifications were carried out with Qiagen's Quantitect SYBR Green PCR kit at the following cycling conditions: 94° C hold for 10 min, followed by 40 cycles of denaturation at 94 ° C for 20 s, annealing at 60 ° C for 30 s and extension at 72 ° C for 60 s. Specificity of amplification was checked by melt curve analysis, MCP-1 expression was normalized to that of GAPDH and fold change over control was determined according to Ct method as described (17).

Western blot

Cytoplasmic or nuclear extracts were collected using the NE-PER (Pierce) kit as per the instructions of the manufacturer. Protein concentrations were estimated by micro BCA method (Pierce). Equal amounts of the samples were then separated in 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane (Bio-Rad). The membranes were blocked in 3.5% skim milk-TBS-T (20 nm Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20) at 4°C overnight, incubated with primary antibodies in skim milk-TBS-T at 4°C overnight, washed three times with TBS-T, and incubated with HRP-conjugated secondary antibody in skim milk- TBST overnight at 4°C. After three washes each in TBS-3T and TBS-T, the blot was developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the instructions of the manufacturer. Actin and histone are used as loading controls for cytoplasmic and nuclear extracts respectively.

***In vitro* kinase assay**

Cells were incubated with cell lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 25mM β-glycerophosphate, 2mM EDTA, 2mM pyrophosphate, 1mM orthovanadate, and 1% Triton X-100, 1mM DTT, 1mM NaF with protease inhibitors) followed by addition of anti IKK-α antibody. Following overnight end-to-end rotation of tubes at 4°C, immunoprecipitates were washed 3 times with lysis buffer and once with kinase buffer (20mM Tris, pH 7.5, 1mM $MnCl_2$, 10mM $MgCl_2$, 20mM β-Glycerophosphate, 0.1mM

sodium orthovanadate, 2mM NaF and 1mM DTT). Immunoprecipitates were finally resuspended in 20.0 µl of kinase buffer containing 5µCi of [γ ^{32}P] ATP and incubated at 30 $^{\circ}$ C for 30 min. 1 µg of GST-IκBα (Santa Cruz Biotech) was used as substrate. The reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized by autoradiography. Cell lysates were also checked for IKK-α expression for normalization.

Luciferase reporter assay

NF-κB transactivation was studied using luciferase reporter plasmids from Stratagene according to manufacturer's instructions. Briefly, vector DNA (pNF-κB-Luc with or without p FC-MEKK positive control plasmid) was allowed to form complexes with Fugene 6 (Roche), which was used at 0.003% v/v (final concentration). Cells were washed once in serum-free medium and the DNA-fugene mixture containing the pNF-κB-luc plasmid was added to 40-50% confluent Caco-2 cells. The transfection continued at 37°C for 6 h, after which the medium was changed to normal growth medium and the cells were allowed to grow for another 48 h. Tansfected cells were treated with soluble amebic proteins for 6 h and luciferase was extracted with Luciferase assay systems (Promega) and the emitted light measured with luminometer.

Statistical analysis

Statistical analysis to check significance was done with Student's t test using Prism software. Graphs plotted were from two to three independent experiments and error bars in all graphs represent mean± SD.

Results

Soluble amebic proteins induce MCP-1 mRNA and protein production

Human colonic epithelial cells were treated with 100 µg/ml of soluble amebic protein for different time points and checked for MCP-1 mRNA expression by real-time PCR and protein production by Western blot. As shown in Fig. 6.1A, SAP induced significant

expression of MCP-1 mRNA as early as 2 h reaching a peak after 8 h. Little protein expression was observed after 6 h but increased steadily thereafter with significant expression seen after 24 h (Fig. 6.1B).

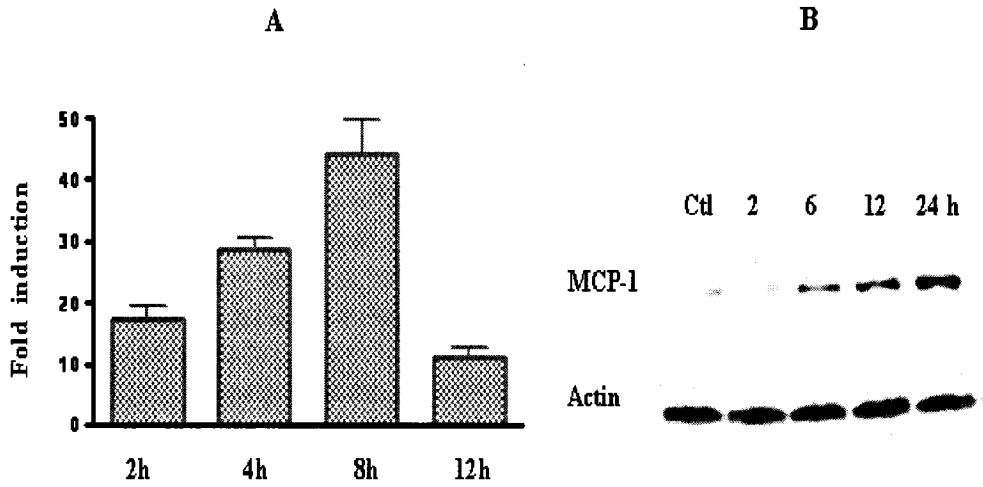

Fig. 6.1: Amebic components induce MCP-1 mRNA and protein production from intestinal epithelial cells. A) Confluent Caco-2 cells were treated with 100 µl/mg of soluble amebic proteins for different time periods, RNA extracted with TriZol reagent and real-time PCR was done as described in methods. B) Caco-2 cells treated as above for different time periods and whole cell lysates were subjected to SDS-PAGE and western blot analysis with anti MCP-1 antibody and actin antibodies.

Ameba induced MCP-1 mRNA induction is independent of IKK activity and IκB-α degradation

As MCP-1 expression is regulated by the transcription factor NF-κB (18), we checked its role in ameba induced MCP-1 induction. Surprisingly, pre-treating cells with the proteasome inhibitor MG132, failed to suppress SAP induced MCP-1 production, while significantly reducing the basal MCP-1 mRNA expression (Fig. 6.2A). SAP treatment also failed to induce IKK activity (Fig. 6.2B) and to phosphorylate IκB (Fig. 6.2C).

Amebic components induce NF-κB transcriptional activity and p65 phosphorylation

Despite the lack of IκB degradation, amebic protein treatment was found to result in around 6-fold increase of the NF-κB transcriptional activity (Fig. 6.3A). To decipher the mechanism underlying the apparently contradicting observations of lack of IKK

activation but increased NF-κB transactivation, we focused on the dynamics of p65 subunit. First we checked its nuclear translocation but did not observe significant accumulation until after 2 h (Fig. 6.3B). To check if amebic components activate NF-κB by post-translational modification, we checked the phosphorylation status of p65 subunit. Indeed, SAP was found to increase the levels of phosphorylated p65 following 30 min treatment (Fig. 6.3C).

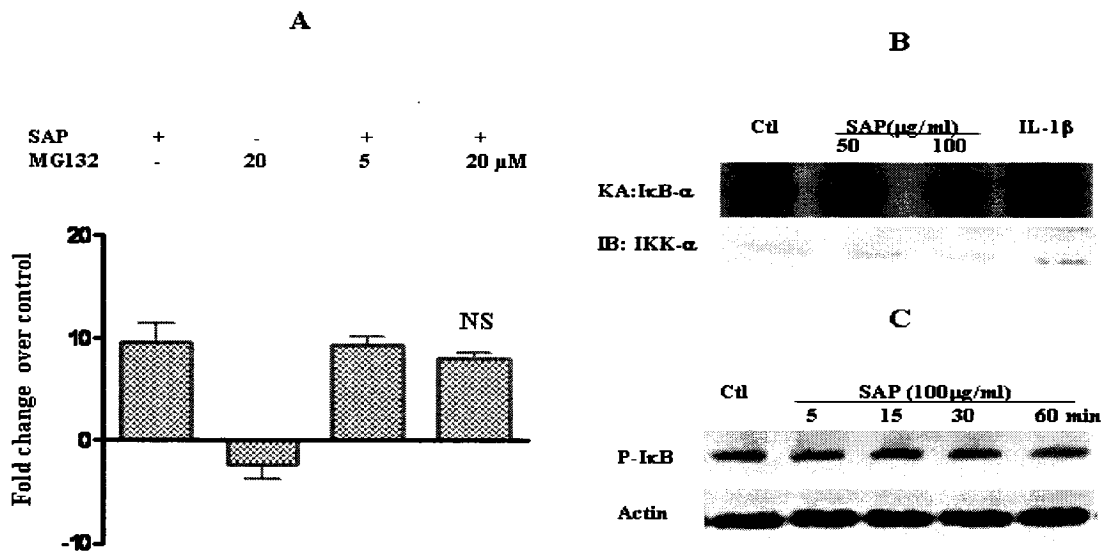

Fig. 6.2: Amebic components do not activate IKK activity. A) Cells were treated or not with MG-132 for 2 h followed by SAP for 2 h. RNA extracted and real time PCR was done as described. B) IEC treated with different concentrations of SAP for 30 min, cell lysates were immunoprecipitated using IKK-α antibody and in vitro kinase assay was done using GST-IκB-α as substrate. Equal quantities (20 μg) of cellular proteins were subjected to western blot to check the IKK-α levels. KA: kinase assay; IB: immunoblot. C) Cells treated with 100 μg/ml of SAP for different time points and cell lysates subjected to SDS-PAGE and western blot with phosphorylated IκB and actin.

E. histolytica activates Akt

As Akt is one of the proposed upstream kinases implicated in p65 phosphorylation (19), its activation was checked with antibody that specifically recognizes phosphorylated Akt. As shown in Fig. 6.4A, SAP treatment rapidly increased P-Akt levels as early as 15 min and this activation was continued for 2 h. Moreover, secreted products from live *E.*

histolytica trophozoites separated by a semi-permeable membrane also activated Akt after 2 h treatment of epithelial cells (Fig. 6.4B).

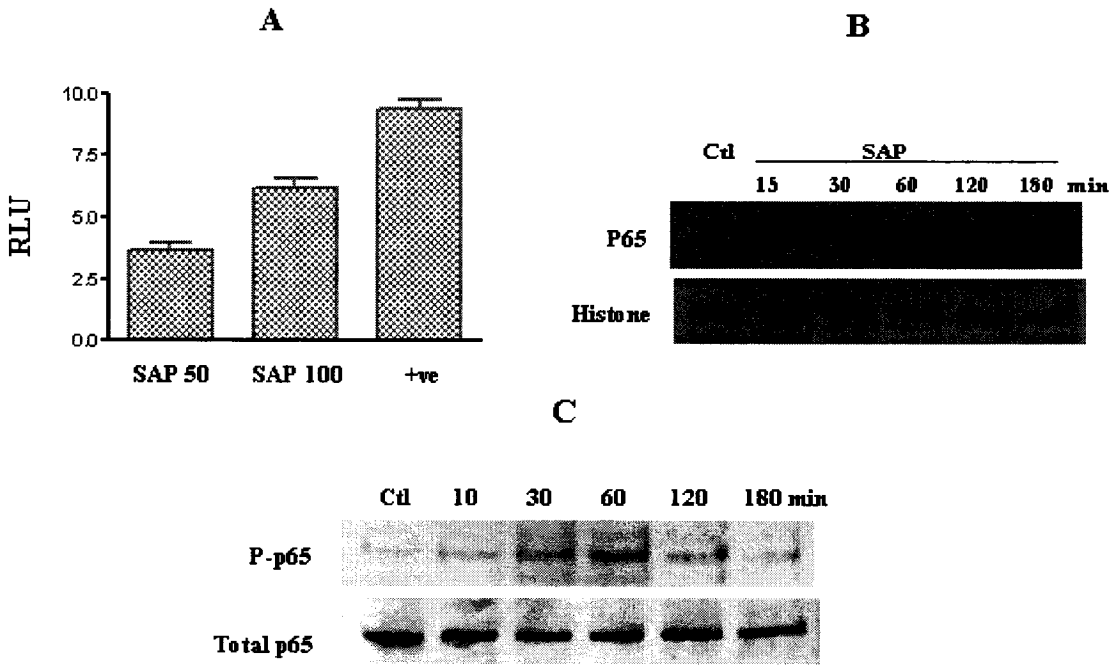

Fig. 6.3: SAP induces NF-κB transactivation and p65 phosphorylation. A) Caco-2 cells were transfected with p-NF-κB-luc plasmid with or without the positive control (p-MEKK) and treated with different concentrations of SAP for 6 h and assayed for luciferase activity as described. RLU- relative luciferase units over the untreated cells. B) Cells were treated with 100 μg/ml of SAP for different time periods and nuclear extracts prepared as described, 10 μg/ml of protein separated by SDS-PAGE and checked for p65 nuclear translocation. The blot was stripped and reprobed with anti histone antibody for normalization. C) Whole cell lysates from cells treated as above were subjected to western blot and probed with antibody specific to phosphorylated p65 and then with antibody against total p65. Representative blots from 3 experiments were shown.

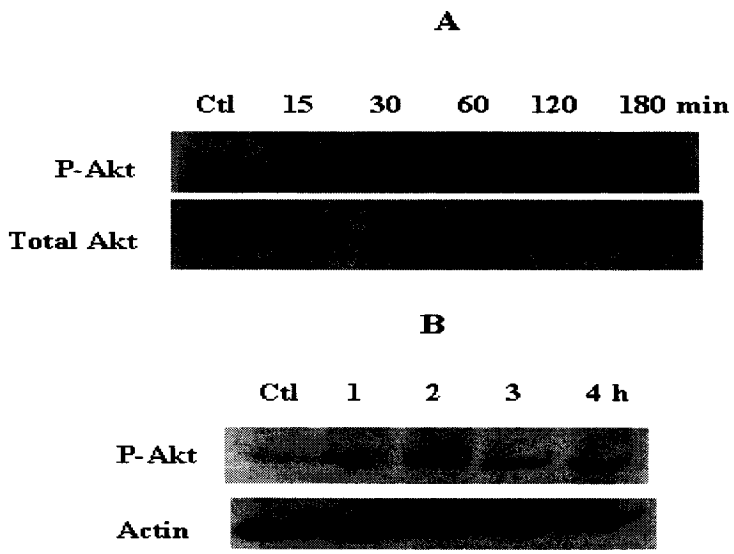

Fig. 6.4: *E. histolytica* activates Akt. A) Caco-2 cells were treated with 100 μg/ml of SAP for different time points and cell lysates were checked for phosphorylation of Akt by western blot. Blots were reprobed with total p65 antibody for normalization. B) 10^6 *E. histolytica* trophozoites were added to the transwell culture with confluent Caco-2 cells at the bottom as described in methods, cell lysates extracted at different time periods and checked for phosphorylated Akt as above. Actin expression was analyzed to check equal loading.

SAP induced P65 activation is PI3 kinase but not MAP kinase dependent

As Akt is a substrate for PI3 kinase, we checked the role of this kinase in SAP induced Akt and P65 phosphorylation. As expected, inhibition of PI3 kinase with LY294002 inhibited SAP induced Akt phosphorylation (Fig. 6.5A) but interestingly also abrogated ameba induced P65 phosphorylation (Fig. 6.5B). To study the role of MAP kinases that are purported to function downstream of PI3 kinase/Akt, we checked their activation by ameba components (Fig. 6.5C) but failed to see activation of ERK, JNK or p38 MAP kinases. Consistently, treatment of colonic cells with inhibitors of ERK and p38 MAP kinases failed to suppress SAP induced p65 phosphorylation (Fig. 6.5D).

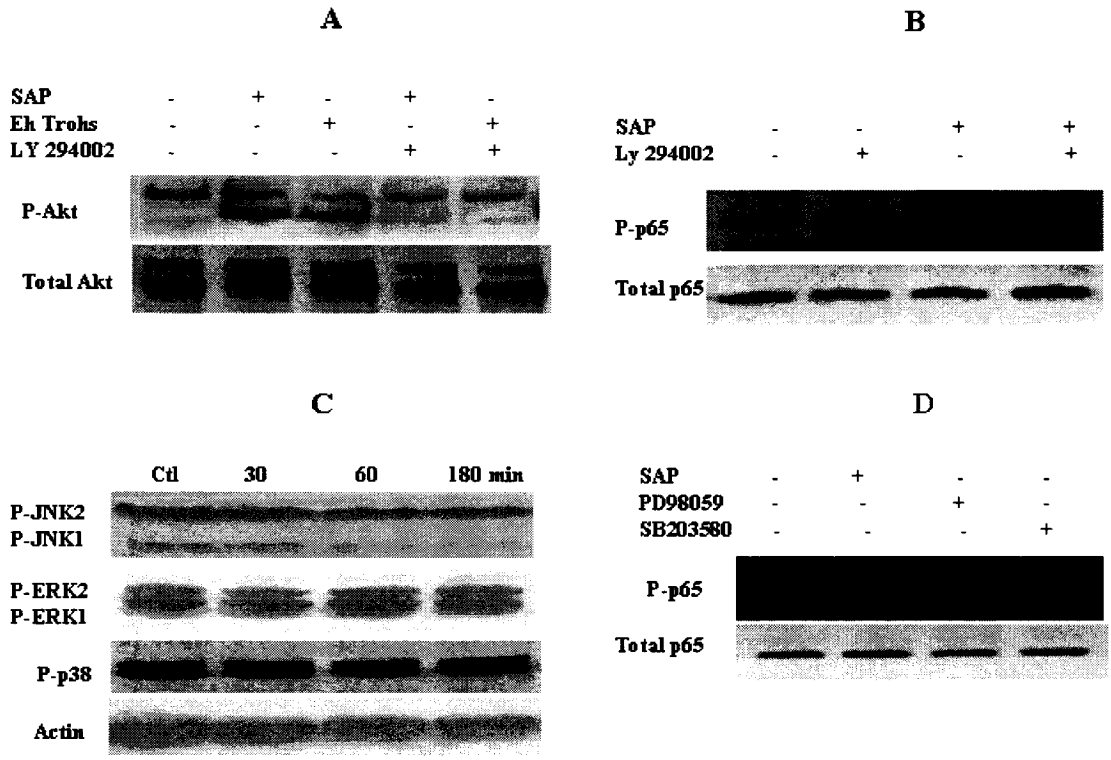

Fig. 6.5: SAP induced P65 phosphorylation is dependent on PI3 kinase but is independent of MAP kinases. A) Caco-2 cells were pretreated with PI3 kinase inhibitor LY294002 for 1 h prior to SAP or trophozoite (in transwells) exposure for 2 h and whole cell lysates checked for phosphorylated Akt levels in relation to total Akt expression. B) Lyates from cells pretreated or not with LY294002 followed by SAP for 30 min were subjected to SDS-PAGE and western blot using anti phospho- and anti total p65 antibodies. C) Caco-2 cells were exposed to SAP for different time periods and cell lysates checked for activation of MAP kinases using phospho-specific antibodies against JNK, ERK and p38 MAP kinases. Actin expression was checked for normalization. D) Cells were pretreated with 50 μM of PD98059 (ERK inhibitor) or SB203580 (p38 inhibitor) for 2 h before incubating with SAP for 30 min and whole cell lysates checked for phosphorylation of p65. Blot was stripped and reprobed with total p65 antibody. Representative blots from at least two different experiments were shown.

PI3 kinase inhibition suppresses SAP induced MCP-1 mRNA expression

Finally we checked if PI3 kinase inhibition can suppress MCP-1 mRNA induction by amebic components. As shown (Fig. 6.6) LY294002 significantly inhibited SAP induced MCP-1 mRNA even at the low concentration of 10 μM and completely abrogated at a high concentration of 50 μM.

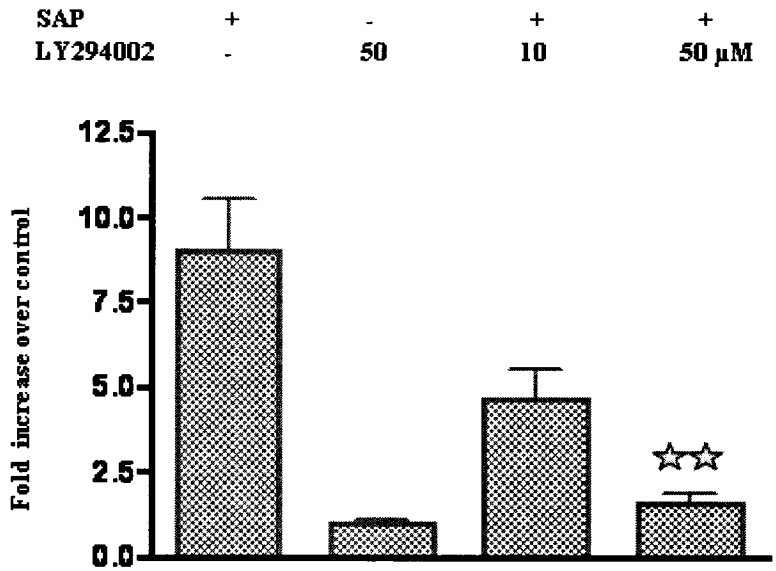

Fig. 6.6: SAP induced MCP-1 induction is PI3 kinase-dependent. Caco-2 cells were pretreated or not with different concentrations of LY294002 for 1 h followed by Sap treatment for 2 h. RNA was extracted and real time PCR done as described. The experiment was repeated three times and fold-increase of MCP-1 over untreated cells was depicted in the graph. (✱✱; p= 0.0088).

Discussion

Herein, we report that soluble amebic components can induce the chemoattractant MCP-1 via a unique mechanism involving PI3 kinase and NF-κB p65 pathway. IEC have been shown to secrete a number of macrophage-specific chemokines like MCP-1 and MIP-1β during several infections that signal macrophages to the site of infection and halt them once they have arrived via macrophage inhibition factor (9). In addition to chemoattraction function, colonic epithelial cells secrete cytokines such as GM-CSF, IL-6, and TNF-α that stimulate macrophage activation, proliferation, and additional cytokine secretion, which further perpetuate the inflammatory response. Macrophages are potent phagocytes and limit the infection at an early stage and in addition to engulfing and destroying pathogens, are also a source of cytokines such as IL-1α/β and TNF.

During the pathogenesis of amebic colitis, early invasive lesions are characterized by infiltration of neutrophils as a result of contact (3) or non-contact (5) dependent secretion

of IL-8 by colonic epithelial cells. However, the role of these cells in ameba killing is doubtful as observed *in vitro,* wherein highly virulent strains of ameba could resist neutrophils at a high ratio of 3000 neutrophils per ameba (20). More so, the neutrophils may contribute to host tissue damage. As invasion progresses, the ulcers extend deep into the submucosa and during this late invasive stage, macrophages and lymphocytes are seen (2). Despite their presence in the lesions, the efficacy of monocytes and macrophages in killing trophozoites is not very clear but probably related to their state of activation (2). Moreover, ameba has been shown to secrete a monocyte locomotion inhibition factor, which has been implicated in the absence of macrophages in severe amebic lesions (21). Apparently, whether macrophages home in and kill trophozoites depends on several factors such as the balance between epithelial MCP-1 production, parasite release of inhibitory factors and the release of other activating factors like TNF-α and IFN-γ. Nonetheless, monocytes and macrophages are present in amebic lesions and might play a role both in the destruction of host tissue and also in killing the trophozoites. As MCP-1 is also able to activate cytotoxic T cells (22) and these cells have been found to kill ameba trophozoites (23), this observation is of great importance to pathogenesis. NF-κB is an important transcription factor that is usually activated by a classical pathway involving IκB degradation and increased NF-κB nuclear translocation or non-canonical pathway involving P65 phosphorylation. While IKK has been shown to be involved in both the pathways, it is mandatory for the former but dispensable for the latter. P65 subunit is phosphorylated at 4 serine residues by several kinases and this post-translational modification is either supplementary to classical IKK-dependent NF-κB activation or is required for the transcription of certain NF-κB dependent genes (12). Some reports suggest phosphorylation of p65 enhances nuclear transport through an unknown mechanism but most probably via decreased affinity to IκB-α (24) and this could be the reason for the delayed increase of nuclear expression of p65 in response to ameba components and the delayed peak induction of MCP-1 mRNA at 8 h. Nonetheless, rapid induction at 2 h without apparent increase of p65 is most probably related to the phosphorylated p65-mediated gene transcription seen in the luciferase reporter studies. MCP-1 is constitutively regulated by the classical NF-κB pathway as observed by its inhibition by the proteasome inhibitor, MG-132 in cells not treated with SAP. It could be

reasonably argued that NF-κB dependent genes are under the regulation of redundant pathways and could be activated in a stimulus-dependent manner. Interestingly, it has been shown before that ameba proteins induce IL-8 production via a post-transcriptional regulation, which is inhibited by dexamethasone (4). This suggests that the host has developed multitude of mechanisms to sense the pathogen and signal the immune system. We previously observed that prolonged treatment with ameba proteins inhibit NF-κB activation via a negative regulation of IKK activity by stress proteins (25).

The pathway involving PI3 kinase and p65 phosphorylation could be an alternative mechanism by which colonic epithelial cells activate the important transcription factor NF-κB to sense the pathogen and signal the immune system. Indeed the DNA damaging agents such as doxorubicin and etoposide induce IκB degradation independent of IKK and also phosphorylate p65 via ribosomal S6 kinase 1 (24). We failed to identify if PI3 kinase or Akt directly phophorylates p65 subunit in IEC in response to ameba components. While previous reports implicate different kinases such as IKK-α, IKK-β, p38 MAP kinases as mediators of PI3 kinase induced p65 phosphorylation (26, 27), we did not find activation of IKK-α in a kinase assay and also failed to see p38 activation by ameba components. Recently, several novel kinases such as Bruton's tyrosine kinase (Btk) and IKK-i/IKK-ε are being found to phosphorylate S536 of p65 subunit (28, 29) and the relation between these kinases and PI3 kinase remains to be explored. It should also be noted that this study does not rule out phosphorylation of other serines and their possible role in regulating SAP induced NF-κB activation. In summary, we demonstrate a novel signaling pathway in colonic epithelial cells for activation of MCP-1 by the gut pathogen, *E. histolytica* and hypothesize that this could have an impact on the outcome of infection (Fig. 6.7).

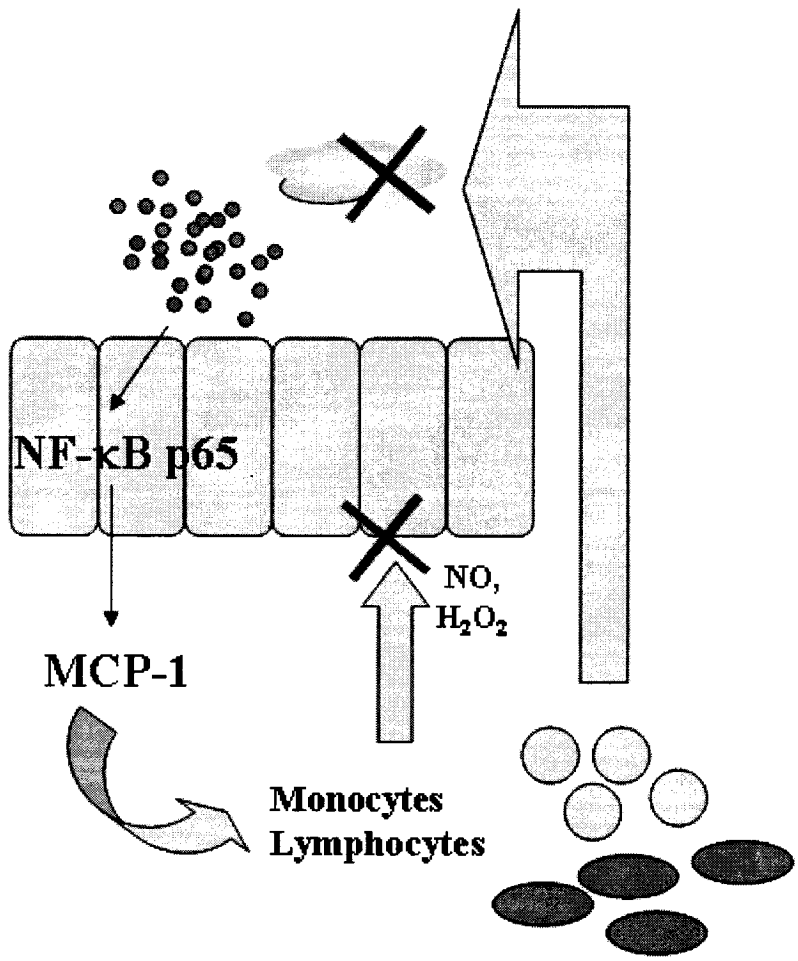

Fig. 6.7: Model for the consequences of MCP-1 production in amebiasis. Intestinal epithelial cells sense ameba and secrete the chemokine MCP-1 via p65 pathway. The resulting homing of macrophages causes release of cytolytic agents such as reactive oxygen species which act both on amebic trophozoites and epithelial cells. Different outcomes are possible; trophozoites are killed and infection eliminated with varying degrees of inflammation; macrophages fail to kill ameba but cause apoptosis of IEC and enhances invasion.

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Connecting Statement II

Despite the homing and activation of immune cells by amebic components that has been reported so far, surprisingly, only a minority of individuals develop symptoms of colonic inflammation. As NF-κB is the principal inflammatory mediator and it has previously been shown that inhibiting this transcription factor reduces amebic colitis, we hypothesized that epithelial cells in the majority of infected hosts respond in a beneficial fashion that can potentially suppresses NF-κB induction. Looking at the other perspective, inhibiting NF-κB is also advantageous for parasite survival. Encouragingly, in our previous work (manuscript II) we observed that amebic components did not activate the classical NF-κB pathway. Hence, we sought to study its suppression by amebic components in IEC. As the stress response is a potent suppressor of NF-κB and its induction during amebic infection has not been studied so far, we studied the stress response and its inhibition of NF-κB by the parasite components.

Chapter 7: Manuscript III

Suppression of NF-κB activation by *Entamoeba histolytica* in intestinal epithelial cells is mediated by heat shock protein 27

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Abstract

Little is known about the pathogenesis of *Entamoeba histolytica* and how epithelial cells respond to the parasite. Herein, we characterized the interactions between *E. histolytica* and colonic epithelial cells and the role macrophages play in modulating epithelial cell responses. Human colonic epithelial cell lines, Caco-2 and T84 were grown either as monoculture or co-cultured in transwell plates with differentiated human THP-1 macrophages for 24 h before stimulation with soluble amebic proteins (SAP). In naive epithelial cells, prolonged stimulation with SAP reduced the levels of heat shock protein (Hsp) 27 and 72. However in THP-1 conditioned intestinal epithelial cells SAP enhanced Hsp27 and Hsp72, which was dependent on the activation of ERK MAP kinase. Hsp synthesis induced by SAP confered protection against oxidative and apoptotic injuries. Treatment with SAP inhibited NF-κB activation induced by IL-1β; specifically, the NF-κB-DNA binding, nuclear translocation of p65 subunit and phosphorylation of IκB-α were reduced. Gene silencing by siRNA confirmed the role of Hsp27 in suppressing NF-κB activation at IKK level. By co-immunoprecipitation studies, we found that Hsp27 interacts with IKK-α and IKK-β and this association was increased in SAP treated conditioned epithelial cells. Over expression of wild type Hsp27 amplified the effects of SAP while phosphorylation-deficient mutant of Hsp27 abrogated SAP induced NF-κB inhibition. In conditioned epithelial cells, Hsp27 was phosphorylated at serine 15 after prolonged exposure to SAP. This mechanism may explain the absence of colonic inflammation seen in the majority of individuals infected with *E. histolytica.*

Introduction

The intestinal protozoan parasite *E. histolytica* affects 50 million people worldwide, causing 100,000 deaths per year (1). Intestinal amebiasis is characterized by colitis and severe dysentery. Despite our knowledge of the role of several pathogen and host factors in causing colonic inflammation, the cell specific response to amebic infection is poorly understood. Moreover, the majority of research done to unravel the mechanism of amebic colitis has been focused on proinflammatory responses (2, 3) by epithelial cells and a role of NF-κB (4) and chemokines such as IL-8 (5) as triggering events for inflammation. However, it is noteworthy that only 10% of *E. histolytica* infected individuals show symptoms of intestinal inflammation (6) and none of the studies addressed the question of why only a minority of infected individuals develops amebic colitis.

Epithelial cells are the first layer of host defense and have been shown to be the effector cells capable of secreting several mediators in response to pathogens (7, 8). Epithelial cells *in vivo* do not respond in isolation but act in concert with several immune and non-immune cells present in the lamina propria. To simulate this, several *in vitro* studies were carried out to assess the epithelial cell responses in the presence of immune cells such as leucocytes and lymphocytes. Under these conditions, a differential response was observed in epithelial cell lines exposed to various pathogenic and non-pathogenic bacteria (9, 10). However, the effect of co-culturing with macrophages on epithelial cell responses has not been well characterized.

The universal response to stress has been the induction of a group of highly conserved family of proteins called heat shock proteins (Hsps) and is commonly referred to as heat shock response or stress response. Several pathogens and their products have been shown to induce various hsps in different cell types including intestinal epithelial cells (11- 13). Hsps serve to protect the cells against several insults such as thermal, toxic or apoptotic stimuli (12, 14-16). Epithelial cell induction of Hsps in response to various pathogens such as *E. coli*, and toxins such as LPS and superantigen has been reported (10, 12, 14). It has also been shown that epithelial Hsp expression is regulated by cytokines and

immune cells such as lymphocytes (13). One emerging concept is that the stress response counters the inflammatory response mediated by NF-κB helping to reduce inflammation and promote healing of damaged tissues (12, 17-20). As proinflammatory cytokines secreted by immune cells have been shown to influence epithelial cell responses (16, 21) and macrophages are a major source of these cytokines, we studied the effect of macrophages on epithelial cell response towards *E. histolytica*. We hypothesized that amebae might be eliciting a protective response whereby inflammation is suppressed in the majority of infected individuals. Thus, we sought to study the stress response induced by ameba components in naïve and macrophage-conditioned colonic epithelial cells and made several interesting and novel observations. For the first time, we showed that macrophage conditioning primes epithelial cells for an augmented Hsp expression in response to amebic components. We identified Hsp27 as the key mediator suppressing NF-κB activation by virtue of its association with IKK complex in intestinal epithelial cells (IEC). We conclude that this could be one of the mechanisms by which colonic inflammation is suppressed in the majority of *E. histolytica* infected individuals, and that lack of such protective responses in susceptible individual could lead to the symptoms associated with amebic colitis.

Materials and Methods

Cell lines, amebic components and reagents

Human colonic adenocarcinomal cell lines T84 and Caco-2 from ATCC (Rockville, MD) were used and maintained in DMEM-F12 and MEM media respectively, supplemented with 10% fetal bovine serum, 100 U / ml penicillin, 100 μg / ml streptomycin sulfate, and 20 mM HEPES (Sigma). Human THP-1 macrophages were maintained at 37 °C and 5 % CO_2 in complete RPMI 1640 (GIBCO) supplemented with 10 % heat-inactivated FCS (Hyclone Laboratories). To obtain adherent macrophages, 2×10^6 cells / well in 6-well culture plates were allowed to differentiate in the presence of 10 nM PMA (Sigma) for 3 days. Macrophages were washed and made quiescent by incubation in complete RPMI 1640 medium for 24 h prior to co-culturing. Soluble Amebic Proteins (SAP) were prepared by three cycles of freeze-thaw lysis of log phase *E. histolytica* virulent strain

HM1:IMSS and quantified by the BCA assay (Pierce). *E. histolytica* secretory components (SC) were prepared as described previously (5). Fas Ligand and Caspase 3 antibody are from Oncogene. Antibody against NF-B p65 & MAP kinases, double stranded NF-κB oligonucleotide (sc-2505) and all siRNA reagents are from Santa Cruz Bio Tech, CA. Anti IKK antibodies are from Cell Signaling Technology. Antibodies against human Hsp27, Hsp72, Hsp60, Hsp90, HSF-1 phospho Hsp27 (S15), phospho Hsp27 (ser 72) and hamster Hsp27 (Hsp25) are from Stressgen, Victoria, Canada. For immunoprecipitation, agarose conjugated Goat antibodies from Santa Cruz Botech. Inc were employed; Hsp27 (sc-1048), Hsp60 (sc-1722), Hsp72 (sc-1060) and Hsp90 (sc-1055). For overexpression studies, hamster Hsp25 was used. PD98059 was obtained from Calbiochem. All other chemicals are from Sigma, St. Louis, MO. The inhibitors galactose, E-64, and cycloheximide were used at slightly higher concentrations than those have been previously demonstrated to have their intended effects (22-24).

Co-culturing of epithelial cells with macrophages

T84 or Caco-2 cells between 10-30 passages grown in either regular or transwell plates for 7-10 days to achieve confluency were used. For siRNA experiments, sub confluent (40-50%) Caco-2 cells, grown for 2-3 days were used. Human monocyte-like cell line THP-1 was differentiated with PMA for 3 days and quiescence for 24 h before use. Transwells with epithelial cells were kept in culture plates containing $2x10^6$ macrophages for 24 h (30h for siRNA studies), removed from co-culture and immediately used for experiments. Epithelial cells were kept under low serum condition (5% fetal bovine serum) during co-culturing and subsequent treatments, and without antibiotics for siRNA studies.

Western blot

Cellular extracts from amebic protein treated epithelial cells were prepared by scraping into the sample buffer containing SDS and mercaptoethanol (ME) and boiled for 10 min, equal volumes were separated in 12% SDS-Polyacrylamide gels and transferred onto nitrocellulose membrane (Bio-Rad, CA). For non-reducing gel run, sample buffer without ME was used. The membranes were blocked in 3.5% skim milk-TBS-T (20mM

Tris-HCl (pH 7.5), 500mM NaCl. 0.1% Tween-20) at 4°C overnight, incubated with primary antibodies in 1% skim milk-TBS-T at 4°C overnight, washed three times with TBS-T and incubated with HRP-conjugated secondary antibody in skim milk-TBST overnight at 4°C. After three washes each in TBS-T and TBS-3T, the blot was developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Electrophoretic mobility shift assay

Nuclear extracts were collected using the NE-PER kit (Pierce) and protein quantified by BCA assay. Annealed double stranded HSE oligonucleotide -107 to -83 of the human Hsp70 gene (5'-GAT CTC GGC TGG AAT ATT CCC GAC CTG GCA GCC GA-3') (Sigma Genosys, ON) or NF-κB consensus oligonucleotide (Santa Cruz Biotech) was labeled with [^{32}P]-ATP, using T4 polynucleotide kinase (Invitrogen). Unlabeled nucleotides were removed using Sephadex G-25 columns. The binding reaction consists of 20 l total volume of 0.5 ng of DNA probe, 5 g of nuclear extract, 1 g of poly dI-dC in the binding buffer (12 mM HEPES, 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM of DTT and 12% glycerol) (pH 8.0), and incubation for 30 min at room temperature. DNA-protein complexes were resolved by electrophoresis on 6% poly-acrylamide gels at 4°C in TBE buffer (90 mM Tris-borate, 2mM EDTA). Gels were subsequently dried and autoradiographed with intensifying screens at -70°C.

Neutral red assay

Neutral red (Sigma) was reconstituted in serum free medium and added to cells at 1.14mM concentration. After 2 h of incubation, the medium was removed and the cells washed twice with PBS; finally, the incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 2 ml of the extraction buffer containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the dye taken up, the cell lysis products were centrifuged and supernatants spectrophotometrically measured at 540 nm.

Co-Immunoprecipitation

Cells were lysed in 1% CHAPS buffer (10mM Tris-HCl, pH 7.4, 150mM NaCl with 1% CHAPS) with protease inhibitor cocktail (Roche). 500 μg of cell extracts were incubated overnight with 5 μg of agarose conjugated anti HSP antibodies (Santa Cruz Biotech). Precipitate was washed thrice with lysis buffer, dissolved in 2x Lammeli buffer, boiled and separated by SDS-PAGE, transferred to nitrocellulose membrane and detected by western blot analysis using ECL (Amersham Biosciences).

RNA interference

Caco-2 cells were transfected with small interfering RNA (siRNA) against HSF-1 (sc-35611), Hsp27 (sc-29350), Hsp70 (sc-29352), or control siRNA (sc-37007) from Santa Cruz Biotech as per the manufacturer's protocol. Briefly, sub-confluent (40-50%) Caco-2 cells were transfected using siRNA transfection reagent for 30 h during the co-culture at a siRNA concentration of 40nM. Cells were immediately used for SAP and subsequent treatments.

Over expression of wild type and phosphorylation deficient-mutant Hsp27 in M-IEC

Plasmids (pcDNA3.1) encoding wild type hamster Hsp25 (WT) and phosphorylation deficient-mutant Hsp25 (AA) in which ser 15 and ser 90 are replaced by alanine were kindly provided by Dr. T. Tomako, McGill University, Montreal. Caco-2 cells were transfected with empty plasmid (vector), WT and AA by Fugene reagent (Promega) overnight according to the manufacturer's instructions. A transfection efficiency of 50-60% was observed using a GFP control. Cells were recovered for 24 h while simultaneously co-cultured with differentiated THP-1 and then at the end of 24 h, conditioned IEC was treated with SAP and IL-1β as described before.

***In vitro* kinase assay**

Cells were incubated with cell lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 25mM β-glycerophosphate, 2mM EDTA, 2mM pyrophosphate, 1mM orthovanadate, and 1% Triton X-100, 1mM DTT, 1mM NaF with protease inhibitors) followed by addition of

anti IKK-α antibody. Following overnight end-to-end rotation of tubes at 4°C, immunoprecipitates were washed 3x with lysis buffer and once with kinase buffer (20mM Tris, pH 7.5, 1mM $MnCl_2$, 10mM $MgCl_2$, 20mM β-Glycerophosphate, 0.1mM sodium orthovanadate, 2mM NaF and 1mM DTT). Immunoprecipitates were finally resuspended in 20.0 µl of kinase buffer containing 5µCi of [γ ^{32}P] ATP and incubated at 30 ° C for 30 min. 1 µg of GST-IκBα (Santa Cruz Biotech) was used as substrate. The reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized by autoradiography. Cell lysates were also checked for IKK-α expression for normalization.

TNF-α ELISA

Culture supernatants collected from cells treated or not with SAP (for 12 h) and IL-1β (for 8 h) and quantified for TNF-α protein. DuoSet ELISA development system from R&D systems was used to check the TNF-α secretion from IEC according to manufacturer's protocol. Briefly, ELISA plates were coated with capture antibody overnight, washed with PBS-T, incubated with 100 µl of supernatnant from the culture medium for 2h at room temperature followed by wash and further incubation with detection antibody for another 2 h at room temperature. Streptavidin-HRP was then added to wells for 20 min followed by substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine). Stop solution (2N H_2SO_4) was added and optical density measured immediately at 450nm and 540nm. Readings at 540nm were suubstracted from those at 450nm. Protein estimation in samples was done from the standard curve as per the protocol.

Statistical analysis

Blots were scanned and densitometric values obtained by using Image J program. Statistical analysis to check significance was done with Student's t test using Prism software. Graphs plotted were from two to three independent experiments and error bars in all graphs represent mean ± SD.

Results

Differential induction of Hsp27 and Hsp72 in naive and macrophage conditioned epithelial cells

As the induction of Hsps is a universal stress response against various insults and has been widely shown to have an anti-inflammatory effect, we checked the expression of Hsps representing four families. While soluble amebic protein (SAP) treatment suppressed Hsp27 and Hsp72 in naïve IEC (Fig. 7.1A), the same two Hsps were significantly over expressed in macrophage-conditioned Caco-2 and T84 colonic epithelial cells (Fig. 7.1B). Expression levels of Hsp60 and 90 remained unaltered in both naïve and conditioned IEC (not shown). Surprisingly, even though both secretory components (SC) and SAP elicited this stress response, SAP was consistently found to be twice as potent as SC (Fig. 7.1C) and was therefore used for all further studies. It should be noted that co-culturing alone did not affect the basal HSP expression significantly (Fig. 7.1B). Also, treatment of IEC with 24 h THP medium was sufficient to prime epithelial cells for ameba induced Hsp72 induction (Fig. 7.2) without the need for co-culturing. This confirms that macrophage secretions can alter epithelial cell responses to pathogen.

Ameba induced Hsp72 expression is ERK MAP kinase dependent

Mitogen activated protein kinases (MAPK) play important roles in regulating both inflammatory and stress responses (12) and HSPs have been shown to be regulated by different MAPK in intestinal epithelial cells (16, 25). Hence, we checked if ameba induced expression of Hsp27 and 72 are regulated by MAPK. As shown in Fig. 7.3A, when the conditioned epithelial cells was pre-treated with PD98059, an ERK MAPK inhibitor, SAP-induced Hsp expression was significantly inhibited. Correspondingly we observed the activation of ERK MAPK by amebic proteins as early as 30 min (Fig. 7.3B).

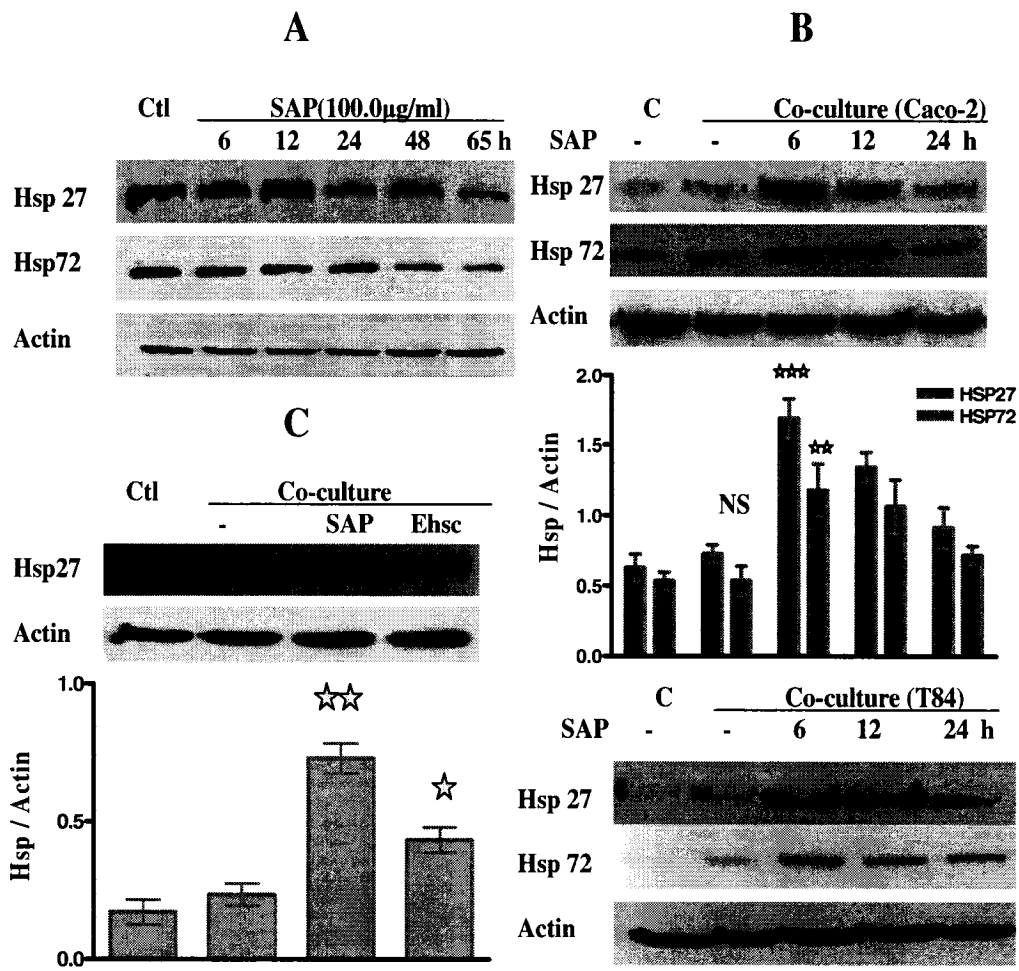

Fig. 7.1: Differential regulation of Hsps in naive and macrophage conditioned epithelial cells. A) Expression of Hsps in response to amebic lysate in naive epithelial cells. 100 µg/ml of soluble amebic proteins (SAP) were added to naive Caco-2 cells and cell lysate collected at different time points and checked for the Hsp expression by Western blot as described in methods. B) Caco-2 cells were co-cultured with differentiated THP-1 cells for 24 h and immediately stimulated with 100 µg/ml of SAP for different time periods and equal volumes of cell extracts in Laemelli sample buffer were subjected to SDS-PAGE and Western blot. A representative blot from 3 independent experiments is shown. (*** P<0.001; ** P<0.01). Bottom panel shows representative blots from T84 cells. C) Caco-2 cells were co-cultured with THP-1 as described and stimulated with 100 µg/ml of SAP or ameba secretory components for 12 h and checked for Hsp27 expression. (** P<0.01; * P<0.05)

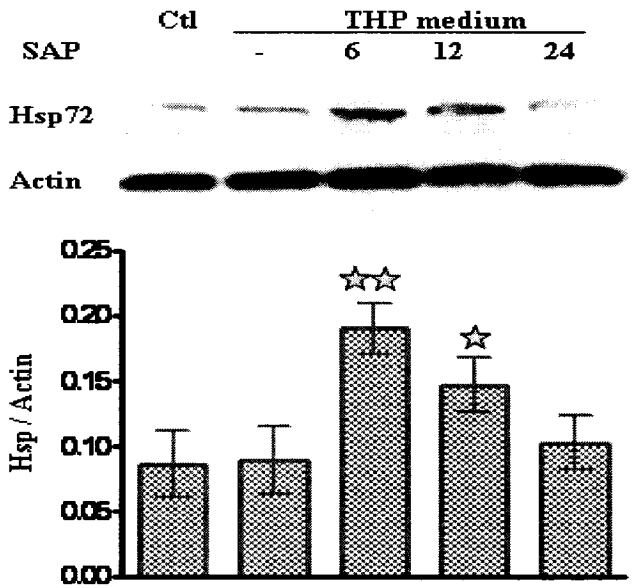

Fig. 7.2: Macrophage secretions prime IEC for Hsp induction. Caco-2 cells were treated with 24 h macrophage culture medium for 12 h before stimulating with SAP for different time points and checked for Hsp72 expression by Western blot. Representative blots from two experiments are shown. (✱✱, $P<0.01$; ✱ $P<0.05$)

E. histolytica induction of Hsp72 is independent of Gal-lectin and cysteine proteinase

The three well recognized virulent factors in *E. histolytica* are the surface adhesin Gal/GalNac lectin, cysteine proteinases and amebapore. The pathogenic effects of the Gal-lectin and cysteine proteinases have been extensively documented (26) and it was of interest to determine if the up-regulation of Hsp72 is mediated by these virulent factors. As shown in Fig. 7.4, pretreatment of SAP with either galactose or E-64 failed to inhibit Hsp72 expression indicating that Hsp induction by SAP is independent of Gal-lectin and cysteine proteinases. In contrast, significant abrogation of Hsp72 induction was noted by boiled SAP, suggesting a yet to be characterized protein moiety might be responsible for this effect.

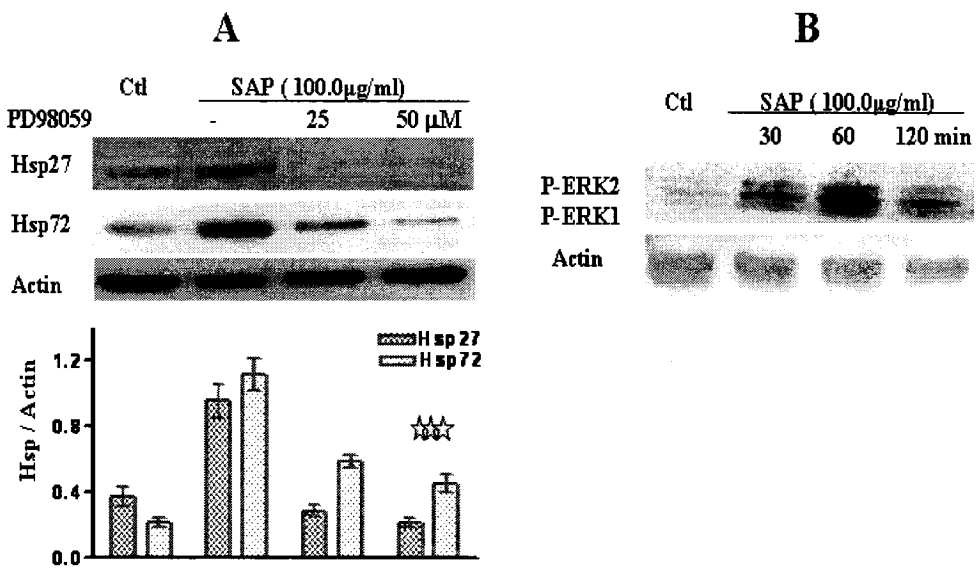

Fig. 7.3: Ameba induced Hsp expression in macrophage-primed IEC is dependent on ERK MAP kinase. A) Macrophage conditioned IEC (M-IEC) were pretreated with ERK MAP kinase inhibitor PD98059 at different concentrations for 2 h and then stimulated with SAP (100 µg/ml) for 12 h. (*** P< 0.001). **B)** M-IEC treated with SAP for different time points and cell extracts probed with antibody against ERK MAP kinase. The blot shown is representative of three independent experiments.

Amebic components induce Hsp72 via heat shock factor (HSF)-1

We then studied the mechanism of up-regulation of Hsp72 by amebic components. Quercetin is a flavanoid compound and a known inhibitor of Hsp synthesis by inhibiting HSF-1 (27, 28) and it was of interest to determine if it could inhibit SAP-mediated Hsp72 induction. As shown in Fig. 7.5A, quercetin (25 µM) significantly inhibited Hsp72 expression. Quercetins at high concentrations are known to inhibit protein synthesis. Thus, to confirm that quercetin inhibits SAP induced Hsp72 expression by inhibiting HSF-1 and not by inhibiting Hsp protein synthesis, we treated the cells with an equi-molar concentration of cycloheximide, an inhibitor of protein synthesis. Under these conditions cycloheximide treatment failed to inhibit SAP induced Hsp72 expression, confirming that amebic components, indeed, induce Hsp synthesis by activating HSF-1. To conclusively prove the role of HSF-1 in ameba induced Hsp synthesis, we silenced the HSF-1 gene by siRNA (Fig. 7.5B) and observed inhibition of Hsp72 expression by SAP in macrophage treated epithelial cells (Fig. 7.5C). Consistent with its role in regulating Hsp72 expression, HSF-1 was activated by amebic proteins as shown by Western blot

analysis (Fig. 7.5D). We found that amebic components were able to induce trimerization of HSF-1 in macrophage conditioned IEC (M-IEC) at a time point of 3 h. There was a corresponding increase in the DNA binding activity of HSF-1, which was inhibited by pre-treatment with quercetin (Fig. 7.5E). These studies confirm that amebic components activate HSF-1which in turn is inducing Hsp gene expression.

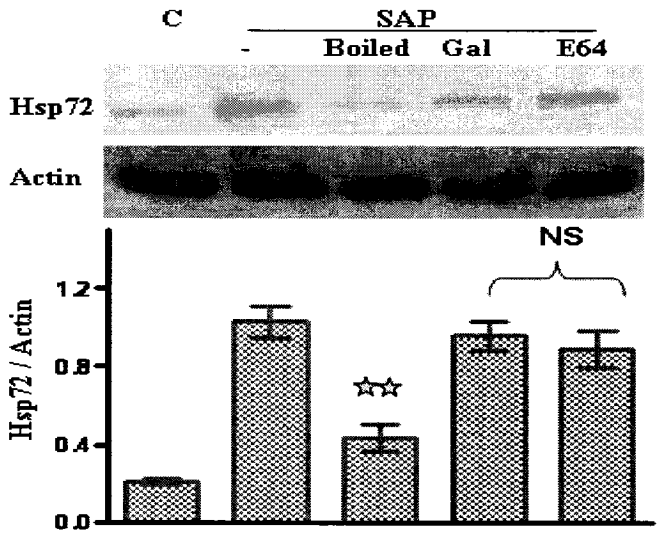

Fig. 7.4: SAP induced Hsp72 induction is independent of Gal-lectin and cysteine protease. SAP was treated with 150mM of D-Galactose or 100μM of E-64 for 1 h or boiled at 100° C for 5 min before adding to the M-IEC for 12 h. Western blot was done as described in experimental procedures. NS= Not significant. (✱✱ $P<0.01$)

Heat shock response by amebic components inhibits NF-κB activation and TNF-α secretion induced by IL-1β

As NF-κB was shown to be the key mediator of colonic inflammation in amebic infection (4) and stress response is increasingly being shown to inhibit this molecule (12, 17- 20), we studied the activation of NF-κB following amebic protein treatment. For this we treated M-IEC with SAP for 12 h and then stimulated with IL-1β, a prototypical NF-κB activator. As shown, pre-treatment with amebic proteins inhibited NF-κB-DNA binding activity (Fig. 7.6A), nuclear translocation of NF-κB p65 subunit (Fig. 7.6B) and IκB-α phosphorylation (Fig. 7.6C) induced by IL-1β in macrophage conditioned colonic epithelial cells. We also checked the effect of SAP treatment on TNF-α secretion. As

shown (Fig. 7.6D), pretreatment of M-IEC with SAP significantly inhibited IL-1β induced TNF-α secretion.

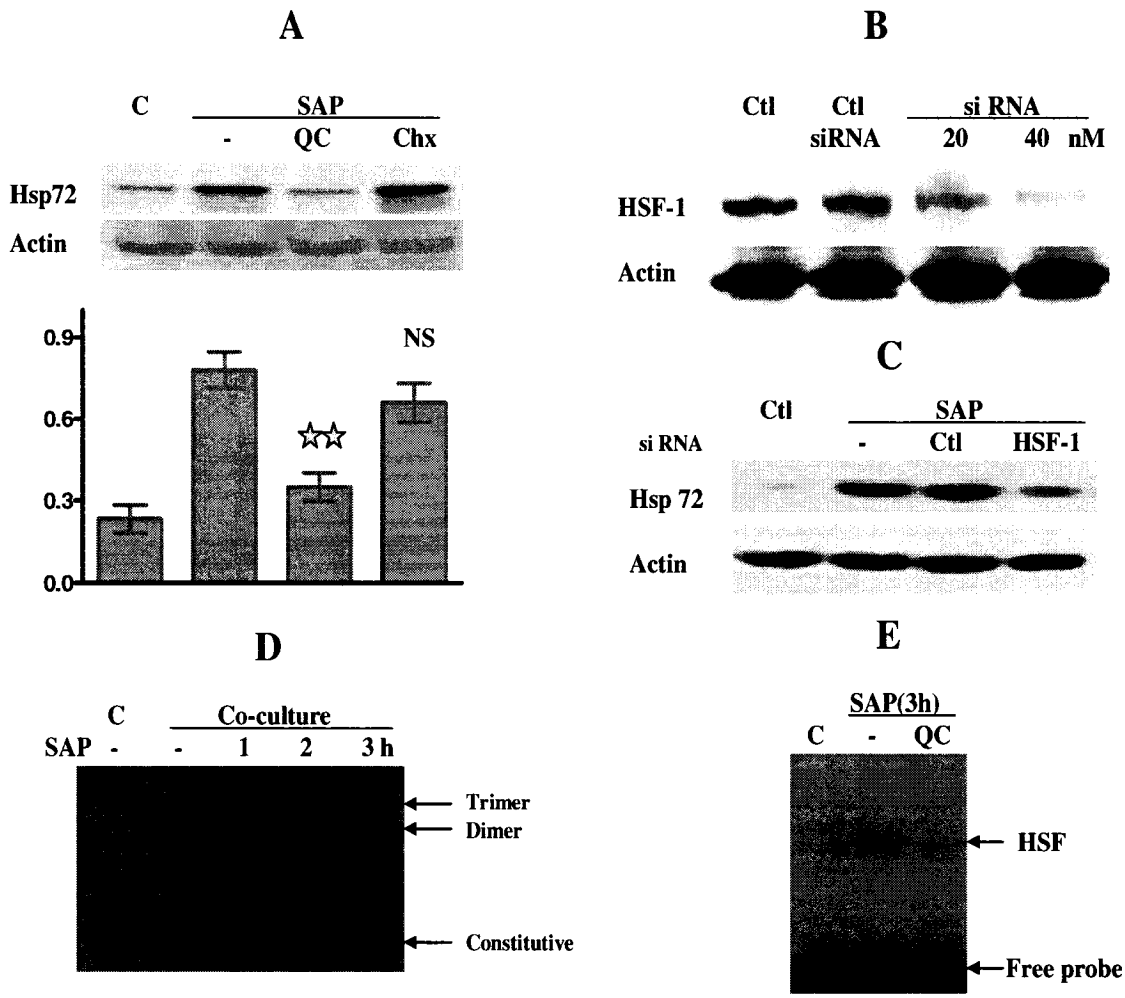

Fig. 7.5: Amebic protein induced Hsp72 expression is mediated by HSF-1. A) M-IEC were treated with 25μM of Quercetin (QC) or cycloheximide (chx) for 2 h before stimulating with SAP for 12 h. (✱✱ $P<0.01$). B) M-IEC transfected with different concentrations of siRNA against HSF-1 for 30 h as described in Methods. Cell lysates subjected to SDS-PAGE and Western blot was done with HSF-1 antibody. C) M-IEC cells were transfected or not with 40 nM HSF-1 siRNA for 30 h before treating with SAP for 12 h and checked for Hsp72 expression by Western blot as described. D) M-IEC was treated with SAP for different time points and cell extract collected and ran in a non-reducing gel and Western blot done with HSF-1 antibody as described. Arrows indicate activated dimeric and trimeric forms of HSF-1. E) M-IEC was incubated or not with quercetin (25μM) for 2 h prior to treatment with SAP (100μg/ml) for 3 h. 10.0μg of nuclear extracts subjected to EMSA as described. Blots shown are one of two to three replicates.

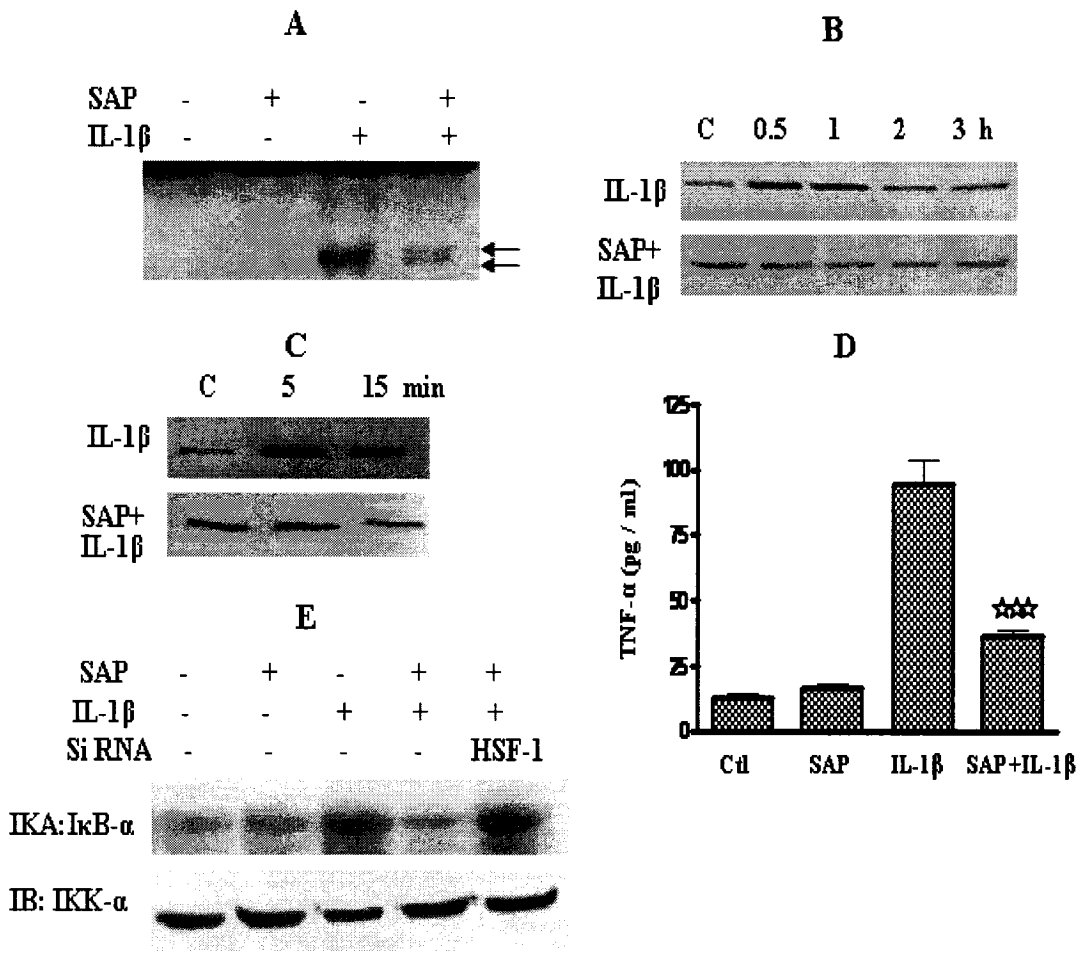

Fig. 7.6: **Amebic proteins inhibit IL-1β induced NF-κB activation and TNF-α secretion.** A) M-IEC were treated with SAP (100μg/ml) or medium for 12 h before stimulating with 5.0ng/ml of IL-1β for 30 min. 10.0μg of nuclear extract was subjected to EMSA using NF-κB consensus oligonucleotide as described in the experimental procedures. Arrows indicate NF-κB-DNA complexes. B) Cells were treated with IL-1β with or without prior treatment with SAP and nuclear extract collected at different time points and Western blot done to check the NF-κB p65 subunit. Top panel shows p65 nuclear translocation with IL-1β alone. Bottom panel was from cells pretreated with SAP. Membranes were stripped and re-probed with anti-histone antibody for normalization (not shown). C) Western blots of whole cell extracts showing the phosphorylation status of IκB with IL-1β alone (top panel) or following SAP treatment and IL-1β stimulation (bottom panel). D) Culture supernatants tested for TNF-α by ELISA as described in methods. *** ($p<0.005$). E) M-IEC transfected or not with HSF-1 siRNA was treated with SAP or medium alone for 12 h before stimulating with IL-1β for 30 min. *In vitro* kinase assay was done following immunoprecipitation with IKK-α as described in methods. Top panel shows kinase assay using GST-IκB as substrate. Bottom panel is Western blot of cell lysate to confirm equal quantities of IKK-α. All blots shown are one of two to three experiments.

Ameba induced stress response inhibits IKK activity via Hsp27

As IκB is phosphorylated by IκB kinase (IKK), we checked IKK activity in an *in vitro* kinase assay. As shown in Fig. 7.6E, amebic pretreatment suppressed IL-1β induced IKK activity. Moreover, when HSF-1 gene was silenced by siRNA, this suppression was abrogated suggesting that stress response is involved in this suppression. Thus, to precisely identify which HSP mediates this effect we silenced Hsp27 and 72 and checked the IKK activity. As shown in Fig. 7.7A, silencing Hsp27 significantly restored IKK activity while Hsp72 did not have a significant effect on this suppression. We observed a significant suppression in the expression of both Hsp27 (not shown) and Hsp72 (Fig. 7.7B) by siRNA.

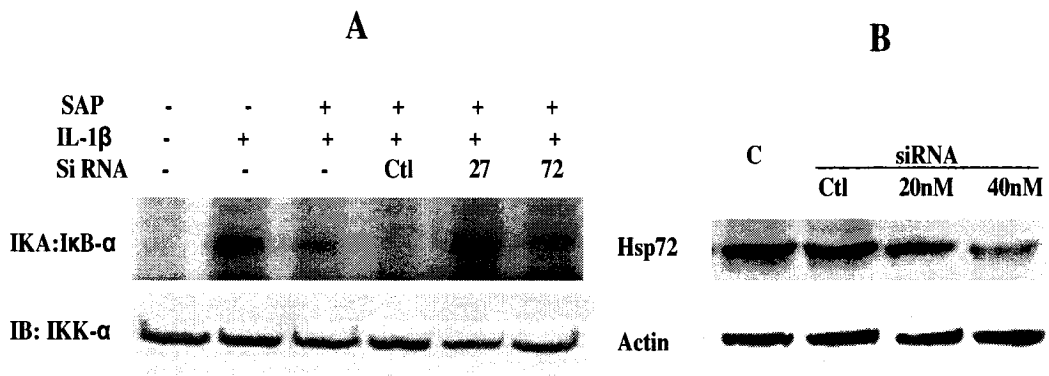

Fig. 7.7: Hsp27 mediates amebic protein induced inhibition of IKK activity. A) M-IEC transfected with control, Hsp27 or Hsp72 siRNA were treated with SAP for 12 h followed by 30 min stimulation with IL-1β. Kinase assay was done using IκB as substrate (top panel) and IKK-α level checked for normalization (bottom panel). **B)** M-IEC were transfected with either control or Hsp72 siRNA for 30 h as described and cell extracts probed with Hsp72 antibodies.

Heat shock proteins associate with IKK complex in IEC and amebic components enhance this interaction

Hsps are known to associate with IKK complex and modulate its activity in other cell lines (20, 29). Hence, for the first time we checked the interaction between Hsps and IKK in intestinal epithelial cells and found that Hsp27, 60 and 90 associate with IKK-α (Fig. 7.8A) and treatment with SAP increased the association of Hsp27 with IKK-α by twofold (Fig. 7.8B). As expected, siRNA of Hsp27 reduced this association. Results from Figs. 7.7A and 7.8B together confirm that Hsp27 negatively regulates IKK activity. All the Hsps tested, Hsp27, 60, 72 and 90 interacted with IKK-β in Caco-2 IEC (Fig. 7.8A) and

again Hsp27 interaction with IKK-β was also enhanced by SAP treatment (data not shown).

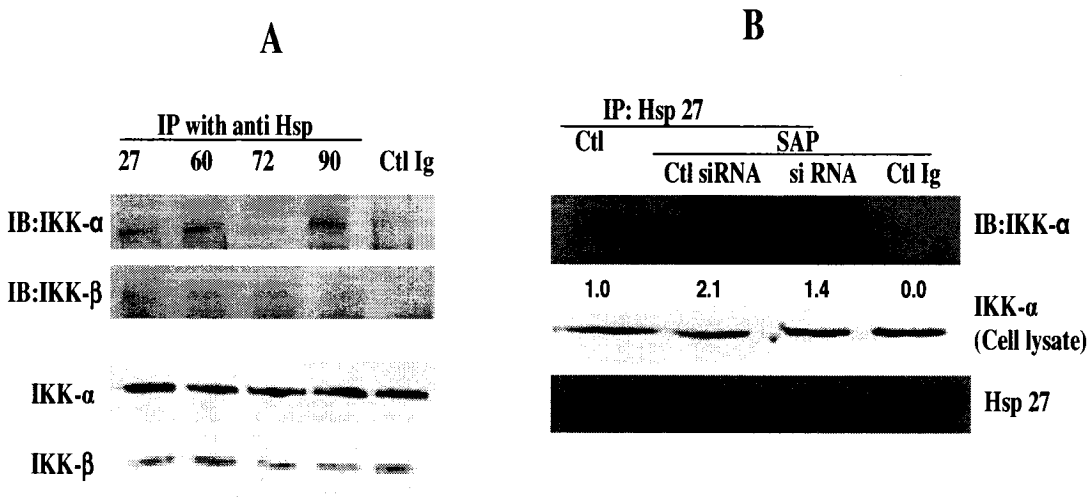

Fig. 7.8: Hsps associate with IKK complex in intestinal epithelial cells. A) Co-immunoprecipitation of IKKs with Hsp antibodies. Macrophage conditioned Caco-2 cell lysates were immunoprecipitated with antibodies against different Hsps ran on a reducing gel and probed with anti IKK-α or IKK-β antibody. Whole cell lysates checked for IKK-α and IKK-β expression (bottom 2 panels). B) Amebic proteins enhance the interaction between Hsp27 and IKK-α. M-IEC transfected or not with Hsp27 siRNA were treated with SAP (100μg/ml) for 12 h, lysates coimmunoprecipitated with Hsp27 and checked for IKK-α by SDS-PAGE and Western blot. Relative densitometric values of the bands are shown. Equal quantity of cell lysates was probed with Hsp27 and IKK-α (bottom panels). All experiments were repeated two to three times and representative Western blots for each are shown.

Phosphorylation of Hsp27 is required for interaction with and inhibition of IKK

In order to confirm that Hsp27 mediated IKK inhibition in IEC and to fully understand the mechanism involved, we overexpressed wild type (WT) and phosphorylation deficient mutant of hamster Hsp25 (AA) in conditioned epithelial cells (Fig. 7.9A). Transfected M-IEC was treated with SAP and checked for interaction between Hsp25 and IKK-α. As shown in Fig. 7.9B, while over expression of Hsp25WT enhanced SAP induced association with IKK-α, Hsp25AA significantly inhibited this interaction. Consistent with the negative regulation of IKK by Hsp27, this decreased interaction between IKK-α and Hsp27 abrogated the inhibitory effect of SAP on IKK activity, seen as restored IκB phosphorylation and NF-κB p65 nuclear translocation (Fig. 7.9C).

Consistently, we observed that SAP induced a delayed but significant phosphorylation of ser 15 Hsp27 in M-IEC (Fig. 7.9D).

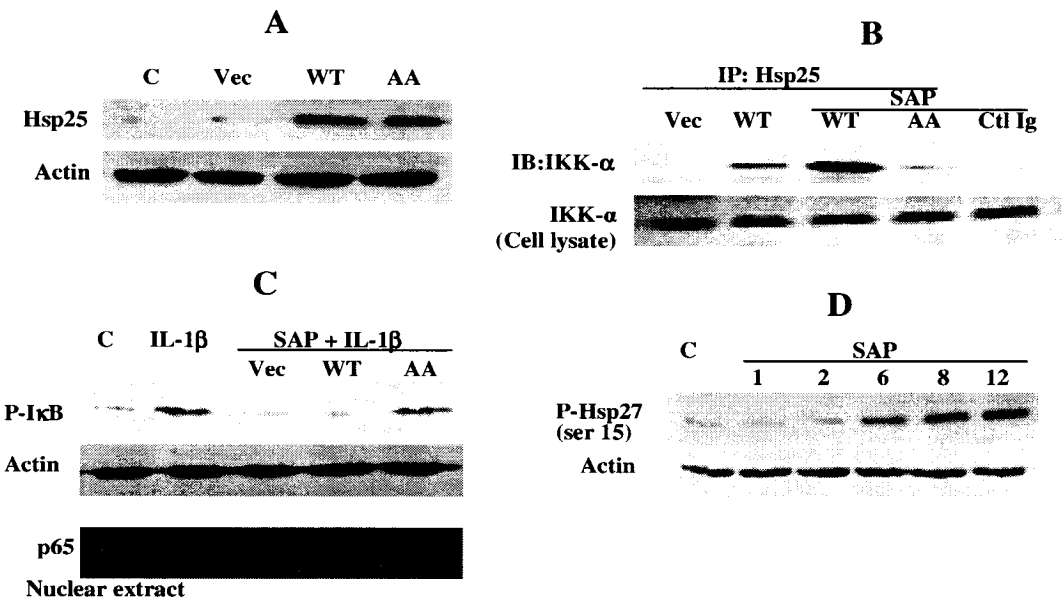

Fig. 7.9: NF-κB inhibition by SAP requires phosphorylation of Hsp27. A) Conditioned IEC were transfected with vector, wild type or mutant Hsp25 plasmids during co-culture as described and whole cell lysates were subjected to SDS-PAGE and probed with antibodies against hamster Hsp25 and actin. B) M-IEC over expressing hamster Hsp25 plasmids were treated with SAP for 12 h and lysates subjected to immunoprecipitation using anti hamster Hsp25 or control rabbit IgG antibody followed by Western blot using IKK-α antibody. Cell lysates from same cells were probed with IKK-α. C) Conditioned IEC transfected with vector, wild type or mutant Hsp25 plasmids were pre- treated or not with SAP and IL-1β and whole cell lysates checked for phospho IκB-α by Western blot as described. Bottom panel: 10 μg of nuclear extracts from cells treated similarly were probed with anti p65 antibody. D) M-IEC treated with SAP for different time periods and cell lysates probed with phospho ser 15-Hsp27 and actin. Representative blots from at least two different experiments were shown.

Ameba induced stress response in conditioned epithelial cells has cytoprotective function

As Hsps also have cytoprotective abilities in different cells, we checked if amebic proteins can protect IEC against injuries caused by diverse agents. As shown in Fig. 7.10A, pretreatment with SAP significantly increased cell viability following oxidative injury by hydrogen peroxide and reduced caspase-3 cleavage induced by the apoptotic agent, Fas L (Fig. 7.10B).

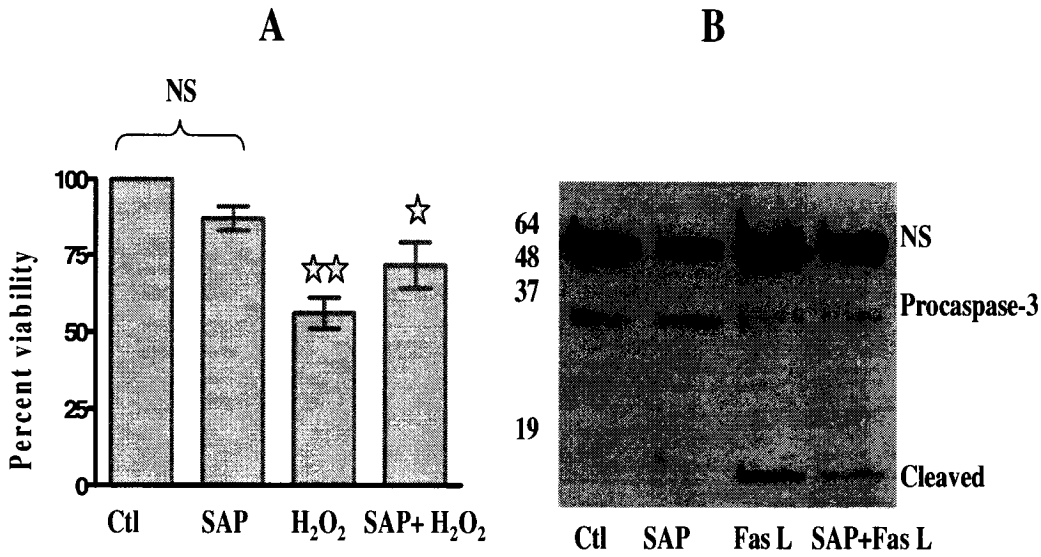

Fig. 7.10: Amebic proteins confer protection against oxidative and apoptotic injuries. A) M-IEC were treated with SAP (100μg/ml) for 12 h and then incubated with 10mM of hydrogen peroxide for further 12 h and cell viability was checked by neutral red assay as described. ✱✱ P<0.01 compared to control; ✱ P<0.05 compared to H_2O_2 alone. NS= Not significant. B) M-IEC (T84 cells) were exposed to SAP (100μg/ml) for 12 h before treating with 1.0 μg/ml of Fas L for 12 h. Cleavage of Pro caspase-3 was examined by Western blot. The experiment was repeated three times and one representative blot is shown.

Discussion

In this study we show that SAP have protective effects mediated by Hsps on intestinal epithelial cells. Interestingly, the Hsps were induced only in epithelial cells that have been exposed to macrophage secretions for 24 h but not in the naive IEC. We made 2 novel observations: one, this is the first report on the protective effect of amebic proteins, and secondly, that macrophage secretions can prime epithelial cells to elicit stress responses.

Co-culture systems have been popular owing to their better simulation of *in vivo* situation wherein epithelial and immune cells exist in close proximity and respond to each other components. Several studies reported a differential epithelial response, mostly with respect to cytokines, following exposure to immune cells. Two recent studies reported the induction of Hsp25 and Hsp72 in mouse intestinal epithelial cells co-cultured with

lymphocytes and IL-2 was found to be the mediator (13, 16). We made the novel observation that intestinal epithelial cells express Hsps in response to amebic proteins following exposure to macrophage secretions.

Notably, basal Hsp expression remains unchanged in macrophage conditioned IEC suggesting that epithelial cells are only primed rather than activated by macrophage secretions. The cross talk between macrophages and IEC could be bi-directional in the sense that macrophages also respond to epithelial secretions such as MCP-1 and TGF-β (30). However, amebic proteins could induce Hsp72 in epithelial cells that have been incubated with macrophage secretions without co-culturing; suggesting that epithelial cell modification of macrophages is not required to elicit a differential epithelial response. As differentiated THP-1 cells are shown to constitutively produce a variety of cytokines such as TNF-α, IL-1β, IL-6, IL-8 etc. (31) and IL-1β induce Hsp expression (32), we checked the role of these cytokines in priming the IEC. Adding neutralizing antibodies against TNF-α and IL-1β in the macrophage medium did not significantly inhibited ameba induced Hsp expression (data not shown). Previous studies (33) have shown that co-culture with activated PBMC altered T84 epithelial cell physiology and that exogenously added TNF-α or IFN-γ did not mimic the changes induced by immune cells. This suggests that mediators in immune cell secretions have an effect (both adverse and beneficial) on epithelial responses but their identities are not known.

IEC are known to express Hsps in response to diverse insults such as pathogens, pathogen specific molecules and chemicals and immune cells can alter this response. Accordingly, we checked the expression of four Hsps belonging to different families and found specific up-regulation of Hsp27 and 72 but not Hsp60 and 90 (data not shown) by SAP. Our observation of involvement of ERK MAP kinase in ameba induced Hsp expression is consistent with previous reports (16, 34, 35) and extends the current thinking that MAP kinases play an important role in regulating both inflammatory and stress pathways. After establishing that amebic proteins induce Hsps in macrophage primed epithelial cells by activating heat shock transcription factor (HSF)-1, we proceeded to check the functional significance of this phenomenon. As our objective was

to understand how colonic inflammation was absent in the majority of infected individuals and given the evidence that Hsps suppress NF-κB activation, we critically analyzed if and how Hsps induced by amebic proteins inhibits IL-1β mediated NF-κB activation. We chose IL-1β for two reasons; one is it is a prototypical NF-κB inducer and secondly, it was shown to play a role in ameba induced colitis (2, 36). Moreover, inhibiting NF-κB significantly reduces amebic colitis in a mouse model of intestinal amebiasis (4). NF-κB is a ubiquitous transcription factor that regulates a number of genes involved in inflammation and immune response (37). Activation of this transcription factor is critically regulated at multiple steps. Recently, the inhibitory effects of Hsps on NF-κB activation are increasingly being demonstrated in different cell systems. Hsp72 has been found to associate with P65 subunit of NF-κB and inhibits the latter's nuclear transport in T-cells (38) and Hsp27 has been shown to be a ubiquitin binding protein regulating the degradation of IκB expression thereby indirectly influencing NF-κB activation (39). Recent studies show that IKK complex is the potential target for Hsp inhibition of NF-κB pathway (17, 18, 40). In particular, one study showed that Hsp27 interacts with IKK complex and negatively regulates its activation by TNF-α (20) in HeLa cells. As SAP increased Hsp72 expression and inhibited IL-1β induced NF-κB p65 nuclear translocation, we checked for their interaction in IEC but failed to see any association (data not shown). We found that IκB phosphorylation was also inhibited which was a direct result of reduced IKK activity by SAP treatment. SiRNA technology has rapidly become a revolutionary tool for efficient silencing of gene expression in a variety of experimental settings (41). We exploited this powerful system to silence Hsp genes to understand their role in ameba mediated suppression of IKK activation and found that silencing HSF-1 or Hsp27 but not 72 resulted in significant abrogation of this inhibition. As different Hsps interact with and regulate IKK complex, we checked if amebae-induced Hsps interacted with the IKK subunits in intestinal epithelial cells. For the first time, we reported an association between IKK and Hsps in IEC. Hsp27, 60 and 90 constitutively interact with IKK-α and the interaction of Hsp27 with IKK-α was strongly enhanced following SAP treatment. While Hsp27 and Hsp90 have been previously shown to associate with the IKK complex, this is the first report of the

interaction between Hsp60 and IKK. Our observation that all Hsps tested interacted with IKK-β is surprising but could be attributed to the use of mild detergent (CHAPS) in the cell lysis buffer for co-immunoprecipitation. We found that Hsp27 association with IKK-β was also increased by amebic protein treatment (not shown). Previous report (20) showed that Hsp27 association with IKK-β but not with IKK-α increased in response to TNF-α. The reason(s) for this selective interaction is unclear.

As posttranslational modifications of Hsp27 are known to regulate its biological activity (20), we tested the role of phosphorylation of Hsp27 in its ability to inhibit NF-κB activation. Overexpression of phosphorylation deficient mutant of hamster Hsp25 (at serine 15 and 90) in IEC resulted in significant reversal of NF-κB inhibition by amebic proteins. There are two sites phosphorylated in hamster Hsp27 (Ser15, Ser90) and three in human (15, 78, 82). Ser78-Ser82 of human Hsp27 probably works in tandem as the equivalent of the unique Ser90 in hamster Hsp25. We also observed that SAP induces significant phosphorylation of serine 15 (Fig.7.9D) but not ser72 (data not shown) after prolonged treatment in M-IEC. As Hsp27 phosphorylation depends on p38 MAP kinase (20), this could have resulted from a delayed activation of p38 MAP kinase as we did not find either p38 (data not shown) or Hsp27 phosphorylation in the early time periods of 1 h. Nonetheless it is clear that overexpression of Hsp27 amplified the SAP effects while blocking the Hsp27 phosphorylation significantly abrogated the amebic inhibitory effect on NF-κB activation. These studies confirm and further strengthen the concept that post-translational modifications, particularly, the phosphorylation of Hsp27 plays an important role in NF-κB regulation via inhibiting IKK activity. Extending our observations on the protective effects of ameba induced stress responses we also showed that prior treatment with amebic proteins protected the epithelial cells against hydrogen peroxide and Fas L treatment. This is not surprising in view of the potent apoptotic inhibitory effects of Hsp72 (42-43). In fact, both Hsp27 and 72 are powerful chaperones and inhibit key effectors of the apoptotic machinery including apoptosome, caspase activation complex and apoptosis inducing factor (44). While this observation supports the anticipated protective effects of stress response, its functional significance with respect to ameba pathogenesis is difficult to surmise. Contact dependent apoptosis induction by *E.*

histolytica was reported in immune cells such as neutrophils, T-cells and erythrocytes (23, 45, 46). It was also shown that *E. histolytica* preferentially ingests apoptotic Jurkat T- cells. But hitherto no data is present on epithelial cell apoptosis by the parasite. Assuming a similar phenomenon for epithelial cells, it is reasonable to argue that stress response by the host is an efficient way to circumvent parasite induced cell death. At present, it is not known what soluble amebic molecule(s) is responsible for the induction of Hsp. In summary, we have shown that amebic proteins can inhibit NF-κB activation and promote cell survival via stress protein expression in macrophage primed intestinal epithelial cells (Fig. 7.11). These studies for the first time, demonstrate a potential mechanism by which intestinal inflammation induced by *E. histolytica* might be inhibited in majority of infected individuals and suggests that amebic colitis could result from the lack of such protective responses to suppress pro-inflammatory cytokine induction in a minority of susceptible individuals.

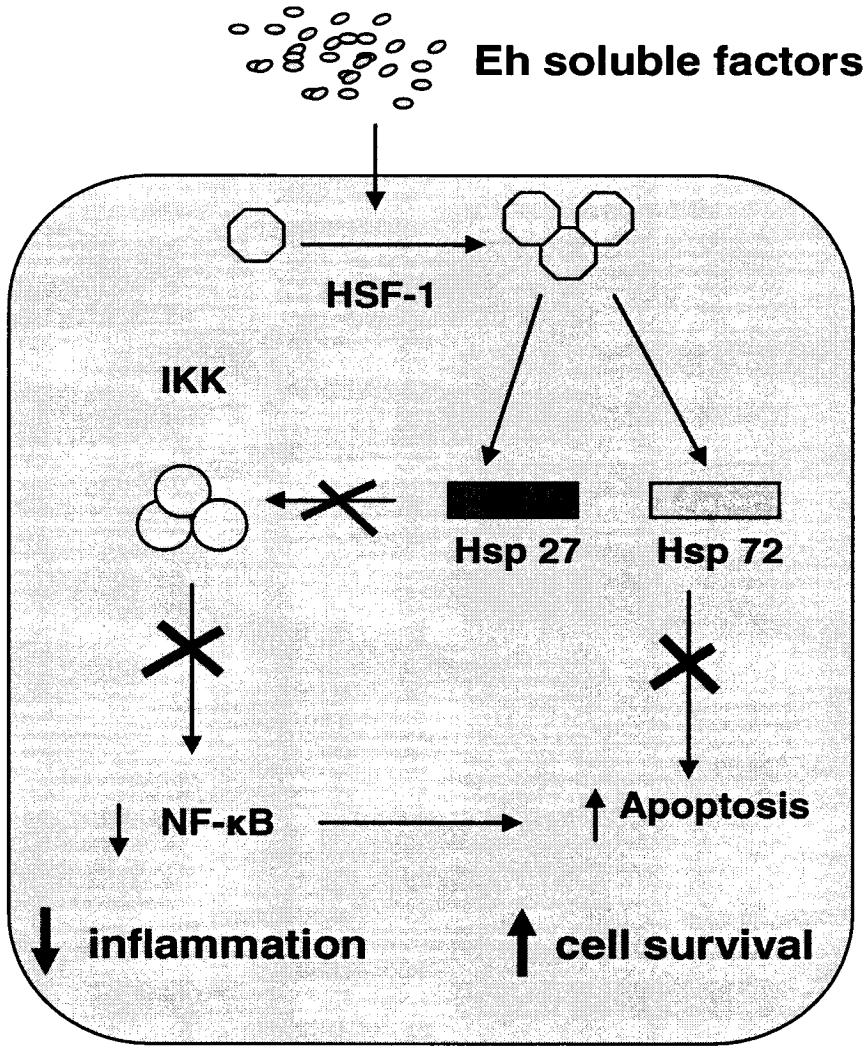

Fig. 7.11: Schematic model for suppression of amebic colitis. Soluble amebic proteins activate the heat shock transcription factor-1 to form trimers from its inactive monomers. Activated HSF-1 induces the expression of Hsp27 and Hsp72, which perform different functions. Phospho-Hsp27 associates with IKK and inhibits its activity thereby suppressing NF-κB activation induced by the proinflammatory cytokines released in response to amebic infection. Suppression of NF-κB favors increased apoptosis. However, Hsp72, which apparently does not play a role in NF-κB signaling in IEC, might function to promote cell survival through its potent anti apoptotic activity. Together the stress response induced by amebic proteins functions to inhibit intestinal inflammation and promote cell survival.

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SECTION III: GENERAL DISCUSSION

In this work, I addressed some of the unsolved puzzles in the early interactions between the enteric protozoan parasite *E. histolytica* and host cells. The parasite virulence factor Gal-lectin plays a key role in the colonization of the pathogen and host cell killing. In this regard, its properties as surface adhesin and as a receptor to initiate intracellular signaling within the parasite have been well documented. However, its immunological properties are increasingly being appreciated. It is highly antigenic and able to activate host immune system. For these reasons, its potential as a vaccine candidate is studied extensively and indeed a majority of researchers reported successful protection against amebic infection in various models of amebic liver abscess. One recent report showed a protection in a burgeoning mouse model of intestinal amebiasis (1). While Gal-lectin induces good antibody response, this alone fails to explain its efficiency as vaccine, as humoral immunity was not found to be sufficient for protection against the disease. In our laboratory attempts were made to study the ability of the Gal-lectin to activate cell mediate immunity. Indeed we found that purified native Gal-lectin is able to induce inflammatory cytokines such as TNF-α and IL-12 from macrophages. As these cytokines can activate immune cells to kill ameba, their induction by Gal-lectin is an important means of conferring resistance to host.

Discovery of Toll like receptors (TLR) is a major breakthrough in our understanding of innate immune system. Multiple signaling cascades are activated by TLRs that in majority of cases result in the activation of NF-κB. NF-κB is a ubiquitous transcription factor which regulates the expression of a number of genes involved in inflammation, immune response and apoptosis. Its activation could be a double edged knife. A mild inflammation mediated by NF-κB could eliminate the pathogens and signal the adaptive immune system. However an uncontrolled severe inflammation leads to indiscriminate host tissue damage. Interestingly, expression of some TLRs is also regulated by this transcription factor. Its elevated expression during inflammatory bowel disease suggests it plays a dominant role in gut inflammation (2).

Our observation that the Gal-lectin could up-regulate TLR-2 expression in macrophages has dual significance. Increased TLR-2 makes the immune cells more responsive to a number of opportunistic pathogens in gut. This results in amplifying the Toll signaling, NF-κB activation and inflammatory cytokine production by lamina propria immune cells, thus initiates or aggravates the inflammation. The cytokines secreted by lamina propria immune cells could also alter epithelial response to intestinal pathogens or commensals (3). Gal-lectin could come in contact with underlying immune cells by two means; either by translocation of Gal-lectin from lumen to lamina propria through epithelial cells (4) or following the breach of epithelial layer by the amebic trophozoites. Recent report showed that another amebic molecule lipophosphoglycan (LPPG) activates both TLR-2 and TLR-4 to induce TNF-α, IL-12 and IL-8 in monocytes (5). Thus, increased TLR-2 expression by Gal-lectin could act synergistically with LPPG and amplify the ameba induced colonic inflammation. Destruction of mucosal barrier by trophozoites exposes immune cells to amebic Gal-lectin or LPPG and other microbial TLR-2 ligands which aggravates the existing inflammation. Hence, we propose that this occurs in 1% of individuals who show the severe symptoms of dysentery or extra-intestinal amebiasis, which are associated with extensive colonic tissue destruction. Another aspect of Gal-lectin induced up-regulation of TLR-2 is with regard to the use of the lectin as a vaccine to induce protective Th1 responses. Activation of immune cells via TLRs has been shown to skew the immune response towards Th1 type and hence it is logical to think that increased Toll signaling would confer better protection against amebiasis. In this context, a combined Gal-lectin and LPPG vaccine would work better than either of them alone. Even in the absence of LPPG, increased TLR-2 expression by the Gal-lectin could enhance TLR activation by ligands present widely in gut commensals. Despite all the observations about the Gal-lectin ability to activate immune cells, its receptor remained elusive. Indeed, efforts are now on in our laboratory to check if TLR-2 is a potential receptor for the Gal-lectin. In short, our work unraveled a new facet of TLR involvement in the pathogenesis of amebiasis.

Our observation of monocyte chemotactic protein (MCP)-1 induction by amebic components is not surprising. MCP-1 is chemoattractant for monocytes/ macrophages

and T-cells. While infiltration of neutrophils early in the amebic lesion is explained by induction of IL-8, the mechanism for subsequent homing of monocytes, lymphocytes or eosinophils remains unanswered. We showed for the first time that amebic soluble factors can induce MCP-1 production in IEC, resulting in the recruitment and activation of macrophages. While this alone is exciting, the mechanism of MCP-1 induction is quite novel. Amebic activation of PI3 kinase phosphorylates Akt and p65 subunit of NF-κB, thus inducing MCP-1 gene expression. However, it is not clear from our studies whether PI3 kinase or Akt directly phosphorylates p65 subunit or do so via other kinases. Further studies are required to completely understand the signaling events activated by the parasite in intestinal epithelial cells. Previously it has been shown that ameba induces IL-8 via post-transcriptional regulation. Together, these studies suggest that the host developed diverse means to sense the presence of the parasite and to signal the immune system. The secretion of chemokines by IEC is beneficial during the non-invasive phase of infection, as the recruited immune cells would rapidly kill any pathogen that tries to breach the barrier. This could also result in host tissue damage and form the foci of infection for the trophozoites. Indeed, depletion of neutrophils has been shown to make the mice more susceptible to intestinal amebiasis (6) and the *in vivo* significance of our observation needs a similar investigation. Nonetheless, it is clear that macrophages do home to lesion and could possibly play a role in pathology. We propose this could be occurring in the 10% of the individuals that show the symptoms of colitis and diarrhea.

Most of the pro-inflammatory molecules such as TNF-α, iNOS, COX-2 etc. are regulated by the classical NF-κB activation involving IKK activation. Our failure to see any IKK or MAP kinase in the previous study leads us to study if amebic components could suppress NF-κB, the key inflammatory mediator. As 90% of infected hosts remain asymptomatic, we hypothesize that anti-inflammatory responses operate in majority of cases. Even in biopsy samples of late invasive lesions from amebiasis patients, trophozoites are found in the muscular layer in the absence of any tissue damage and inflammatory infiltrate (7). Keeping these observations in view, our primary objective was to begin exploring the protective host responses that could inhibit inflammation. We focused on stress response as a potential means to suppress NF-κB for many reasons; it is a universal response to

diverse stimuli including pathogens, there are confirmed reports on the ability of heat shock proteins to inhibit NF-κB in different cell types including IEC. When we failed to see the induction of Hsp expression in naive epithelial cells, we undertook the transwell system in order to mimic the *in vivo* situation where the baso-lateral surface of IEC is exposed to the secretions of immune cells. While previous studies utilized peripheral blood mononuclear cells, we employed differentiated THP-1 cells which more closely mimic the tissue macrophages. Also, using specific cell type would give better mechanistic details on cellular interaction. For the first time we reported that macrophages can prime IEC for stress response during infections. This is also the first observation that ameba can elicit a protective host responses. So far studies have been attempting to understand how the disease occurs (which is only in 10% cases) rather than why there is no disease (90% of cases) and focused on pro-inflammatory responses from host cells. Our observation that heat shock protein induction in response to amebic components inhibiting NF-κB and apoptosis in IEC is significant both for the amebic pathogenesis and epithelial biology. This is the first report demonstrating the anti-inflammatory function of Hsp27 in intestinal epithelial cells. The precise mechanism of Hsp27 mediated suppression of IKK activity needs extensive biochemical characterization. The most plausible explanation seems to be a steric hindrance of catalytic domain but inhibition of IKK-α or IKK-β in different cell types suggests additional factors might play a role. While in Hela cells, phosphorylated Hsp27 associates with IKK-β following TNF-α treatment, in IEC we observed an increased association with both IKK-α and IKK-β in response to amebic components. Subtle differences in the physical assembly of IKK complex in different cell types might also account for these differences. Suppression of TNF-α by amebic components is a novel finding that could potentially explain the absence of inflammation. This cytokine has been implicated in gut inflammation during various conditions such as IBD and also amebiasis. Importantly, this cytokine is primarily regulated by the classical NF-κB activation that requires IKK activation, IκB degradation and nuclear translocation of p65/p50 dimer. By suppressing IKK activity while maintaining the post-translational activation of p65, intestinal epithelial cells appear to maintain a tight regulation of NF-κB and consequently the set of genes that can be activated in response to a specific stimulus; as demonstrated in our

work wherein IEC produce MCP-1 but not TNF-α in response to ameba. Other NF-κB regulated genes such as iNOS that require IKK activation might also be suppressed in IEC and we propose this could be occuring in 90% of amebic infections. It should be mentioned that from a recent *in vivo* mouse chimera studies, Hamano *et al.* (8) demonstrated that hemopoeitic cell derived IL-10 is required for non-hemopoeitic cell mediated protection against intestinal amebiasis. This work confirms the concept that immune cells alter epithelial cell responses to the parasite and futher justifies the *in vivo* validity of our observations. To thoroughly decipher the mechanism underlying the observations of Hamano's work, an *in vitro* approach as done in our work, should be undertaken by employing transwell cultures of hemopoeitic/non-hemopoeitic cells from different KO mice.

One critical issue to be resolved is to identify the specific factor(s) that are responsible for induction of chemokines and stress proteins from IEC. The principle immune cell activator Gal-lectin was not able to induce any response from IEC. This is not surprising given the normal hyporesponsive status of IEC. It is not clear if a single or multiple moieties in amebic components induce IEC responses similar to those reported using soluble mediators from probiotic bacteria (9). Clearly, proteomic studies should be undertaken to identify and characterize the factors responsible for these beneficial effects.

Putting these three observations together, we envisage a model (see Fig. III) whereby epithelial cells sense the ameba and respond by producing chemokines with the resultant homing of immune cells to mucosal tissue. Immune cells could be successful in clearing the infection or fail to kill the trophozoites with varying degrees of tissue destruction; depending on several factors such as activation state of immune cells or virulence of the invading ameba. A leaky epithelium might result in immune cell activation by Eh Gal-lectin and other opportunistic microbes via TLRs, aggravating the inflammation. Immune cell secretion of lytic agents such as NO and H_2O_2 can cause apoptosis of IEC and form foci of infections. Such a situation might result in systemic invasion of pathogens. However, the host has developed several mechanisms such as stress response to avoid such unwarranted inflammatory response and apoptosis. A disturbance and imbalance in

any of these events will give rise to different scenarios of asymptomatic, intestinal and systemic disease states in individuals infected with amebic trophozoites.

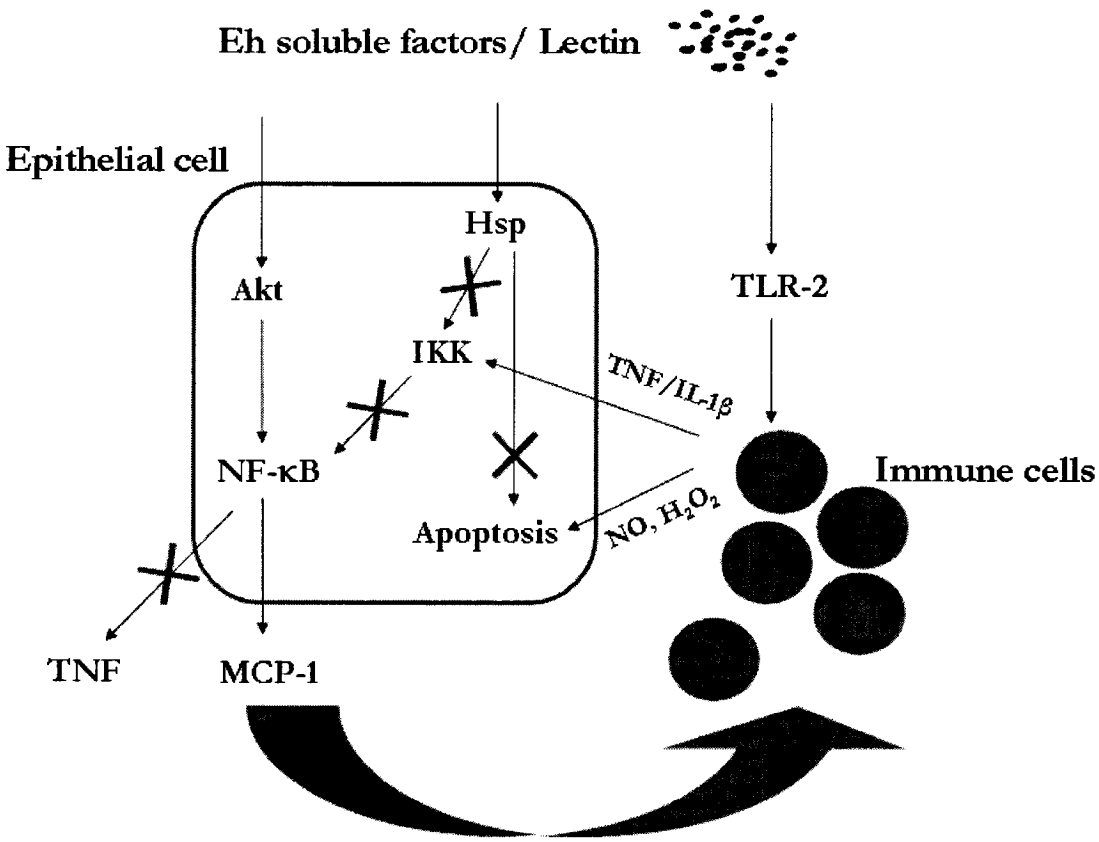

Fig. III: Model for dynamic host-parasite interactions and the outcomes during amebiasis: Parasite activating a non-classical pathway of NF-κB via Akt induces chemokine, MCP-1 secretion from IEC. Homing of macrophages and T cells to the site exposes them to Gal-lectin and other microbes, results in enhanced TLR-2 signaling via secretion of pro-inflammatory cytokines IL-1β and also cytolytic agents such as H_2O_2. Cytokines induce pro-inflammatory molecules such as TNF-α from IEC via the classical NF-κB pathway and the ROS induce apoptosis in IEC. However, ameba induced heat shock proteins suppress IKK activity and suppress inflammatory response of IEC. Hsp also rescue IEC from apoptotic effects of ROS.

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activate MAPKs and induce cytoprotective heat shock proteins in intestinal epithelial cells. *Am J Physiol Cell Physiol* 290:C1018-1030.

Appendix

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

The required documents are appended in the following pages.